Phosphorylation of huntingtin at residue T3 is decreased in Huntington’s disease and modulates mutant huntingtin protein conformation

Cristina Carilo1,*, Lucia Azzolinib,*, Margherita Veranib, Paola Martufib, Roberto Boggioa, Anass Chikib, Sean M. Dequierec, Marta Cherubinid,e, Silvia Ginesed,e, J. Lawrence Marshf, Paola Conforthb, Elena Cattaneobh, Iolanda Santimonef, Ferdinando Squitiere, Hilal A. Lashulecf,2, Lara Petricca2,3, and Andrea Caricasolea,b,3

1Department of Neuroscience, IRBM Science Park, 00071 Pomezia, Rome, Italy; 2IRBM Promidia, 00071 Pomezia, Rome, Italy; 3Laboratory of Molecular and Chemical Biology of Neurodegeneration, Brain Mind Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland; 4Department of Biomedicine, Facultad de Medicina, Instituto de Neurociencias, Universidad de Barcelona, 08035 Barcelona, Spain; 5Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain; 6Department of Developmental and Cell Biology, University of California, Irvine, CA 92697; 7Laboratory of Stem Cell Biology and Pharmacology of Neurodegenerative Diseases, Department of Biosciences, University of Milan, 20122 Milan, Italy; 8Istituto Nazionale Genetica Molecolare (INGM) Romeo ed Enrica Invernizzi, Milan 20122, Italy; and 9Huntington and Rare Diseases Unit, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Casa Sollievo della Sofferenza, 71013 San Giovanni Rotondo, Italy

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Posttranslational modifications can have profound effects on the biological and biophysical properties of proteins associated with misfolding and aggregation. However, their detection and quantification in clinical samples and an understanding of the mechanisms underlying the pathological properties of misfolding- and aggregation-prone proteins remain a challenge for diagnostics and therapeutics development. We have applied an ultrasensitive immunoassay platform to develop and validate a quantitative assay for detecting a posttranslational modification (phosphorylation at residue T3) of a protein associated with polyglutamine repeat expansion, namely huntingtin, and characterized its presence in a variety of preclinical and clinical samples. We find that T3 phosphorylation is greatly reduced in samples from Huntington’s disease models and in Huntington’s disease patients, and we provide evidence that bona-fide T3 phosphorylation alters huntingtin exon 1 protein conformation and aggregation properties. These findings have significant implications for both mechanisms of disease pathogenesis and the development of therapeutics and diagnostics for Huntington’s disease.

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Significance

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C.C. and L.A. contributed equally to this work.

L.P. and A. Caricasole contributed equally to this work.

To whom correspondence may be addressed. Email: hilal.lashuel@epfl.ch, l.petrice@irbm.it, or a.caricasole@irbm.it.

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of the N-term region of HTT (23–25). Interestingly, regions flanking the polyQ domain, including the first 17 N-terminal amino acids of HTT (N17) and a polyproline-rich C-terminal to the polyQ region, have been shown to influence the propensity of short N-term HTT fragments to misfold and aggregate (26–30). Recent studies demonstrated that phosphomimetic mutations at specific residues (S13/S16) within the N17 region can modulate the subcellular localization, stability, aggregation, and toxicity properties of mutant HTT in different preclinical models (29, 31–33), raising interest in these PTMs as possible modifiers of mutant HTT biological and biophysical properties. Significantly, studies on HTT exon 1-like peptides bearing phosphorylated S13/16 residues or S/D mutations at the same residues demonstrated substantially reduced aggregation in vitro compared with the unphosphorylated or wild-type (WT) counterparts, suggesting that N17 phosphorylation modulates the capacity of mutant HTT N-term fragments to self-assemble and aggregate (34–36). Thus, increasing HTT N17 phosphorylation may represent a meaningful therapeutic approach aimed at ameliorating the toxic properties of mutant huntingtin. However, a critical requirement to address this hypothesis is the availability of quantitative, sensitive assays to measure N17 HTT phosphorylation and identify genetic and/or pharmacological modulators of phosphorylation and aggregation. To interfere with aggregation of purified mutant HTT exon 1 protein (36), suggesting that its modulation may impact a pathologically relevant aspect of HD, namely mutant HTT misfolding and aggregation. However, further analysis of this PTM’s relevance for HD and functional/mechanistic studies require the capacity to quantitatively and sensitively measure this PTM in preclinical and clinical samples, which is extremely challenging with current methods. Therefore, we developed a quantitative immunoassays to detect and quantify phosphorylation on HTT N17, starting with pT3 and leveraging the availability of synthetic HTT exon 1 proteins bearing a bona-fide pT3 modification (35) in both WT and mutant forms, as well as a corresponding anti-pT3 HTT antibody, which we previously characterized (35, 40). Importantly, these pg/mL assays uncovered a strong effect of polyQ expansion on pT3 HTT levels in mutant HTT in HD cells, HD mice, and HD patients and indicate that the presence of a mutant polyQ results in greatly decreased immunoreactive T3 phosphorylation on HTT. Mechanistically, using semisynthetic HTT exon 1 (HTT EX1) proteins, we provide evidence that phosphorylation on HTT T3 can influence the conformation and decrease the aggregation properties of mutant HTT in vitro, consistent with other findings reported elsewhere (36). Collectively, our data point to restoration of pT3 levels in mutant HTT as a therapeutically relevant candidate approach for HD and enable investigations aimed at identifying genetic and pharmacological modulators of phosphorylation on T3 in HTT protein.

