Published in final edited form as:

J Proteome Res. 2017 August 04; 16(8): 2692–2708. doi:10.1021/acs.jproteome.6b00991.

Post-Translational Modifications (PTMs), Identified on Endogenous Huntingtin, Cluster within Proteolytic Domains between HEAT Repeats

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Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00991. Supplementary Figure S1. Purification of endogenous FL-Htt for mass spectrometry from mouse brain. Supplementary Figure S2. Validation of the specificity of antibodies to PTMs. Supplementary Figure S3. Subcellular localization of expanded Htt is modulated by PTMs. Supplementary Table S1. Synthetic peptides used to target PTMs on mouse Htt for identification and quantitation in TMT-based mass spectrometry experiments. Supplementary spectra for identification of PTM sites shown in Table 1. (PDF)

The authors declare no competing financial interest.

The mass spectrometry identification data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD005753 and 10.6019/PXD005753.

Post-translational modifications (PTMs) of proteins regulate various cellular processes. PTMs of polyglutamine-expanded huntingtin (Htt) protein, which causes Huntington's disease (HD), are likely modulators of HD pathogenesis. Previous studies have identified and characterized several PTMs on exogenously expressed Htt fragments, but none of them were designed to systematically characterize PTMs on the endogenous full-length Htt protein. We found that full-length endogenous Htt, which was immunoprecipitated from HD knock-in mouse and human post-mortem brain, is suitable for detection of PTMs by mass spectrometry. Using label-free and mass tag labeling-based approaches, we identified near 40 PTMs, of which half are novel (data are available via ProteomeXchange with identifier PXD005753). Most PTMs were located in clusters within predicted unstructured domains rather than within the predicted α -helical structured HEAT repeats. Using quantitative mass spectrometry, we detected significant differences in the stoichiometry of several PTMs between HD and WT mouse brain. The mass-spectrometry identification and quantitation were verified using phospho-specific antibodies for selected PTMs. To further validate our findings, we introduced individual PTM alterations within full-length Htt and identified several PTMs that can modulate its subcellular localization in striatal cells. These findings will be instrumental in further assembling the Htt PTM framework and highlight several PTMs as potential therapeutic targets for HD.

Graphical abstract

Polyglutamine (polyQ) expansion in huntingtin (Htt) protein causes Huntington's disease (HD), a genetically inherited autosomal dominant neurodegenerative disorder.^{1,2} Previous studies have indicated that PTMs of expanded Htt protein are important modulators of HD pathogenesis.^{3–5} While mutant Htt RNA and repeat-associated non-ATG (RAN) translation products may contribute to HD pathogenesis,^{6–11} the polyQ-expanded Htt protein is believed to be a major source of toxicity for target cells. As a scaffold protein with no known enzymatic activity, Htt appears to be a recalcitrant target for small-molecule therapeutics. Thus substantial effort has been focused on the identification and characterization of PTMs on Htt because they are likely to be catalyzed by enzymes and could offer targets for therapeutics.

Two recent reviews^{12,13} summarize the current body of knowledge and the several PTMs of Htt that have been documented. Most of the PTMs characterized so far are located in the N-terminal region of Htt protein. Phosphorylation of Htt at threonine 3 and serines 13 and 16 were reported to modulate aggregation and toxicity^{4,14} and regulate additional PTMs, including ubiquitination, SUMOylation, acetylation, altering nuclear localization, clearance and cleavage of Htt, and its interactions with nuclear pore proteins.^{15,16} Phosphorylation at serines 13 and 16 was also found to control cytoplasmic/nuclear shuffling of Htt induced by cellular stress,^{17,18} with the N17 domain of Htt acting as a reactive oxygen species sensor that regulates Htt phosphorylation and localization.¹⁹ Acetylation of lysine residues within the N17 domain was reported to modulate both Htt aggregation and its membrane attachment,²⁰ which was also mediated by palmitoylation of Htt by HIP14 at cysteine 214.²¹ Phosphorylation of Htt at serine 421 by AKT and SGK kinases was found to reduce Htt toxicity and restore axonal transport in neurons.^{22,23} Phosphorylation at serine 536 was demonstrated to modulate Htt cleavage by calpain as well as mutant Htt toxicity.²⁴ Acetylation at lysine 444 reportedly facilitated trafficking of mutant Htt into autophagosomes, improved clearance of the mutant protein by macroautophagy, and reduced Htt toxicity in cell models and *C. elegans* models.²⁵ Finally, phosphorylation of Htt at serines 1181 and 1201 by cyclin-dependent kinase 5 (CDK5) induced by DNA damage was found to be protective against polyQ-induced toxicity.²⁶

A number of studies have identified several PTMs on Htt using recombinant proteins expressed in cell models.^{24,27–29} However, there has been considerably less attention toward the identification of such modifications in the context of the full-length normal and polyQ-expanded Htt endogenously expressed in vivo in HD mouse models and in HD human brain. Human post-mortem HD brain tissue is especially pertinent as a source for potential modifications relevant to the disease. Detection of PTMs in post-mortem brain material may present a challenge because these modifications may be labile and subject to reversal, especially phosphorylation. There has been little previous study of Htt PTMs in the human brain. Hayden et al. were able to detect endogenous phosphorylation of Htt at serine 421 in one human frontal cortex sample.³⁰

This study demonstrates that full-length endogenous Htt, purified by immuno-precipitation from HD mouse brain and from human post mortem brain, is suitable for the detection of PTMs by mass spectrometry (MS). We used prescreened well-preserved cases³¹ for our analysis to ensure the detection of modifications to the endogenous human Htt and to

minimize variability due to post-mortem autolysis. Using label-free and tandem mass tag (TMT)-based MS techniques, we identified 34 PTMs on the endogenous Htt, including 18 novel PTMs (10 serine and 1 threonine phosphorylation and 7 lysine acetylation sites). To further validate our findings and to address a potential role of Htt modifications in HD, we assessed and identified the changes in the PTM stoichiometry induced by the polyQ expansion in HD mouse model. MS identification and quantitation were verified using phospho-specific antibodies for selected PTMs. As a first step toward deciphering the PTM code of the full-length Htt, we introduced alterations to prevent these modifications. The goal was to establish whether amendment of a single PTM site could affect the overall functional properties of the full-length Htt protein, as manifested by a change in its subcellular localization. In the current study and in a parallel study (Arbez et al., manuscript submitted), we were able to identify several PTM sites that can modulate expanded Htt toxicity and its subcellular localization. Notably, these sites appear to cluster within predicted proteolytic domains between HEAT domains. Our studies validate PTMs on Htt as potential therapeutic targets for HD.

EXPERIMENTAL SECTION

Purification of Endogenous Htt from Mouse and Human Brain and Western Blotting

HD and normal control tissues were prepared using total cell homogenates from whole mouse brain (KI Q175 and WT controls at 6 months of age) or human superior frontal gyrus (500 mg of frozen brain tissue). This was accomplished 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. Lysates were precleared by incubating with Protein G-Sepharose beads (GE Healthcare) for 1 h at 4 °C, followed by incubation overnight at 4 °C with the primary anti-Htt antibody (MAB 2166, Millipore) to IP normal Htt or with MW1 polyQ-specific antibody (Hybridoma Bank, University of Iowa) to IP expanded Htt. These were then incubated with Protein G-Sepharose for 1 h at 4 °C. The IPs were washed three times with the lysis buffer, and Htt proteins were eluted from the beads with heating (80 °C) in 2× SDS Laemmli sample buffer (BioRad), followed by fractionation on NuPAGE 4–12% bis-tris polyacrylamide gels (PAGE, GE Healthcare). Htt protein bands were visualized with SimplyBlue Safe Stain (ThermoScientific). For Western blot analysis, aliquots (~10% of the sample) were fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies to Htt (MAB2166, N17), polyQ-specific antibody MW1, or phospho-specific antibodies to selected PTMs of Htt. N17-PO4 (to pS13/16) was a gift from Ray Truant.¹⁷ Huntingtin phosphorylation site-specific affinity purified rabbit polyclonal antibodies against four sites (S1181, S1201, S1864, and S1876) were generated by New England Peptide (Gardner, MA). In short, KLH-coupled peptides bearing the specific phosphorylated residue (pS1181-Ac-PSL(pS)PIRRKC-amide, pS1201Ac-ASVPL(pS)PKKGSC-amide, pS1864 Ac-CKRH(pS)LSSTKL-amide, and pS1876 Ac-CLSPQM(pS)GEE-amide) were injected into two rabbits each. Booster doses were given on days 14 and 28. Production bleeds were checked for antibody titer by ELISA using purified phosphopeptides. High-titer bleeds (40–50 mL) were subjected to a double-

affinity purification. The first step involved negative selection with corresponding nonphosphorylated peptide backbones, and the flowthrough was then subjected to a second cycle of purification (positive selection) using an affinity column with phosphorylated peptides. For Western blotting, immunoblots were incubated with peroxidase-conjugated secondary antibodies (GE Healthcare), and protein bands were visualized using enhanced chemiluminescence (ECL-Plus detection reagent, GE Healthcare) and a Molecular Imager Gel Doc XR System (BioRad).

Sample Digestion, Phosphopeptide Enrichment, and Mass Tag Labeling

Normal and polyQ-expanded Htt protein bands were cut out of the gel and subjected to in-gel digestion with trypsin, chymotrypsin, or Lys C, as previously described.³² For phosphopeptide mapping, 50% of the extracted peptides were enriched for phosphopeptides using titanium dioxide (TiO₂) according to Larsen et al.³³ Both enriched and nonenriched fractions were analyzed by tandem MS. For peptide quantification, extracted peptides were labeled with TMT reagents (ThermoScientific) according to the manufacturer's instructions. In brief, extracted peptides from each gel band were labeled with a unique isobaric tag by adding a 10-plex TMT reagent dissolved in 41 μ L of anhydrous acetonitrile at room temperature for 1 h. The reaction was quenched with 8 μ L of 5% hydroxylamine. After labeling, all samples were mixed, dried to a volume of 200 μ L, and fractionated into three fractions 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 analyzed by tandem MS.

Tandem Mass Spectrometry

Peptides before and after phosphopeptide enrichment or after bRP fractionation were loaded in 0.2% formic acid onto a C18 trap and separated by reverse-phase liquid chromatography 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), which was packed in house with Magic C18AQ (5 μ m, 120 $\text{\AA}$, www.unichrom.com). A gradient of 0–60% acetonitrile/0.1% formic acid was applied over 70 min at 300 nL/min on a NanoAcquity UPLC (Waters) interfaced with a Q-Exactive Orbitrap mass spectrometer (ThermoScientific). Eluting peptides were sprayed directly into the Q-Exactive at 2.0 kV. 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 collision energy based on dynamic exclusion times of 31 and 30 s. Precursor and the fragment ions were analyzed at 70 000 and 17 500 resolution, respectively.

Peptide Identification and Quantification

Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra extracted with and without deconvolution using the Thermo Scientific MS2 processor and Xtract software. Mascot software (Version 2.2 www.matrixscience.com/) interfaced with Proteome Discoverer 1.4 (<http://portal.thermo-brims.com/>) was used to identify and quantify peptides by searching the MS data against the Refseq mouse 2012 database (concatenated with the reverse database) using the following criteria: sample species; trypsin chymotrypsin or Lys C as the enzyme allowing one missed cleavage;

methionine oxidation, asparagine, and glutamine deamidation; serine, threonine, and tyrosine phosphorylation; and lysine acetylation as variable modifications. For TMT-labeled samples, cysteine methylthiomethane and 10-plex TMT on lysine and N-terminus were also included as fixed modifications. Peptides were identified with a false discovery rate of 1% as a confidence threshold based on a concatenated decoy database search. For quantification of the PTM stoichiometry, we developed a new procedure called Targeted Isobaric Mass Tag Analysis (TIMTA) analysis. This approach includes the use of TMT-labeled synthetic modified peptides (Table S1) added to the samples to increase the probability of detecting the peptides containing PTMs of interest. The procedure is described in detail in the Results section and is depicted in Figure 5. In the first step of data analysis for PTM quantification, reporter ion spectra with isolation interference 30% were excluded. Manual annotation of peptides with multiple PTMs was performed based on raw spectral data to select peptides identified by high quality spectra. Adjustments for different sample preprocessing and amounts of material loaded in the channels were carried out using a modified method from Herbrich et al.³⁴ In this algorithm, the log₂ reporter ion intensities for each spectrum were “median-polished”: The log₂ median reporter ion signal per sample (channel) was determined for all spectra matching peptide sequences in Htt. The global log₂ median was subtracted from the log₂ median observed for each sample to calculate the normalization factor per sample. Each sample’s normalization factor was applied to the reporter ion of each modified (phospho- and acetyl-) Htt peptide in that sample to create values normalized across all samples. To normalize for differences between reporter ions values from different spectra (for the same PTM), the linear ratios of the normalized reporter ion values were calculated for each sample. To assess the differences between modification states of specific sites, the statistical analysis comparing all eight samples’ normalized ratios between the HD and WT groups was performed (using SigmaPlot software). We used paired difference t-tests or a Mann–Whitney Rank sum test if the Shapiro–Wilk normality test failed. The analysis yielded both relative peptide fold changes and *p* values representing the statistical significance of differences between modification states of specific sites.