Results

Development of a Singulex-Based Immunoassay for Huntingtin pT3 and Specific Detection of Huntingtin T3 Phosphorylation in Cells. Immunoassay development requires the availability of antibodies capable of specifically recognizing the epitope and availability of the purified antigen protein to assess specificity and sensitivity in a controlled context. Recently, we reported the development of semisynthetic strategies that enabled the generation of HTT EX1 proteins with bona-fide phosphorylation on residue T3 (pT3) as well as a rabbit polyclonal antibody (pAb) specific for pT3 HTT (35, 40). We were therefore in a position to attempt the development of quantitative immunoassays to detect and measure levels of pT3 in HTT protein in different biological contexts using these reagents (Fig. 1A and B). Because a specific PTM may affect only a fraction of the total cellular steady-state pool of the protein of interest, we turned to one of the most sensitive immunoassay platforms available (43), an application of which was recently employed to develop an ultrasensitive immunoassay for detection of mutant HTT in cerebrospinal fluid (44). The assay is essentially a quantitative fluorescent sandwich immunoassay coupled to single-molecule counting technology (Singulex Erenna immunoassay). Two antibodies are therefore required (one for capture

Fig. 1. Profiling of anti-pT3 HTT antibody. (A) The pT3 signal is specific for the presence of a phosphorylated T3 residue. Western immunoblotting profiling of anti-pT3 antibody on semisynthetic HTT Q23 EX1 protein, HTT EX1 Q23 protein with phosphorylated T3 residue, and the same proteins treated with alkaline phosphatase. (B) The anti-pT3 antibody detects a specific signal on WT HTT EX1 Q16 overexpressed in HEK293T cells lysates, but not on the T3A mutant form by Western immunoblotting analysis. (C) Diagram illustrating the position (i) and epitopes (ii) of antibodies used in the present study. (D) Strategy adopted for the development of sandwich assays specific for total HTT and pT3 HTT, employing antibody MW1 as a capture antibody for both HTT forms.
and concentration bearing no phosphorylation, a phosphorylated antibody allowing detection of both WT and mutant HTT in a phosphorylated T3 residue (pT3), or the same pT3 protein subjected to phosphatase treatment. A serial dilution of each protein was produced and analyzed using the Singulex Erenna platform with the MW1/pT3 antibody pair (Fig. 2A) or with the MW1/2B7 pair (Fig. 2B). The MW1/pT3 pair was clearly able to detect the HTT EX1 bearing a pT3 residue, but not the phosphatase-pretreated protein or its unphosphorylated equivalent, thus demonstrating specificity toward pT3 (Fig. 2A). Key parameters for the MW1/pT3 and MW1/2B7 Singulex immunoassays, namely Limit of Detection (LoD), Lower Limit of Quantification (LLOQ), and Upper Limit of Quantification (ULOQ) (48) are summarized in Fig. 2A and B. Collectively, the data obtained using purified HTT EX1 proteins indicated that the MW1/pT3 and MW1/2B7 Singulex immunoassays were specific, sensitive, and robust. The specificity of the MW1/pT3 and MW1/2B7 Singulex immunoassays was next tested in HEK293T cells through RNAi (knockdown of HTT expression on pT3 HTT and total HTT levels under endogenous or transfected HTT expression) or through specific mutation (effects of T3A mutation on pT3 HTT and total HTT levels with HTT expressed from a plasmid construct). Endogenous HTT expression in HEK293T cells is detectable by WB (Fig. 3A), and these cells have been widely employed to study various huntingtin and mutant huntingtin characteristics, including its phosphorylation status (37, 40, 42). First, we investigated the effects of RNAi [using either a siRNA targeting a specific HTT sequence or a scrambled, control (CTRL) siRNA] on HTT and pT3 HTT protein levels in HEK293T cells and in HEK293T cells expressing HTT with specific polyQ repeat domain (Fig. 3A and B). Using these lysates, we confirmed by densitometric analysis (Fig. S2A–D) that specific HTT mRNA levels was verified by quantitative real-time PCR (Fig. S2A). As shown in Fig. 3A, HTT protein expression was clearly detected in WB by MAB2166, a specific anti-HTT monoclonal antibody widely employed to detect HTT in different samples (49–53), and was specifically reduced in cells transfected with the HTT-specific siRNA, as confirmed by densitometric analysis (Fig. S2A). Endogenously expressed HTT protein is phosphorylated on T3 as detected in WB by the anti-pT3 pAb. A reduction was observed in cells transfected with the specific HTT siRNA, thus further confirming the specificity of the anti-pT3 pAb. We then interrogated these lysates using the MW1/pT3 and MW1/2B7 Singulex immunoassays to examine the effects of specific genetic knockdown of HTT expression. As shown in Fig. 3B, comparable reductions in pT3 HTT and total HTT protein levels were achieved using genetic knockdown of HTT expression, irrespective of the context (endogenous HTT or overexpressed HTT). These results indicated that the signal obtained from the MW1/pT3 and MW1/2B7 immunoassays in a biological matrix is specific for pT3 HTT and total HTT proteins, respectively.

Next, we interrogated the effect of a specific T3 mutation (T3A), abrogating the possibility of phosphorylation on HTT T3, on the signal obtained from the MW1/pT3 and MW1/2B7 immunoassays. As HEK293T cells bearing endogenous T3A HTT alleles are not available, we opted for a transient transfection context. Plasmid constructs encoding HTT EX1 with different polyQ repeats representing a WT expansion (Q16), an HD expansion (Q39), a juvenile-type HD expansion (Q72), or their T3A variants were transiently expressed in HEK293T cells. The specificity of the anti-pT3 pAb was first confirmed by PhosTag SDS/PAGE WB (Fig. 3C), as previously shown using HTT EX1-EGFP fusions in the same cell line (40). HTT EX1 protein levels were assessed using 4C9, a monoclonal Ab specific for the polyproline repeat domain (Fig. 3C). Using these lysates, we determined the effect of the T3A mutation on pT3 HTT and total HTT signal obtained with the Singulex MW1/pT3 and MW1/2B7 immunoassays, respectively. The absence of the T3A mutation in the transiently transfected HTT EX1 constructs resulted in a strong reduction of pT3 HTT levels without any effect on total HTT levels (Fig. 3D and Fig. S2C), which was significant for all tested polyQ repeat lengths. Collectively, the data indicated that the Singulex MW1/pT3 and MW1/2B7
immoassays were sufficiently specific and sensitive to allow investigations of pT3 HTT levels in biological samples of relevance to HD.