Plasmid Generation and Mutagenesis

Mammalian expression constructs encoding full-length Htt with the N-terminal PTM alterations (of S13, S16) were generated in two steps. As the first step, these alterations were introduced within a previously described Htt-N586-82Q (1–586) plasmid³⁵ (obtained from D. Borchelt). This was carried out using site-directed mutagenesis and the QuikChange II XL kit (Stratagene) according to the manufacturer’s protocol, except that Stbl2 competent cells (ThermoFisher Scientific) specifically designed for cloning of unstable inserts were used throughout this study. The presence of PTM mutations and the entire N586-82Q sequences were confirmed by sequencing. As the second step, the fragments comprising the first 171 amino acid (aa) residues of Htt with corresponding mutations were generated from the N586 constructs using digestion at 5′ flanking NotI and Htt-internal *Xho*I restriction sites. These N-terminal fragments were subcloned into a vector comprising the rest of the downstream sequence of full-length Htt. This vector was constructed by subcloning of the *Xho*I/*Sa*I downstream Htt fragment (of the original synthetic plasmid containing the entire full-length Htt-23Q) into the *Xho*I site of the pcDNA vector. The synthetic FL-Htt-23Q plasmid was generated (DNA 2.0) previously and was checked for expression of full-length

Htt protein in our lab. PTM alterations of T271, S421 and S434 were also introduced within an existing Htt-N586-82Q plasmid as described above. Next, the fragments containing N586-82Q with corresponding mutations were generated by PCR of the Htt-586-82Q constructs using the primers incorporating *KpnI* restriction sites on both 5' and 3' ends. Purified PCR products were digested with the enzyme and subcloned into a vector comprising the rest of the downstream sequence of the full-length Htt prepared upon digestion with *KpnI* using 5' flanking and Htt-internal *KpnI* sites. The rest of the PTM alterations (C-terminal of aa 586) were generated using a newly synthesized big C-term Htt fragment (downstream of aa 586) with incorporated convenient unique restriction sites (DNA2.0), which allowed for the isolation of five domains separated by these restriction sites. These domains were subcloned into the pJ cloning vectors (DNA2.0) and used to introduce alterations by site-directed mutagenesis and the QuikChange II XL kit (Stratagene). These mutagenized domains were subcloned into the big C-term Htt fragment using unique restriction sites (swapping with corresponding domains without mutations). In the final step, we reintroduced the N-terminal 586 aa fragment with polyQ into the mutagenized C-terminal fragment for each PTM alteration.

Subcellular Fractionation

Striatal STHdh neuronal progenitor lines generated in the MacDonald lab from E14 striatal primordia of wild-type (Q7) mouse embryos³⁶ were grown in DMEM (with 4.5 g/L D-glucose, Invitrogen) supplemented with 10% FBS, 100 µg/mL geneticin, 100 units/mL penicillin, and 100 units/mL streptomycin. Q7 cells were transfected with constructs encoding full-length Htt-82Q with and without PTM alterations using Lipofectamine 2000 reagent (ThermoFisher Scientific) according to the manufacturer's protocol. At 48 h after transfection, cells were harvested with trypsin-EDTA and collected by centrifugation at 500g for 5 min, and the cell pellet was washed with PBS. Cytoplasmic and nuclear fractions were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific) according to the manufacturer's protocol. Protein concentrations were estimated using the BCA method (Biorad).

For Western blot analysis, aliquots (15 µg of protein) were fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies to expanded Htt (MW1), cytoplasmic markers (β -tubulin, Santa-Cruz Biotechnology), and nuclear markers (Histone 1, Santa-Cruz Biotechnology). Protein bands were visualized using a Molecular Imager Gel Doc XR System and quantified using ImageJ software. The quantification results are presented as a ratio of nuclear to cytoplasmic mean intensity values ($\pm$ SEM) normalized to histone H1 (nuclear) and β -tubulin (cytoplasmic) as a loading control for each PTM alteration.

RESULTS

Purification of Endogenous Htt from Mouse and Human Brain and Detection of PTMs

To identify PTMs on the endogenous Htt, we purified Htt proteins from mouse (homozygous zQ175 knock-in mice and WT controls at 6 months of age), and from human HD and control brain using immunoprecipitation (IP). We have used one whole mouse brain or 500 mg of

human superior frontal cortex per sample (50 mg of total protein) to obtain enough material to detect Htt protein bands on the gel with protein stains following IP. The homozygous zQ175 knock-in mouse (KI Q175) model is derived from a spontaneous expansion of the CAG copy number in CAG 140 knock-in mice and has been characterized extensively.^{37–40} The zQ175 KI model shows early onset HD pathology (at 3 to 4 months of age), including brain atrophy, robust motor deficits, and electrophysiological and histopathological features. The age of 6 months was chosen based on the observed moderate mutant Htt aggregation in zQ175 mouse brain at this age, which increases dramatically by 12 months⁴⁰ and could potentially cause difficulties in the purification of soluble Htt proteins from the brains of older mice. Human frontal cortex tissues were selected from previously characterized well-preserved cases to ensure adequate detection of PTMs. Human tissue selection and quality control are described in detail in our previous publication.³¹

Using anti-Htt antibody (to amino acids 181–810, MAB 2166), we were able to pull down ~50% of Htt protein from WT mouse brain lysate. However, the recovery of expanded Htt from mouse HD brains with 2166 was poor (Figure S1A), although detection of normal and expanded Htt by Western blotting using this antibody was comparable. In contrast, MW1 antibody (polyQ-specific) pulled down ~50% of full-length expanded Htt from homozygous Q175 mouse brains (Figure S1B). On the basis of these results, 2166 antibody was routinely used to pull down normal Htt, and MW1 antibody was used for expanded Htt from mouse samples in all further experiments. MW1 antibody was also used to selectively pull-down expanded Htt from human HD brains for the downstream analysis of PTMs on expanded Htt, while 2166 antibody was used for control brains. Other antibodies tested for IP (exon1, 909, N17) did not produce an adequate yield of the full-length Htt and showed some nonspecific binding, which interfered with the clear separation of Htt band on stained SDS gels. For additional quality control, we also evaluated the preservation of phosphorylation signal using small aliquots of IP material and detection with anti phospho S13 and S16 antibodies (N17-PO4).

To prepare samples for MS, Htt proteins were eluted from the beads and fractionated on SDS-PAGE. The Htt bands were then visualized by Coomassie protein staining. Figure 1 shows examples of IPs of full-length Htt from mouse and human brain as well as HEK293 cells. Htt protein bands were cut out of the gel and subjected to in-gel digestion with trypsin, chymotrypsin, or Lys C. Next, 50% of each sample was used for titanium dioxide enrichment for phosphorylated peptides. The phosphorylated peptides in both enriched and nonenriched fractions were analyzed using nanoflow liquid chromatography ESI-MS/MS on an Orbitrap mass spectrometer. We have obtained ~70% Htt sequence coverage from human brain and 80% coverage from mouse brain, with 350–550 unique peptides identified in a typical experiment. For confirmation of some low stoichiometry sites, we also used human Htt expressed in HEK293 cells, where the coverage of the Htt sequence was up to 99% with ~2000 unique peptides identified. As a result, we identified 34 phosphorylation and acetylation sites on either mouse or human (or both) endogenous Htt, including at least 18 novel sites (Table 1). There were four additional PTMs that were only detected on the full-length Htt expressed in HEK293 cells.

Table 1 presents a summary of PTMs that were detected in a number of MS experiments from at least four different HD cases and matching normal controls and from four HD and eight WT mouse brains. Initial PTM identifications were performed using label-free MS with or without enrichment for phosphorylated peptides. The identification of some low-abundance PTMs (low-confidence sites) was confirmed using TMT-labeling methods, with further validation using a targeted approach with synthetic peptides containing the PTMs of interest (see Table 1 and below).

One PTM site that has been studied extensively in cell and mouse HD models is serines 13 and 16 (S13/S16) phosphorylation,^{4,16–19} but no spectral evidence of endogenous Htt modification at this site has been previously reported. In our initial experiments using trypsin, we were unable to detect phosphorylation at this site *in vivo* because trypsin digestion does not yield any peptides within the N-terminal 17 amino acids that are suitable for identification. A short peptide, ES₁₃LKS₁₆F, was predicted to arise from chymotrypsin digestion, but it was also not detected using shotgun MS with DDA. To confirm that phosphorylation at this important site occurs *in vivo*, we targeted this peptide's calculated *m/z* value for MS analysis and compared the fragmentation spectra with those of a synthetic diphosphopeptide standard, EpSLKpSF (Figure 2). This approach confirmed the presence of this diphosphopeptide in a chymotryptic digestion of endogenous mouse Htt. As shown in Figure 2A, the standard diphosphopeptide elutes off our reverse-phase column at 25.6 min as a doubly charged ion at 435.66 *m/z* (right panel). Within 12 s of the same time (25.4 min), a low-intensity 435.66 *m/z* doubly charged ion was detected from a separate MS analysis of a chymotryptic digest of endogenous mouse Htt (left panel). The fragmentation spectra of the standard peptide were manually compared with the spectra of the peptide (435.66 ± 0.95 Da) isolated from the endogenous sample (Figure 2B, top panel). The result shows a virtually identical fragmentation pattern in both the fragment ion masses and the relative intensities. The additional ions in the endogenous peptide's fragmentation spectrum are due to interference from another coeluting peptide, close in mass to the 435.66 ion, that was captured in the ±0.95 isolation window (Figure 2A).

We have also established the first MS-based confirmation of the phosphorylation of serine 421 (S421) occurring *in vivo*, which we detected consistently in human brain (Table 1 and Figure 3A). However, in mouse brain, modification at this site was only detected using a targeted approach with synthetic peptide. The most prevalent phosphorylation of mouse endogenous Htt in this region appears to be at serines 432 and 434 (S432 and S434, Figure 3C), with multiple supporting spectra present in each experiment (Table 1). Figure 3B shows an example of MS/MS tandem mass spectra identifying a novel human-specific Htt phosphorylation of serine 2342.

We also identified several novel lysine acetylation sites on endogenous Htt from mouse and human brain. To identify acetylation sites with confidence, we applied additional criteria, such as the presence of acetyllysine immonium ions (*m/z* 126.1 or 143.12) in the spectra and a delayed retention time for acetylated peptides due to increased hydrophobicity relative to unmodified peptide present in the sample. Figure 4 illustrates the identification of two novel lysine acetylation sites found in human brain. More spectral evidence of PTM identification is shown in the Supporting Information.

Out of 34 PTMs identified on the endogenous Htt, 28 sites were found in mouse brain, while only 23 sites were identified in human brain (including five sites detected only using the targeted approach, Table 1). Although previously we did not observe any obvious correlation between the post-mortem delay and the integrity of Htt protein in brain tissues analyzed,³¹ the discrepancy between mouse and human PTM identifications may be due to the temporary nature of modifications and their potential reversal during post-mortem autolysis of human brain tissues. However, there were several PTMs detected more robustly (with more identifying spectra and in several samples) in human brain, and some were not detected in mouse tissues at all, for example, S421 phosphorylation. Notably, most PTMs detected from either species were present on both normal and polyQ-expanded Htt. These observations suggest that differences in the PTM stoichiometry between HD and the control are likely to be subtle and need to be evaluated using quantitative MS methods.

Stoichiometry of PTMs on Htt Is Different in HD and WT Mouse Brain

A high degree of conservation in PTMs between mouse and human Htt (Table 1) validates the use of a mouse model for the relative quantitation of the stoichiometry of PTMs. To determine whether polyQ expansion leads to changes in PTM stoichiometry in homozygous KI Q175 HD mouse, we developed a new method for relative quantification of PTMs on normal and expanded Htt, which we term targeted isobaric mass tag analysis (TIMTA, Figure 5). The method is based on stable isotope labeling using TMT reagents. Discovery proteomics usually relies on nonsupervised DDA. This strategy is focused on protein identification but has limitations with respect to quantitative applications⁴¹ because low-abundance modified peptides may be missed during MS analysis due to poor ionization or poor fragmentation. The targeted data acquisition (TDA), where the preselected peptides are used as surrogates and are consistently measured across a multitude of samples, have been used previously to overcome the under-sampling issue of DDA and to address the bias toward the most abundant components.⁴² In this study, to increase the sensitivity of detection for PTMs of interest to provide robust spectral evidence of PTM quantification, we included TMT-labeled synthetic modified peptides in amounts that are readily detected by DDA. The corresponding low-abundance endogenous peptides in our brain samples (labeled with different TMT tags) are coisolating with the synthetic peptides and generate reporter ions for relative quantification. Similar strategies utilizing spiked-in internal standard peptides have been used previously to enable robust sampling of target peptides detected by TDA.^{41,43,44} Our method, which uses a DDA approach, also allows us to acquire data for the quantitation of the off-target peptides, thus enriching the data set. This approach can also detect the changes in targeted peptides due to different sets of modifications, which are not included in the spiked standards. Another feature of directed MS method described here is the use of an inclusion list, compiling the precursor ions of interest, which is loaded into the computer controlling the mass spectrometer to ensure that the instrument reproducibly selects for the ions with relevant attributes, such as the precursor-ion charge state, m/z ratio, and retention time.⁴² For our targeted experiments, we have selected a group of PTMs that are consistently detected in mouse and human brain and have been confirmed by high confidence (1% FDR) spectral data. Because we know the exact m/z mass, retention time, and fragmentation patterns of these synthetic peptides, we were able to selectively target these masses (inclusion list for data acquisition), as well as interpret their fragmentation spectra. Table S1

shows the complete list of synthesized modified peptides used to target these sites in mouse brain.