**Huntingtin T3 Phosphorylation Is Strongly Decreased in HD Mice and in Human HD Samples.** We first examined the effect of expanded polyQ on pT3 HTT levels in the brain, using the Hdh Q111 mouse model (S4–S6) where the mouse exon 1 sequence has been replaced with a human exon 1 sequence with either a Q7 or a Q111 repeat (Q7/Q111 and Q111/Q111; HD mouse strains). Mutant huntingtin is therefore expressed in a fully physiological manner. Two brain regions (cortex and cerebellum) were investigated from 6-month-old mice (an age at which EM48-positive nuclear inclusions are not yet detectable) (S4, S5). Animals included four homozygous WT (Q7/Q7) mice, six heterozygous mutant (Q7/Q111) mice, and one homozygous mutant (Q111/Q111) mouse. pT3 HTT and total HTT levels were interrogated by SDS/PAGE WB analysis and by Singulex immunoassays. Levels of pT3 HTT as detected by the anti-pT3 pAb in SDS/PAGE WB are clearly decreased in brain cortex from heterozygous and homozygous mutant mice but not in cortex from WT mice (Fig. 4A (46)). Coherent results were obtained in pooled whole brains from WT (Q7/Q7) mice and mutant (Q7/Q111) mice (Fig. S3B). Next, pT3 and total HTT levels were assessed using the MW1/pT3 (Fig. S4A, i) and the companion MW1/2B7 Singulex immunoassay (Fig. S4A, ii) in a brain cortex sample. To exclude a possible confounding influence of known HTT PTMs on total HTT detection using 2B7, two additional total HTT Singulex immunoassays were employed, based on MAB2166 (epitope amino acids 442–457; Figs. S4A, iii and S3C) or HDB4E10 (epitope amino acids 1,831–2,131; Figs. S3C and S5A). All three total HTT immunoassays (MW1/2B7, MW1/MAB2166, and MW1/HDB4E10), independent of the detection antibody employed, displayed higher total HTT signals in heterozygous mutant samples than in WT samples, consistent with a degree of selectivity for expanded polyQ displayed by MW1 when used as a capture antibody in these assays (Figs. S4 and S5). Importantly, upon normalization of pT3 signal for total HTT signal (irrespective of the anti-HTT detection antibody used to measure total HTT in the Singulex assay), the analysis confirms a robust decrease of pT3 immunoreactivity when polyQ is expanded (Fig. 4 and Fig. S5B), consistent with WB data. The specificity of the pT3 signal detected in Q7/Q7 and Q7/Q111 brain samples was confirmed by immunodepleting HTT protein using 2B7 (Fig. S6A) and then assessing pT3 HTT and total HTT levels in the Q7/Q7 and in Q7/Q111 contexts (Fig. S6 B and C, respectively) where immunodepletion resulted in a clear, comparable decrease of MW1/pT3 and MW1/MAB2166 signals relative to controls. We also assessed an independently produced and previously characterized rabbit pAb specific for pT3 HTT (39) in the same assay platform, using MW1 as a capture Ab and the anti-pT3 Ab as the detection Ab. This alternative pT3 HTT Singulex immunoassay behaved comparably to the MW1/pT3 Singulex immunoassay under all conditions tested (Fig. 4C and Fig. S4). To determine if the difference in pT3 HTT levels is observable in another brain area, we investigated the cerebellum from these mice by both WB (Fig. 5A, i; see also Fig. S3A) for total HTT detection using the alternative anti-HTT antibody, D7F7 (Fig. 5A, ii) and Singulex immunoassays (Fig. 5A, ii and Fig. S7A) and found comparable results as those obtained in mouse cortex. The striatum was also investigated with similar results (Fig. S8). We also investigated pT3 HTT levels in cortex from knock-in Hdh mice bearing a humanized HTT exon 1 with different polyQ expansions (Q7, Q20, Q50, Q92, and Q111; Fig. 5B, i and ii and Fig. S7B) to confirm data obtained in Q7/Q7 and
Q7/Q111 mice. As shown in Fig. 5 B, ii, normalized pT3 levels are clearly decreased in the presence of a polyQ expansion of Q50 or greater. Therefore, pT3 levels in the brain of mice expressing a mutant huntingtin protein are decreased relatively to the same brain region of mice expressing WT huntingtin protein. As this effect is observed on the denatured protein (SDS/PAGE) as well as on the native protein (Singulex immunoassay), it is likely due to a bona-fide decrease in phosphorylation levels on the residue rather than a conformational change masking the pT3 epitope as a result of polyQ repeat expansion.

The findings in Hdh Q111 mice prompted us to interrogate HD samples of human origin to determine if the effect of mutant polyQ on HTT pT3 levels observed in animal models is relevant to human HD. Recently, the HD consortium reported the generation of induced pluripotent stem cell (iPSC) lines from HD patients and controls (57). A number of these iPSC lines can be engineered to express a temperature-dependent change in the α-helicity of HTT’s N terminus, which increases with increasing polyQ length (24, 25). Briefly, the ratio of the signals produced by the TR-FRET antibody pair on HTT proteins at the two test temperatures is inversely proportional to the length of the polyQ repeat and is paralleled by a temperature-dependent change in the α-helix of HTT’s N terminus, which increases with increasing polyQ length (24, 25). We therefore interrogated the effects of pT3 on the conformation of semisynthetic HTT EX1 with WT (Q23) or mutant (Q43) expansions using the 2B7/MW1 conformational immunoassay, taking advantage of the recent availability of semisynthetic HTT EX1 with Q43. In this assay, pT3 had no significant conformational effect on HTT EX1 Q23 as detected by the 2B7/MW1 TR-FRET immunoassay, with the temperature ratio of the 2B7/MW1 signals from the pT3-modified HTT EX1 Q23 and the unmodified HTT EX1 Q23 proteins being comparable (Fig. 7 A, i). However, pT3 modification of the mutant EX1 HTT Q43 resulted in a significant increase in the temperature ratio of the 2B7/MW1 signals obtained from the EX1 HTT Q43 protein, approximately halving the conformational constraint imposed by the mutant polyQ expansion (Fig. 7 A, ii). Interestingly, semisynthetic HTT Q23 and Q43 proteins bearing another PTM associated with HTT’s N17 region [acetylated K6, AcK6 or acetylated K9, AcK9 residues (32)] produced no effect irrespective of the length of the polyQ region, suggesting that the observed conformational effect is specific for pT3 (Fig. 7 A, i and ii). Additionally, a control TR-FRET immunoassay [2B7/4C9, which does not interrogate the polyQ region and is not sensitive to temperature and polyQ length.