We have conducted six TMT experiments targeting PTMs on mouse Htt using four homozygous KI Q175 and four WT mouse brains for each experiment. Endogenous Htt proteins were purified from the whole mouse brains as described above, using 2166 antibody to pull down normal Htt and MW1 antibody for expanded Htt. Because neither antibody was raised against a specific PTM, therefore, although we cannot exclude a potential bias due to preferential affinity toward a certain PTM form by either antibody, it is only a slight concern. The gel bands containing Htt were digested with trypsin and subjected to quantitative MS using TMT labeling. All eight samples were processed in one 10-Plex TMT experiment, including one channel used for synthetic peptides, corresponding to mouse endogenous PTMs spiked into the samples (Table S1 and Figure 5). The quantitative MS data underwent the normalization process described in the Experimental Section to normalize for differences in the amount of Htt per sample and for differences between reporter ion values from different spectra for the same PTM. The statistical analysis comparing all eight samples' normalized ratios between the HD and WT groups yielded both relative peptide fold changes and *p* values representing the statistical significance of differences between phosphorylation and acetylation states of specific sites. Significant differences between HD and WT mouse tissues were most notably found for phosphorylation of serines 1181 and 1201 (S1181 and S1201), which appear to be increased in HD, and acetylation of lysines 818, 1204, and 1246 (K818, K1204 and K1246), as well as phosphorylation of serine 1876, significantly decreased in HD mouse brain, relative to control (Table 2).

Validation of MS PTM Identification and Quantitation with Phospho-Specific Antibodies

To further characterize and validate the PTMs identified on the endogenous Htt *in vivo*, we generated a series of phospho-specific peptide antibodies to the selected PTMs. The generation and characterization of these antibodies are described in detail by Shin et al. (manuscript in preparation). The specificity of the antibodies is demonstrated in Figure S2: HEK293 cells were transfected with the FL-Htt constructs with and without phospho-null alterations. Only very minimal cross-reactivity of the corresponding antibodies was observed with these Htt mutants.

For detection of Htt phosphorylation in mouse brain, endogenous Htt was pulled down from homozygous KI Q175 and WT mouse brains as described above, and PTMs were detected by Western blotting using phospho-specific antibodies to pS1181, pS1201, pS1864, and pS1876. As shown in Figure 6, we were able to confirm the presence of phosphorylation at these sites. Consistent with our MS-based quantification, a significant increase in the phosphorylation signal for S1181 and S1201 was observed in the brains of HD mice versus WT animals. In contrast, the stoichiometry of S1876 phosphorylation was significantly decreased in HD, while S1864 phosphorylation appeared to be similar in HD and WT brains. Although it is difficult to estimate the exact levels of total normal and polyQ-expanded Htt pulled down from mouse brains (because of potentially different reactivity of the antibodies), these levels appear comparable in HD and WT mice based on the detection

of total Htt using Htt-specific antibody (2166 MAB). The results demonstrate a good correlation between MS-based quantitation and Western blotting analysis (Figure 6E).

Subcellular Localization of Expanded Htt Is Modulated by PTMs

Modifications within the N-terminus can affect the subcellular localization of Htt.^{14,16–19,45,46} In addition, phosphorylation of Htt at serine 421 increased Htt colocalization with microtubular motors,²² while acetylation at lysine 444 was shown to facilitate trafficking of mutant Htt into autophagosomes.²⁵ To further validate the MS-based identification of PTMs and investigate the effects of more C-terminal PTMs on the Htt cytoplasmic/nuclear distribution, we introduced mutations that prevent modifications of the amino acids that we have found to have different PTM stoichiometry in HD versus WT mouse brain (Table 2). Because Htt is a very large protein, it was not possible to introduce mutations within the full-length Htt in one step. Therefore, we used a multistep strategy to generate PTM alterations throughout the full-length Htt (see Experimental Section). The high degree of conservation in PTMs between mouse and human Htt (Table 1) supports the use of mouse striatal neurons for the assessment of PTMs. Full-length Htt expression constructs with PTM alterations were transfected into mouse neuronal progenitor cells STHdh Q7/Q7, and cytoplasmic and nuclear fractions were prepared. We compared the amount of soluble Htt retained in each fraction based on the reactivity with polyQ-specific MW1 antibody (Figure 7 and Figure S3). The data are presented as the ratio of nuclear to cytoplasmic Htt levels, which may reflect an increase or decrease in nuclear localization or a variation in Htt levels in the cytoplasm due to altered degradation or aggregation. We have included some of the previously identified PTMs within the N-terminus of Htt (such as S13/S16 and S421 phosphorylation). Although extensively studied, these sites have not been previously characterized in the context of full-length Htt. Consistent with a previous report that used N17-Htt protein,¹⁷ we found an increase in nuclear localization caused by phosphomimetic S13S16D alteration within full-length Htt, while S13S16A mutation slightly but significantly increased cytoplasmic Htt. Several other PTM alterations significantly influenced cytoplasmic and nuclear distribution of Htt. The most striking increase in nuclear localization was observed for S1201A, presumably due to phospho-null alteration.

DISCUSSION

In view of the structural complexity, intramolecular interactions, and potential PTM crosstalk within the huge Htt protein, it is essential to characterize and study PTMs of Htt in the context of the full-length protein. Identification of PTMs on endogenous Htt expressed in vivo in HD mouse and human brain is a unique and challenging approach, which has potential to uncover the most relevant mechanisms and markers for HD. In neurodegenerative diseases, phosphorylation of disease proteins such as α -synuclein has been shown to play an important role in disease pathogenesis.⁴⁷ Another protein modification that has been studied extensively in human postmortem material is the phosphorylation of microtubule-associated protein tau, which is well known to be hyperphosphorylated in Alzheimer's disease and related disorders.⁴⁸ Recently, TDP-43 phosphorylation has also been documented in human post-mortem brain material.⁴⁹