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Fig. 5. Confirmation of mutant HTT hypophosphorylation on residue T3 in Hdh Q111 knock-in HD mice cerebellum and cortex from Hdh HD knock-in mice with different polyQ expansions. (A, i) Western immunoblot of homogenates from Hdh knock-in mouse (6 mo) cerebellum, analyzed using anti-pT3 (for HTT pT3) and mAb 2166 (for total HTT), and showing that mutant HTT is hypophosphorylated relative to its Q7 counterpart. (A, ii) Relative HTT pT3 Singulex signal in brain cerebellum samples from WT (Q7/Q7) and heterozygous mutant (Q7/Q111) Hdh HD animals, normalized to total HTT measured with the MW1/2B7 Singulex immunoassay. Means and SD of four Q7/Q7 samples and six Q7/Q111 samples, unpaired t-test (two-tailed; ***p < 0.001). (B, i) Analyzed brain cortex samples from Hdh Knock-in mice expressing mutant HTT bearing different expanded polyQ lengths. (B, ii) Relative HTT pT3 levels in these samples, confirming hypophosphorylation on residue T3 in mice expressing mutant HTT. Means and SD of four Q7/Q7, four Q7/Q20, five Q7/Q50, four Q7/Q92, and five Q7/Q111 samples; one-way ANOVA test (***p < 0.005).

(24, 25)] did not produce temperature- and polyQ-dependent differences in TR-FRET signals (Fig. 7B). These results suggest that pT3 can influence mutant HTT EX1 conformation, apparently ameliorating the conformational rigidity imparted by the mutant polyQ expansion. In this light, pT3 might therefore be a protective modification. To further investigate this hypothesis, we interrogated the effect of pT3 on semisynthetic mutant HTT EX1 aggregation using a standard filter trap assay (63). A notable difference in the SDS-insoluble fraction present in semisynthetic HTT EX1 Q43 and Q43 pT3 was detectable, suggesting that the presence of T3 phosphorylation may reduce aggregation in these semisynthetic proteins (Fig. 7C). A filter trap assay confirmed these observations, with a reduction of HTT EX1 Q43 filter-retained material observed when the protein was phosphorylated (Fig. 7 C, iii), consistent with data obtained by other methodologies. As expected, HTT EX1 Q23 did not produce significant signal in the filter trap assay. Collectively, these data suggest that T3 phosphorylation can ameliorate the conformational constraint imposed by the HD mutation on HTT protein and reduce aggregation, at least in the in vitro context examined.

Discussion

Mutant HTT self-association and toxicity are believed to be primary determinants of HD pathology (13). Accumulating evidence indicates that the first 17 amino acids of HTT (N17) play a key role in modulating mutant HTT toxicity (30, 64). This domain can adopt an α-helical structure (65–67) and appears to be a key cis-acting modifier of polyQ aggregation (28), subcellular localization, and toxicity (29, 31, 33, 68). The N17 domain can be postranslationally modified at several residues including pT3 (39) and S13/S16 (32, 33). Introduction of mutations that abolish (S → A) or mimic (S → E/D) phosphorylation at S13 and S16 residues has been shown to affect the conformation of HTT’s N17 domain as well as subcellular localization, aggregation, and toxicity of mutant HTT (29, 33). Studies employing HTT exon 1-like peptides containing pS13/pS16 have confirmed the modulatory effects of phosphomimetic S13/S16 mutations on HTT aggregation and suggest that pS13/pS16 may impart a thermodynamic barrier to the initial phases of oligomerization by interfering with the packing and stabilization of oligomers or by affecting overall oligomer stability (34). The collective evidence therefore argues that increasing or mimicking phosphorylation within HTT N17’s domain reduces mutant HTT toxic properties (29, 33, 69), and significant potential exists for therapeutic strategies aimed at addressing HD through the modulation of these PTMs. Despite this, our capacity for testing the hypothesis that HD pathology can be modulated by leveraging HTT PTMs is presently hindered by the paucity of quantitative, sensitive, and robust assays capable of profiling these PTMs across disease models, of confirming their relevance to human HD, and of enabling the identification of genetic and pharmacological tools for proof-of-concept studies.

We therefore set out to develop HTT assays to interrogate HTT PTMs, focusing initially on phosphorylation of the N17 domain on residue T3. This HTT PTM was chosen for assay...
Relative pT3 HTT levels (normalized with MW1/2B7)

![Graph showing relative pT3 HTT levels](Image)

Fig. 6. Mutant HTT is hypophosphorylated on residue T3 in HD iPSC-derived neuronal populations and in peripheral (blood) PBMCs isolated from HD individuals. (A) Relative HTT pT3 Singulex signal in iPSC-derived neuronal population protein lysate from WT and HD individuals, normalized to total HTT measured with the MW1/2B7 Singulex immunoassay. Means and SD of three CTRL iPSC lines (#CTRL21: CAG status 21/18; #CTRL28: CAG status 28/18; #CTRL33: CAG status 33/18) and two HD iPSC lines (#HD60: CAG status 60/18; #HD109: CAG status 109/19). Unpaired t-test (two-tailed; *P < 0.05). (B) HTT pT3 Singulex signal in PBMCs isolated from HD individuals, relative to PBMCs from normal individuals and normalized to total HTT measured with the MW1/2B7 Singulex immunoassay. Means and SD of three control and three HD samples, unpaired t-test (two-tailed; *P < 0.05).

development for a number of reasons. First, by analogy with the effects of S13 and S16 modification on HTT’s N17 structure and function, it seemed plausible that phosphorylation of T3 may also play a role in regulating HTT N17’s structure and function, and indeed recent structural evidence argues that T3 is strategically positioned to regulate α-helical content in the N17 domain of HTT (70–72). Importantly, recent studies showed that pT3 inhibits the aggregation of mutant HTT Ex1 (36), and pT3 is the phosphorylation event most readily detected in the N-terminus of HTT by mass spectrometry and WB, at least when HTT is overexpressed in cells (39, 40). Also, HTT T3 phosphorylation has been profiled previously in HD models by standard immunoblotting methods to a more detailed extent than other HTT N17 phosphorylations (39). Finally, relevant tools such as semi-synthetic HTT proteins bearing a phosphorylated T3 residue and specific antibodies are available (35, 36, 39, 40).