Among the important findings of the current study is the first spectral evidence of the presence of S13/S16 and S421 phosphorylation of Htt in vivo. These are by far the best-characterized PTMs on Htt, which were confirmed by MS in cell models.^{16,24} Their potential role in HD pathogenesis has been demonstrated by introducing phospho-mimetic and phospho-null mutations in HD mouse models.^{4,5} However, there has been a lack of direct MS evidence of the occurrence of these modifications in mouse or human brain. Comparison of the PTMs detected on mouse and human endogenous Htt demonstrates a high degree of conservation, although some sites do appear to be robustly detected in only one species (Table 1), which may be an indicator of different stoichiometry in mouse and human brain. For example, S421 phosphorylation was detected consistently in human brain, but this site appears to have low stoichiometry in mouse brain because it was only detected using a targeted approach with synthetic peptide. Thus S421 phosphorylation, shown previously to be important for Htt function and HD pathogenesis, may represent an example of human-specific PTM-mediated regulation. A recent report⁵ demonstrates that phosphorylation at this site regulates Htt toxicity in BACHD mouse model, which expresses human HTT gene with S421 mutations; however, it is hard to account for the contribution of human HTT gene context toward S421-mediated HD pathology in these mice. Thus it would be important to further assess the role of phosphorylation at this site using human HD models, such as HD iPS cells.

We have discovered 18 novel PTMs on the endogenous Htt, including several novel lysine acetylation sites (Table 1). Acetylation at lysine 444 has previously been implicated in the regulation of Htt by macroautophagy, and it reduced Htt toxicity in HD models.²⁵ However, this site has only been documented by MS on recombinant Htt expressed in cell models.^{25,50} We were able to confirm the presence of this modification on the full-length Htt expressed in HEK293 cells but failed to detect it in vivo (Table 1). In addition, we identified seven novel lysine acetylation sites on the endogenous Htt, including five sites detected in the human brain. Alterations in several of these PTMs have been shown to modulate mutant Htt toxicity (Arbez et al., submitted). Several of the newly discovered acetylation sites are positioned next to previously characterized functional phosphorylation sites; for example, acetylated lysine 1190 lies in between serines 1181 and 1201, and phosphorylation at these sites was found to be protective against polyQ-induced toxicity.²⁶ These various modifications positioned near each other provide a potential for PTM crosstalk in the regulation of Htt function and modulation of expanded Htt cellular toxicity.

Overall, we have identified around three dozen PTMs on the endogenous Htt from mouse and human HD brain (Table 1). However, our current capacity to detect PTMs far exceeds our ability to understand their biological function and their role in HD pathogenesis. As a further step in this direction, we have employed a newly developed quantitative MS approach to compare the PTM stoichiometry in HD versus normal mouse brain. We detected significant differences in several targeted PTMs (Table 2), although understanding the significance of these variations for HD pathogenesis will require considerable further study. In fact, an increased stoichiometry of modification at a specific site may reveal an important alteration that drives HD pathogenesis, or it may reflect a potential compensatory change.

For further functional assessment of these sites, we have tested the effects of PTM alterations on expanded Htt subcellular localization and found a few modifications that likely affect the cytoplasmic and nuclear distribution of Htt. Htt modifications within the N-terminus have been previously shown to regulate its subcellular localization.^{14,16–19,45,46} We found that the most dramatic redistribution of Htt to the nucleus was induced by S1201A phospho-null alteration (Figure 7). In addition to increased nuclear localization of the S1201A mutant, we observed a protective effect of this alteration in cortical neurons expressing expanded Htt (Arbez et al., manuscript submitted). The protective effect of the S1201A alteration, that induces nuclear localization, is intriguing because previous evidence highlights the nucleus as the main site of Htt-induced cell toxicity. However, more recent studies from Truant's group demonstrate that N17-phosphorylated Htt localizes to chromatin and plays a vital role during the normal stress response, which seems to be impaired in HD.^{17,18} S1201 also appears to be hyperphosphorylated in HD mouse, as determined using both quantitative MS and phospho-specific antibody (Table 2 and Figure 6). On the basis of these data, our current hypothesis is that prevention of phosphorylation of expanded Htt at this site (by S1201A alteration) promotes nuclear localization of Htt by an unknown mechanism, which may facilitate Htt-mediated stress-response and thus may be beneficial in HD. Further studies are warranted to test this hypothesis.

The crosstalk between PTMs in proteins regulates various protein functions and cellular signaling.^{51,52} The complexity of studying PTM code stems from its dynamic nature, with different PTM combinations present on the same protein depending on its subcellular localization or associated with the activation of certain cellular pathways. The range of functional interaction between different PTMs within one protein or within multiprotein complexes is largely determined by protein structure, which is, in turn, essentially determined by PTMs. The 3D structure of Htt has not been resolved: Modeling based on the other members of the HEAT/HEAT-like repeat family (huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1) suggests that the protein contains multiple α -helical HEAT domains interspersed with more flexible unstructured domains. This secondary structure and the large number of protein interacting partners for Htt have led to suggestions that the Htt protein may function as a multiprotein scaffold for the assembly of protein complexes involved in many cellular functions, including axonal transport.^{53–55} Sequence-based structural predictions (<http://emboss.bioinformatics.nl/cgi-bin/emboss/pepfind>) position the proteolysis-prone PEST domains (regions enriched in proline, glutamic acid, serine, and threonine) in Htt between HEAT repeat domains, where several currently known caspase and calpain recognition sites in Htt are located.⁵⁶ The notion that most of the reported PTMs of Htt have been found in these PEST domains was first suggested by Hayden.¹² Since then, several new PTMs on Htt have been identified, including those in the current study. As shown on Figure 8, the majority of the PTMs detected on the endogenous Htt in our study are located between HEAT repeats, with many positioned within protease-sensitive domains

Seong's group recently reported additional insights into the structure of Htt molecules.⁵⁷ Using electron microscopy and biophysical methods, this study found that Htt can adopt a spherical α -helical solenoid shape with the N-terminal and C-terminal regions folding together in close proximity to form a confined central cavity. Cross-linking MS analysis

revealed multiple intramolecular contacts within full-length Htt, including short-range and midrange interactions along with long-range contacts between the C-terminal and N-terminal residues. Physical interaction between the N-terminal (1–586) and the C-terminal (587–3144) fragments of Htt has also been reported in transfected cells and human striatum.⁵⁸ In a parallel study (Arbez et al., manuscript submitted), we found several clusters of PTMs within the full-length Htt, which modulate its functional or toxic properties. Notably, it appears that the clusters that mostly affect Htt properties are grouped within predicted protease-sensitive regions near multiple short- and midrange intramolecular contacts that are shared by the normal and extended Htt, as defined by cross-linking MS analysis.⁵⁷ This supports the importance of these regions for internal folding of Htt itself as well as likely providing interfaces for binding other proteins. To conclude, the findings of our current study will be instrumental in further assembly of the Htt PTM framework. Most importantly, such “hot spots” within expanded Htt are promising targets for the development of HD therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by CHDI Foundation, Inc., a nonprofit biomedical research organization exclusively dedicated to develop therapeutics that will substantially improve the lives of HD-affected individuals. T.R and C.A.R. also received support from NINDS (5R01 NS076631). We thank Olga Pletnikova and Juan C. Troncoso (Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD) for assistance with human brain dissections and Marcy MacDonald (Massachusetts General Hospital, Boston, MA) for helpful discussions. We also thank Ray Truant (McMaster University, Hamilton, Ontario, Canada) for N17-PO4 antibody.

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Figure 1.

Purification of endogenous FL-Htt for mass spectrometry from mouse (A) and human (B) brain and from HEK293 cells (C). Total cell lysates from frozen whole brains of normal WT controls or homozygous KI Q175 mice (A), from superior frontal gyrus (500 mg of frozen brain tissue) of normal controls and HD cases (B), or from HEK293 cells transfected with normal and polyQ-expanded Htt (C) were prepared as described in the Experimental Section. Htt proteins were immunoprecipitated with either anti-Htt 2166 or with anti-polyQ MW1 antibodies, as indicated, and fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels. Representative preparative gels stained with SimplyBlue Safe protein stain for control

and HD tissues are shown on left panels. Htt bands (marked with arrows) were cut-out from the gel and subjected to MS analysis. Small aliquots of IP material were analyzed by Western blotting with antibodies to Htt (MAB2166, polyQ-specific MW1, and N17-PO4 antibody to phosphorylated S13/S16).

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Figure 2.

Detection of S13/S16 phosphorylation in vivo. (A) Precursor ion spectra of diphosphopeptide ES₁₃-phosphoLKS₁₆-phosphoF, produced by chymotryptic digest of endogenous Htt purified from mouse brain (left) and standard diphosphopeptide of the same sequence (right). (B) Fragmentation spectra of the endogenous (top) and standard (bottom) diphosphopeptide ES₁₃-phosphoLKS₁₆-phosphoF. Experiment was replicated two times.

A, S421-phospho, Htt from human brain

B, S2342-phospho, Htt from human brain

C, S432, S434-phospho, Htt from mouse brain

Figure 3.

Examples of serine phosphorylation sites identified by MS. (A) ESI-MS/MS tandem mass spectra of phosphorylated peptide SGS₄₂₁-phosphoIVELIAGGGSSCSPVLSR produced by tryptic in-gel digestion of endogenous Htt purified from human brain, followed by MS/MS. (B) Tandem MS/MS spectra of phosphorylated peptide AIS₂₃₄₂-phosphoEEEEVEVDPTNQNP, produced by tryptic in-gel digestion of endogenous Htt purified from human brain, followed by MS/MS. (C) ESI-MS/MS tandem mass spectra of phosphorylated peptide SGSIVELLAGGGSSC-S₄₃₄-phosphoPVLRS, produced by tryptic in-gel digestion of endogenous Htt purified from mouse brain, followed by MS/MS. The tables of predicted

fragmentation ions show blue and red highlighted masses present in the spectra within 0.03 Da, as shown in the lower panels below each table. Four HD and eight WT mouse brains were used for MS identification, and the experiment was repeated three times. For human brain four HD and four control cases were used, and the experiment was repeated three times with separate brain preparations.

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A, K815-Ac, Htt from human brain RT 12.90 **Corresponding unmodified peptide, RT 11.90** **B, K1190-Ac, Htt from human brain RT 21.19** **Corresponding unmodified peptide, RT 18.81** **Figure 4.**

Two novel Htt acetylation sites were identified by MS. ESI-MS/MS tandem mass spectra of acetylated peptides K₈₁₅-Ac-TLKDESSVTCK (A) and E_{K1190}-Ac-EPGEQASVPLSPK (B), produced by tryptic in-gel digestion of endogenous Htt purified from human brain, followed by MS/MS. Acetyllysine immonium ions (*m/z* 143.12), confirming acetylation of K815 and K1190, were present in both spectra. On the right panels, the ESI-MS/MS tandem mass spectra of the corresponding unmodified peptides of the same sequence showing similar ion series are shown. Delayed retention time (RT) for acetylated peptide due to increased hydrophobicity was observed. The tables of predicted fragmentation ions show blue and red

highlighted masses present in the spectra within 0.03 Da, as shown in the lower panels below each table. For MS identification, four HD and four control cases were used, and the experiment was repeated three times with separate brain preparations.

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Figure 5.

Targeted isobaric mass tag analysis (TIMTA) procedure. Optimized TMT (131)-labeled standard peptide mix is added to the labeled peptides produced by tryptic in-gel digestion of endogenous Htt purified from mouse brain.

Figure 6.

Validation of MS PTM identification and quantitation with phospho-specific antibodies. (A–D) Total cell lysates from frozen whole brains of normal WT controls or homozygous KI Q175 mice were prepared as described in the Experimental Section. Htt proteins were immunoprecipitated with either anti-Htt (2166 MAB) or anti-polyQ MW1 antibodies, as indicated, fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels, and analyzed by Western blotting with indicated phospho-specific antibodies to Htt. The blots were stripped and reprobed with 2166 MAB for estimation of total Htt levels. The experiment was repeated three times with three different groups of 4 HD and 4 WT mouse brains, yielding

similar results. Representative images are shown. Graph shows the mean intensity values (+ SEM) of phospho-Htt normalized to total Htt (as detected with 2166 antibody) for each PTM analyzed. Significant changes are shown with asterisk, $p < 0.05$ HD vs WT, $n = 3$ independent experiments. (E) Comparison of the fold change of PTM stoichiometry in HD versus WT obtained using quantitative MS (TMT) and Western blotting.

Figure 7.

Subcellular localization of expanded Htt is modulated by PTMs. (A) Striatal STHdh Q7/Q7 cells³⁶ were transfected with indicated constructs encoding full-length Htt-82Q with and without PTM alterations. 48 h after transfection, cytoplasmic and nuclear fractions were prepared as described in the Experimental Section, fractionated on NuPAGE 4–12% bis-tris polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies to expanded Htt (MW1) and cytoplasmic (β -tubulin) and nuclear (Histone 1) markers. Two representative gels are shown. The panels were assembled from the different parts of the same blot, as indicated by vertical lines. (B) Graph shows the ratio of nuclear to cytoplasmic mean intensity values (+ SEM), normalized to histone H1 (nuclear) and β -tubulin (cytoplasmic) as a loading control, for each PTM alteration analyzed. Significant changes are shown in red, $p < 0.05$ versus Htt 82Q, $n = 3$ independent experiments.

Figure 8.