The approach that we applied for pT3 immunoassay design involved the Erenna Singulex sandwich immunoassay, a version of which, based on 2B7 as a capture Ab and MW1 as a detection Ab, was recently employed to develop an ultrasensitive immunoassay for detection of mutant HTT in cerebrospinal fluid (44). Sandwich immunoassay design involved the concomitant development of immunoassays for the detection of HTT pT3 and “total” HTT based on the same capture antibody. Assay development followed a standard approach and involved preliminary assay qualification using reference standards of high purity and known concentration (semisynthetic HTT Ex1 proteins with/without pT3), followed by a rigorous interrogation of assay specificity using genetic means (specific RNA knockdown and T3A mutations) in a simple cell line context. Following development and validation, the MW1/pT3 and MW1/2B7 Singulex immunoassays were used to interrogate the relevance of T3 phosphorylation for HD pathology, starting with the effect of mutant polyQ expansion on pT3 levels. We have shown that MW1 can efficiently bind native HTT-bearing WT polyQ expansions when used as a capture Ab in the Singulex Erenna immunoassay and that under these conditions MW1’s reported higher affinity for expanded polyQ is significantly attenuated. When quantifying pT3 HTT levels in biological samples, we opted to normalize the signal obtained with the MW1/pT3 Singulex Erenna immunoassay with the signal obtained as a measure of total HTT protein present in the sample using the MW1/2B7 Ab combination, which employs the same Ab (MW1) as a capture IgG. We therefore obtained a pT3 HTT signal that is relative to total HTT measured in the same sample. Intersample comparisons are then performed between relative pT3 HTT levels, thus informing in a polyQ-normalized fashion on the proportion of HTT phosphorylated on residue T3. Naturally, several factors aside from absolute T3 phosphorylation levels may influence apparent PTM levels detected by immunoassays because of the intrinsic nature of the antibody-antigen interaction. These include conformational aspects as well as the presence of additional modifiers such as additional PTMs, which we have tried to address using orthogonal approaches (e.g., the use of WB and of multiple antibodies). Immuno-reactive pT3 HTT levels were examined in one of the most widely employed mouse models of HD, the Hdh Q111 mouse model (34, 55). In brain cortex of Hdh mice, a thorough examination of pT3 HTT levels was performed, comparing different normalization approaches toward total HTT (the selected MW1/2B7 pair plus an alternative pair composed of the MW1/2166 combination), the use of an alternative, independently developed anti-pT3 pAb (39) for HTT pT3 detection, the demonstration of specificity using an immunodepletion approach, and orthogonal validation by WB. In brain cortex, immuno-reactive pT3 HTT levels were clearly decreased in Hdh Q7/Q111 and Q111/Q111 mice relative to Q7/Q7 mice, a result that was mirrored in another brain area, the cerebellum, and observed also in Hdh mice bearing a humanized exon 1 with different mutant polyQ expansions (Q50, Q92, and Q111) relative to mice bearing the same transgene with WT polyQ expansions (Q7 or Q20). Next, we decided to investigate if pT3 HTT levels differed significantly in neuronally differentiated cultures from human iPSC lines derived from control and HD individuals, as patient-derived iPSC lines represent one of the most translationally relevant preclinical HD models (73). Coherent with results obtained in the mouse HD model, relative pT3 HTT levels were significantly lower in differentiated cultures from HD iPSC than in control iPSCs. Finally, we investigated HTT pT3 levels in a clinical sample, in which HD was expressed in a humanized exon 1 with different normalization approaches. Although not representative of the CNS, these peripheral samples can be used to probe aspects of mutant HTT pertinent to HD pathology (47). A clear decrease in immuno-reactive pT3 HTT levels was observed in HD PBMCs relative to PBMCs from control individuals, thus confirming results obtained in preclinical HD models.

Given the lower levels of apparent pT3 phosphorylation in mutant huntingtin relative to its WT counterpart in vivo, we investigated the role of pT3 on mutant HTT behavior. We and others have demonstrated that polyQ expansion results in conformational changes in HTT proteins, either purified or expressed in cells, which can be detected by TR-FRET immunoassays (24, 25). Using this conformational TR-FRET immunoassay, we interrogated purified HTT exon 1 proteins (Q23 or Q43) with or without bona-fide T3 phosphorylation and observed that the presence of a phosphorylated T3 residue appears to mitigate the effect of polyQ expansion on HTT exon 1 protein conformation. Although the effects of T3 phosphorylation on HTT N-terminal conformation require further studies, an N17 modification in HTT may alter the α-helicity of HTT’s N-terminus (33, 36), and indeed a phosphorylated T3 residue was shown to modulate the α-helicity of HTT exon 1 protein (35, 36). As temperature-dependent α-helicity as well as overall α-helicity at any one temperature are greater in mutant HTT than in WT HTT (24, 25), an alteration in α-helicity would be more readily detected by the conformational TR-FRET immunoassay in mutant HTT than in WT HTT, as indeed was observed here (Fig. 7). Consistent with pT3 mitigating the conformational effects of polyQ expansion on an exon 1 HTT protein fragment (36), we also observed a decreased aggregation propensity for semisynthetic pT3 HTT exon 1 Q43 relative to its WT counterpart (Fig. 7). Although the phosphomimetic T3 mutation was previously reported to increase aggregation of mutant HTT exon 1 in ST14A cells and in a Drosophila HD model (39), recent biophysical studies showed that the T3D mutation does not
T3 phosphorylation impacts mutant HTT N-terminal conformational rigidity and decreases HTT EX1 aggregation. (A, i) T3 phosphorylation has no effect on the conformation of soluble semisynthetic HTT EX1 Q23 protein as measured by the 2B7/MW1 TR-FRET conformational immunoassay. (A, ii) T3 phosphorylation decreases the conformational rigidity of HTT EX1 Q43 protein assessed with the same TR-FRET. Other relevant N17 PTMs, specifically acetylation on residues K6 and K9, do not affect the conformational rigidity of HTT EX1 Q43 protein as measured in parallel using the same assay. Summary of three independent experiments (one-way ANOVA test; ***P < 0.0005). (B) The control 2B7/4C9 TR-FRET immunoassay, the signal of which on HTT protein is not temperature- and polyQ-dependent, is not affected by T3 phosphorylation. (C) T3 phosphorylation reduces the aggregation propensity of mutant HTT protein. (i) WB and corresponding densitometric analysis of equivalent nominal amounts of semisynthetic HTT EX1 Q43 protein and its T3 phosphorylated counterpart. Means and SD of three independent experiments, paired t-test (two-tailed; ns, not significant). (ii) Detergent-insoluble material was present in lanes loaded with HTT EX1 Q43 protein, the presence of which was invariably reduced if this protein was phosphorylated on residue T3. Means and SD of three independent experiments, paired t-test (two-tailed; **P < 0.01). (iii) Filter trap analysis of semisynthetic HTT EX1 Q43 protein aggregation in the presence/absence of T3 phosphorylation, confirming reduction of insoluble HTT EX1 Q43 aggregates if residue T3 is phosphorylated. Note that a small difference in soluble Q43 and pT3 Q43 semisynthetic protein levels is observed as measured by densitometry; however, a much larger difference in insoluble protein levels is observed. Means and SD of three independent experiments, paired t-test (two-tailed; *P < 0.05).

Fig. 7. T3 phosphorylation impacts mutant HTT N-terminal conformational rigidity and decreases HTT EX1 aggregation.

Materials and Methods

Antibodies. The MWI1 antibody (developed by Paul Patterson, Caltech, Pasadena, CA and obtained from the Developmental Studies Hybridoma Bank) binds to the polyQ stretch of HTT; the purified form was obtained using Protein G HP Spin Trap (catalog #28-9031-34; GE Healthcare Life Science) following the manufacturer’s recommendations. The 2B7 and 4C9 antibodies recognize the HTT N-Term 17 amino acids and the polyP region in exon 1 of the HTT protein, respectively (74), and were obtained from the CHDI (Cure for Huntington’s Disease Initiative) Foundation. The anti-pT3 antibody was previously described (35, 40). MAB2166 was supplied by a commercial source (catalog #MAB2166; Millipore) and binds to a 15-aa region spanning from amino acids 445 to 459 of the human HTT protein (50). HD84E10 antibody was distributed by Thermo Fisher Scientific and binds the region from amino acid 1,831 to 2,131 of human HTT protein. D7F7 is a commercial antibody supplied by Cell Signaling Technology and recognizes amino acids 2,703–2,911 of human HTT protein. Antibody against GAPDH was distributed by Sigma-Aldrich (catalog #G9545). Secondary antibodies used for WB were goat-anti-mouse IgG HRP-conjugated (catalog #12–349; Merck Millipore) and goat-anti-rabbit IgG HRP-conjugated (catalog #12–348; Merck Millipore). The D2 fluorophore and the terbium cryptate antibody labelings were custom made by CisBio. The Alexa Fluor-647 Monoclonal Antibody Labeling Kit from Thermo Fisher Scientific (catalog #A20186) following the manufacturer’s instructions. MW1 antibody was conjugated to magnetic particles for Singulex assays, following the manufacturer’s recommendations (catalog #03–0077–02; Singulex).

Semisynthetic Proteins. The production of semisynthetic HTT exon 1 proteins with relevant PTMs was described before (35, 36). Pure trifluoroacetic acid was added to the lyophilized protein powder for disaggregation, and proteins were then dissolved in TBS buffer (50 mM Tris 150 mM NaCl) to obtain a final concentration of 20 μM (pH adjusted to 7.2–7.4 using 1 M NaOH). Protein solutions
were filtered through a 100-kDa membrane (Nanosep Centrifugal Devices 100k Omega, catalog #OD100C34; Pall). Each sample was supplied with 1% Tween 20. Deposphorylation of protein was performed using 10 unit/L concentrated alkaline phosphatase (calf intestinal phosphatase, catalog #M02905; New England Biolabs), following the manufacturer’s instructions.

Plasmid and Constructs. CDNAs encoding N-terminal HTT fragments (exon 1) bearing different polyQ lengths (Q16, C39, or Q72) were synthesized by GenScript, quality-controlled by DNA sequencing, and subcloned into pcDNA3.1, which was characterized for its reproducibility in human ES and iPSC lines (University of Milan). The human iPSC lines used (#CTRL21: CAG status 60/18; #HD109: CAG status 109/19) are those reported in ref. 57 and were characterized for its reproducibility in human ES and iPSC lines. mRNA was reverse transcribed using the SuperScript III First-Strand Synthesis (catalog #18080–051; Thermo Fisher Scientific). Real-time PCR was performed using Power SYBR Green PCR Master Mix, Applied Biosystem (catalog #4367659; Thermo Fisher Scientific) with specific HTT primers (FWD 5′-GTGGAGGTTGTCAGGCTG-3′; REV GC8AAATTGGC88AAAGG; Bio-Fab Research Srl). Human ES and iPSC lines were used for amplification and analysis.

IPSC Culture and Sample Processing. Neuronally differentiated iPSC lines from control individuals and HD patients were generated and characterized by the E. Cattaneo laboratory (University of Milan). The human iPSC lines used (#CTRL21: CAG status 21/18; #CTRL28: CAG status 28/18; #CTRL33: CAG status 33/18; #HD06: CAG status 60/18; #HD109: CAG status 109/19) are those reported in ref. 57 and have been differentiated using the striatal differentiation protocol in ref. 58. The protocol was characterized for its reproducibility in human ES and iPSC lines as extensively described in ref. 75, and the cells were harvested and immediately stored as pellets at −80 °C. Frozen-cell pellets were lysed using lysis buffer (composition described above) and cleared after sonication. Animal Tissues. HD knock-in mice with increasing CAG repeat lengths were reported previously (56, 77). For tissue preparation, 6- to 8-month-old mice were employed. Following dissection, mouse brain-tissue samples were homogenized using a FastPrep (BioMedical) in 10 vol (wt/vol) of lysis buffer (composition described above) using PreCellys tubes, and two lysis cycles of 10 min at 6,000 × g were carried out. Homogenates were cleared and stored at −80 °C. Tissues from knock-in Hdh mice bearing a humanized exon 1 with different polyQ expansions (Q7, Q20, Q50, Q92, and Q111) were obtained from the CHDI Foundation.

Human Samples. Peripheral blood lymphocytes from control and HD subjects were obtained as described (78), harvested by centrifugation, and snap-frozen as pellets. Pellets were resuspended in lysis buffer (composition described above) and sonicated, and finally clarified before the total protein quantification. A protocol to collect biological specimens, including blood samples, at Instituto CSS-Mendel, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Ospedale Casa Sollievo della Sofferenza for research purposes, was approved by the Ethical Committee from the Casa Sollievo della Sofferenza Foundation, section of Istituto Tumori Giovanni Paolo II in Bari. Informed consents were obtained from patients and healthy control subjects.

were denatured at 95 °C in 4× Loading Buffer (125 mM Tris HCl, pH 6.8, 6% SDS, 4 M urea, 4 mM EDTA, 30% glycerol, 4% 2-mercaptoethanol and bromophenol blue) and loaded on NuPAGE 4–12% Bis–Tris Gel (catalog #WG14020BX; Thermo Fisher Scientific). Proteins were transferred on PVDF membrane (catalog #162-0177; Bio-Rad Laboratories) using wet blotting. After fixing in 0.4% paraformaldehyde0.4% sucrose solution and blocking with 5% nonfat milk in TBS0.1% Tween-20, primary antibody incubation was carried out overnight at 4 °C, and secondary antibody incubations for 1 h at room temperature. Protein bands were detected using chemiluminescence substrate (SuperSignal West Femto Maximum catalog #34046; SuperSIGNAL West Pico Maximum catalog #34087; Thermo Fisher Scientific) on Chemidoc XR+ (Bio-Rad Laboratories).