PTMs are located in clusters between or in the periphery of predicted HEAT repeats. PTMs, which were consistently detected by mass spectrometry on the endogenous Htt from either human or mouse (or both) brain, are shown. Positions of HEAT domains are indicated (adopted from Warby et al., 2008).⁵⁶ Phosphorylation sites are shown in red; acetylation sites are shown in green.

Table 1

PTM Sites Found on Endogenous Htt from Mouse and Human Brain and on Human Htt Transfected in HEK293 Cells^a

PTM site	Mouse brain					Human brain					HEK293
	ID with label-free MS, spectral counts (samples)	Corresponding peptide without PTM, spectral counts	ID with TMT, # samples	Targeted with synthetic peptides	Confirmed with synthetic peptide	ID with label-free MS, spectral counts (samples)	Corresponding peptide without PTM, spectral counts	ID with TMT, # samples	Targeted with synthetic peptides	Confirmed with synthetic peptide	
S116	–	9	8	yes	+	–		–	yes	–	
S116 and S120	–		8	yes	+	–		–	yes	–	–
T271	2(2)	42		yes	+	4(3)	90		yes	+	+
S419	–		–	no		–		8	no		
S421	–	22	–	yes	+	19(7)	47		yes	+	+
S431	5(2)			no		–		–	no		
S432	4(4)			yes	+	–		–	yes	+	+
S434	25(10)			no		–		–	no		+
S421 and S434	–		8	no		–		–	no		+
K444	–		–	no		–		–	no		+
	–	16	16	no		6(6)	44		yes	+	–
	2(1)		yes	+	1	10		no		+	
	4(4)	56		no		–		8	no		
	16(7)		yes	+	2(2)	68		yes	+	+	
S1181	3(3)	61		yes	+	23(13)	149		yes	+	+
S1201	26(7)	75		yes	+	–			no		+
K1190 and S1201	–		8	yes	+	–		–	no		+
	–		–	no		–		–	no		+
	4(2)	0		yes	+	3(3)	69		no		+
	1	4		yes	+	–		–	yes	+	+
	2(2)	16	16	no		3(3)	5	8	no		+
S1866	–		8	no		–		–	no		+
S1872*	*			no		1			no		+
K1875* and S1876	–	46	16	yes	+	*			no		

PTM site	Mouse brain					Human brain					HEK293
	ID with label-free MS, spectral counts (samples)	Corresponding peptide without PTM, spectral counts	ID with TMT, # samples	Targeted with synthetic peptides	Confirmed with synthetic peptide	ID with label-free MS, spectral counts (samples)	Corresponding peptide without PTM, spectral counts	ID with TMT, # samples	Targeted with synthetic peptides	Confirmed with synthetic peptide	ID with label-free MS
S1876	20(7)			yes	+	92(15)	173		yes	+	+
	—		24	no		—		—	no		
	—	31	16	yes	+	—		—	yes	+	—
1	24		yes	+	—		—	yes	+	+	
S2116	*			no		14(5)	1		no		
	*			no		18(12)	211		yes	+	+
	1	5		no		—		—	no		—
	1	8		yes	+	2(2)	24		no		+
	—			no		—		—	no		+
	*			no		—		—	no		+
S2653	—		—	no		—		—	no		+
S2653 and 2657	1	0	8	no		5(3)	7	16	no		+
	—	51	8	no		—		—	yes	+	
	1		8		—		—	no		+	
	—		8			—		—	no		—
	1		8	no		—		—	no		
	1		16	no		*			no		
	—			yes	+	3(3)	34		yes	+	+

²Novel sites are shown in red. PTMs identified with high confidence are in green cells, those with limited spectral evidence are in yellow, and those with no spectral evidence are in pink. PTMs confirmed in vivo are in bold, and those confirmed in HEK293 cells only are in italic. Spectral counts obtained for PTM identifications (and samples) are indicated. Spectral counts obtained for the corresponding peptide with unmodified residue of interest are also included for qualitative comparison of PTM stoichiometries. However, trends in stoichiometric differences need validation because modified peptides have different ionization efficiencies and elution times from their corresponding unmodified peptides and may coelute with different peptides that produce variable signal suppression. Amino acid numbers are reported based on human HTT sequence with 23 glutamines (NCBI Reference Sequence: NP_002102.4).

* represents amino acids only present in either mouse or human Htt due to differences in sequence.

Table 2

PTM Stoichiometry Is Significantly Changed in HD Mouse Brain versus WT^a

Exp	K818		S1181		S1201		K1204		K1246		S1876	
	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL
1	ND		1.33	0.136	ND		0.21	<0.01	0.64	<0.001	ND	
2	ND		1.27	0.083	ND		ND		0.711	0.001	ND	
3	ND		1.39	<0.001	1.24	0.01	ND		ND		0.66	0.03
4	0.88	<0.001	1.2	0.031	0.93	0.1	0.83	0.2	0.87	0.042	0.87	<0.001
5	0.79	0.042	1.5	<0.001	1.7	0.008	0.85	0.044	1.3	0.15	1.25	0.17
6	0.57	<0.001	1.25	0.016	ND		0.66	0.001	ND		0.75	0.019

Exp	S116		T271		S432/434		K1190		K1875		K2969	
	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL
1	ND		0.84	0.33	1.3	0.06	ND		ND		ND	
2	0.87	<0.01	0.97	0.3	1.18	0.132	ND		ND		ND	
3	0.93	0.17	0.95	0.45	1.57	0.05	1.2	0.2 *	ND		ND	
4	0.84	0.84	0.81	0.024	0.85	0.1	0.74	0.02 *	0.84	0.07	0.93	0.53
5	ND		ND		0.94	0.16	1	0.76	0.78	0.87	0.89	0.22
6	ND		ND		0.9	0.2	0.74	<0.001	0.69	0.06 *	0.86	0.012

Exp	K1190-S1201		S1864		S2116		S2114	
	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL	HD/WT	P-VAL
1	ND		ND		ND		0.45	0.065 *
2	ND		ND		2.22	0.06	0.85	0.8
3	ND		ND		ND		0.7	0.2 *
4	1		ND		1		0.81	0.93
5	0.71	0.04	ND		0.74	0.18	0.91	0.23
6	1.49	0.047	0.75	<0.001	0.63	0.015	0.75	0.008

^aRatio of HD to WT is shown. Each line represents one TIMTA experiment. ND, the peptide was not detected.

* represents low statistical power. Significant changes are shown in color: PTMs that were found more abundant in HD in more than one experiment are shown in bright red, and PTMs that were more abundant in WT brain in more than one experiment are in dark green (top panel). Lighter shades of red and green indicate PTMs that showed trend in most experiments but significance in only one experiment (middle panel). Very light shades (bottom panel) indicate inconsistent results due to limited detection.