Western Blot Assay, Filter Trap Assay, and pTag Gel. For WB, samples were run on a 10% or 12% SDS-PAGE gel and transferred to PVDF membranes. Detection was performed using a ChemiDoc XR+ (Bio-Rad Laboratories). After transfer, membranes were blocked with 5% nonfat milk in TBS/Tween-20 and incubated with primary antibodies for 1 h at room temperature. Secondary antibodies were detected using a chemiluminescence substrate (SuperSignal West Pico Maximum catalog #34046; SuperSIGNAL West Pico Maximum catalog #34087; Thermo Fisher Scientific) on Chemidoc XR+ (Bio-Rad Laboratories). The primary antibody was detected using horseradish peroxidase–conjugated antibodies. Membranes were developed using a Chemiluminescence substrate (SuperSignal West Pico Maximum catalog #34046; SuperSIGNAL West Pico Maximum catalog #34087; Thermo Fisher Scientific) on Chemidoc XR+ (Bio-Rad Laboratories) and scanned using a GelDoc XRS+ (Bio-Rad Laboratories).

Immunoprecipitation. Immunoprecipitation was performed using Dynabeads Protein G (catalog #10004D; Thermo Fisher Scientific) following the manufacturer’s instructions and using an HTT-specific antibody (287) or an unrelated antibody (GFAP, catalog #G9269; Sigma–Aldrich). The pulled-down material was loaded on a SDS/PAGE, and the supernatant (immunodepleted sample) was analyzed by Singulex assay.

Singulex Assays. A total of 50 μL/well of dilution buffer (6% BSA, 0.8% Triton X–100, 3 mM NaCl, and protease inhibitor) was added to a 96-well plate (catalog #49-300; Sigma–Aldrich) and loaded on a 384-well plate (catalog #96-450V-C; Agilent). Samples to be tested were diluted in artificial cerebral spinal fluid (0.3 M NaCl; 6 mM KCl; 2.8 mM CaCl2–2H2O; 1.6 mM MgCl2–6H2O; 1.6 mM Na2HPO4–7H2O; 0.4 mM NaH2PO4–H2O) supplemented with 1% Tween-20 and complete protease inhibitor in a final volume of 150 μL/well. Finally, 100 μL/well of the MV1 antibody coupled with magnetic particles (appropriately diluted in Erenna Assay buffer, catalog #02-6474-00; Singulex) was added to the assay plate and incubated for 1 h at room temperature under orbital shaking. The beads were then washed with Erenna System buffer (catalog #02-0111-00; Singulex) and resuspended using 20 μL/well of the specific detection antibody labeled with D2 fluorophore (or Alexa–647 fluorophore) appropriately diluted in Erenna Assay buffer. The plate was incubated for 1 h at room temperature under shaking. After washing, the beads were resuspended and transferred to a new 96-well plate. A total of 10 μL/well of Erenna buffer B (catalog #02-2027-00; Singulex) was added to the beads for elution and incubated for 5 min at room temperature under orbital shaking. The eluted complex was magnetically separated from the beads and transferred to a 384-well plate (Nunc catalog #265473; Sigma–Aldrich) where it was neutralized with 10 μL/well of Erenna buffer D (catalog #02-0368-00; Singulex). Finally, the 384-well plate was heat-sealed and analyzed with the Erenna Illumina assay.

TR-FRET Assays. A total of 5 μL/well of samples and 1 μL/well of antibody mixtures (287–Tb 1 ng/mL; MW1-D2 10 ng/mL; 4C9–Alexa647 10 ng/mL) were diluted in lysis buffer (composition described above), and the assay was performed as described (24).

Data Analysis. See SI Materials and Methods.

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1. Prabakaran S, Lippers G, Steen H, Gunawardena J (2012) Post-translational modification: Nature’s escape from genetic imprisonment and the basis for dynamic information encoding. Wiley Interdiscip Rev Syst Biol Med 4:585–583.
2. Khoury GA, Baliban RC, Floudas CA (2011) Proteome-wide post-translational modification statistics: Frequency analysis and curvature of the Swiss-Prot database. Sci Rep 1:000990.
3. Sato C (2003) Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci 4:49–60.
4. Seokko DJ (2004) Cell biology of protein misfolding: The examples of Alzheimer’s and Parkinson’s disease. Nat Cell Biol 6:1054–1061.
5. Noble W, Hanger DF, Miller CC, Lovestone S (2013) The importance of tau phosphorylation for neurodegenerative diseases. Front Neurol Sci 4:83.
6. Sabbagh JD, Dickey CA (2016) The metamorphic nature of the tau protein: Dynamic flexibility comes at a cost. Front Neurol 10:3.
13. Ross CA, Tabrizi SJ (2011) Huntington’s disease: From molecular pathogenesis to clinical treatment. Lancet Neurol 10:82–98.

14. Snell RG, et al. (1993) Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington’s disease. Nat Genet 4:393-397.

15. Brinkman RR, Mezei MM, Thielmann I, Almqvist E, Hayden MR (1997) The likelihood of being affected with Huntington disease by a particular age, for a specific CAG size. Am J Hum Genet 60:1200–1212.

16. Sathasivam K, et al. (2013) Aberrant splicing of HTT generates the pathological exon 1 protein in Huntington disease. Proc Natl Acad Sci USA 110: 2366–2373.

17. Gipson TA, Neueder A, Wexler NS, Bates GP, Housman D (2013) Aberrantly spliced HTT, a new player in Huntington’s disease. Proc Natl Acad Sci USA 110: 2366–2373.

18. Hazeki N, Nakamura K, Goto J, Kanazawa I (1999) Rapid aggregate formation of the huntingtin N-terminal fragment carrying an expanded polyglutamine tract. Biochem Biophys Res Commun 256:361–366.

19. Lunkes A, et al. (2002) Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. Mol Cell 10: 259-269.

20. Wang X, et al. (2008) Accumulation of N-terminal mutant huntingtin in mouse and monkey models implicated as a pathogenic mechanism in Huntington’s disease. Hum Mol Genet 17:2378–2381.

21. Landles C, et al. (2010) Proteolysis of mutant huntingtin produces an exon 1 fragment that accumulates as an aggregated protein in neuronal nuclei in Huntington disease. J Biol Chem 285:8808-8823.

22. Barbaro BA, et al. (2015) Comparative study of naturally occurring huntingtin fragments in Drosophila points to exon 1 as the most pathogenic species in Huntington’s disease. Hum Mol Genet 24:913-925.

23. Caron NS, Desmond CR, Xia J, Truant R (2013) Polyglutamine domain flexibility mediates the proximity between flanking sequences in huntingtin. Proc Natl Acad Sci USA 110:14610–14615.

24. Fodaie V, et al. (2014) Polyglutamine- and temperature-dependent conformational rigidity in mutant huntingtin revealed by immunoassays and circular dichroism spectroscopy. PLoS One 9:e112262.

25. Cui X, et al. (2014) TR-FRET assays of Huntingtin protein fragments reveal temperature and polyQ length-dependent conformational changes. Sci Rep 4:45601.

26. Barnell G, Orgel JP, Pahl R, Meredith SC (2007) Flanking polyproline sequences inhibit the conformational switch to aggregation. J Mol Biol 374:688-704.

27. Bugg CW, Isas JM, Fischer T, Patterson PH, Langen R (2012) Structural features and functional role of huntingtin exons in Drosophila points to exon 1 as the most pathogenic species in Huntington disease. Hum Mol Genet 21:2461-2472.

28. Warby SC, et al. (2005) Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion in vivo. J Neurochem 94:1461-1469.

29. Huang B, et al. (2015) Scalable production in human cells and biochemical characterization of mutant huntingtin fragment containing polyQ tract. PLoS One 10:e0121055.

30. Todd J, et al. (2012) Ultraconservative flow-based immunoassays using single molecule counting. Mol Simul 38:1990-1995.

31. Wild DJ, et al. (2015) Quantification of mutant huntingtin protein in cerebrospinal fluid from Huntington’s disease patients. J Clin Invest 125:1979-1986.

32. Ko J, Oo S, Patterson PH (2001) New anti-huntingtin monclonal antibodies: Implications for the huntingtin conformation hypothesis. Hum Mol Genet 10:1678-1688.

33. Klein FA, et al. (2013) Linear and extended: A common polyglutamine conformation recognized by the three antibodies MW1, 1C2 and 3B5H10. Hum Mol Genet 22: 4215-4223.

34. Weiss A, et al. (2012) Mutant huntingtin fragmentation in immune cells tracks Huntington’s disease progression. J Clin Invest 122:3731-3736.

35. Arbrustner DA, Pry T (2008) Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29:549-552.

36. Chen M, et al. (2008) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 6:797-801.

37. Cong SY, Peoples BA, Roos RA, Van Ommen GJ, Dorsman JC (2005) Epitope mapping of monomeric antibody 4C8 recognizing the protein huntingtin. Hybridoma (Larchmt) 24:253-257.

38. Ko J, Oo S, Patterson PH (2001) New anti-huntingtin monoclonal antibodies: Implications for the huntingtin conformation hypothesis. Hum Mol Genet 10:1678-1688.

39. Arndt JR, Chaibva M, Legleiter J (2015) The emerging role of the first 17 amino acids of its glutamine repeats. Mol Genet Metab 115:321-327.

40. Darnell G, Orgel JP, Pahl R, Meredith SC (2007) Flanking polyproline sequences inhibit beta-sheet structure in polyglutamine segments by inducing PPII-like helix structure. J Mol Biol 374:688-704.

41. Bugg CW, Isas JM, Fischer T, Patterson PH, Langen R (2012) Structural features and functional role of huntingtin exons in Drosophila points to exon 1 as the most pathogenic species in Huntington disease. Hum Mol Genet 21:2461-2472.

42. Martindale D, et al. (2012) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nat Genet 18:150-154.

43. Wanker EE, et al. (1999) Membrane filter assay for detection of amyloid-like protein aggregates. Methods Enzymol 317:361-374.

44. Arndt JR, Brown RJ, Burke KA, Legleiter J, Valentine SJ (2015) Lysine residues in the N-terminal huntingtin amphipathic alpha-helix play a key role in peptide aggregation. J Mass Spectrom 50:117-126.

45. Kelley NW, et al. (2009) The predicted structure of the headpiece of the huntingtin protein and its implications on huntingtin aggregation. J Mol Biol 388:919-927.

46. Atwal RS, et al. (2007) Huntingtin has a membrane association signal that can modulate its glutamine repeats. Hum Mol Genet 16:2368-2376.

47. Martindale D, et al. (2012) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nat Genet 18:150-154.

48. Warby SC, et al. (2005) Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion in vivo. Hum Mol Genet 14: 1569-1577.

49. Aiken CT, et al. (2009) Phosphorylation of threonine 3: Implications for huntingtin aggregation and neurotoxicity. J Biol Chem 284:29427-29436.

50. Bastamante MB, et al. (2015) Detection of huntingtin exon 1 phosphorylation by Phos-Tag SDS-PAGE: Predominant phosphorylation on threonine 3 and regulation by IKK. Biochem Biophys Res Commun 463:1317-1322.

51. Di Paolo T, et al. (2014) FTY720 (fingolimod) is a neuroprotective and disease-modifying agent in cellular and mouse models of Huntington disease. Hum Mol Genet 23:2251-2265.

52. Huang B, et al. (2015) Scalable production in human cells and biochemical characterization of full-length normal and mutant huntingtin. PLoS One 10:e0121055.

53. Todd J, et al. (2012) Ultraconservative flow-based immunoassays using single molecule counting. Mol Simul 38:1990-1995.

54. Wild DJ, et al. (2015) Quantification of mutant huntingtin protein in cerebrospinal fluid from Huntington’s disease patients. J Clin Invest 125:1979-1986.