Protein Modification

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One-Pot Semisynthesis of Exon 1 of the Huntingtin Protein: New Tools for Elucidating the Role of Posttranslational Modifications in the Pathogenesis of Huntington's Disease**

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Abstract: *The natural enzymes involved in regulating many of the posttranslational modifications (PTMs) within the first 17 residues (Nt17) of Huntingtin exon 1 (Httex1) remain unknown. A semisynthetic strategy that allows the site-specific introduction of PTMs within Nt17 by using expressed protein ligation (EPL) was developed. This strategy was used to produce untagged wild-type (wt) and T3-phosphorylated (pT3) Httex1 containing 23 glutamine residues (Httex1-23Q). Our studies show that pT3 significantly slows the oligomerization and fibrillization of Httex1-23Q and that Httex1 variants containing polyQ repeats below the pathogenic threshold readily aggregate and form fibrils in vitro. These findings suggest that crossing the polyQ pathogenic threshold is not essential for Httex1 aggregation. The ability to produce wt or site-specifically modified tag-free Httex1 should facilitate determining its structure and the role of N-terminal PTMs in regulating the functions of Htt in health and disease.*

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of the polyQ repeat (>37Q) within the first exon (ex1) of the Huntingtin (Htt) gene,[1] thereby resulting in enhanced Htt aggregation, increased toxicity in animal models of HD, and early disease onset in humans.[2] Despite constituting less than 3% of the total sequence, overexpression of the N-terminal domain of the Htt gene, which comprises residues 1–90 (Httex1), in cellular and animal models is sufficient to replicate some of the key features of HD pathology,[3] thus suggesting that Httex1 and/or other N-terminal fragments might constitute the primary toxic form of Htt.[3,4]

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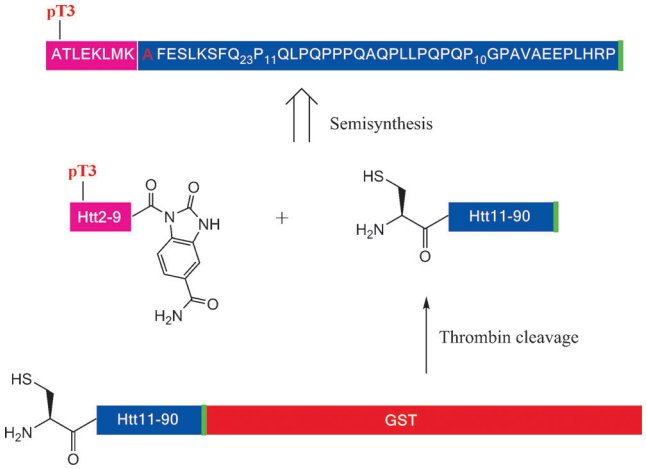
Httex1 contains an N-terminal sequence of 17 amino acids (Nt17) that is highly conserved in all vertebrate species.[4e] Several lines of evidence suggest that Nt17 plays an important role in regulating Httex1 structure,[5] aggregation,[6a,b,7] and subcellular localization,[8] as well as its interactions with organelles[4e,9] and other proteins. Nt17 also contains several residues that undergo posttranslational modifications (PTMs) including acetylation, phosphorylation, SUMOylation, and ubiquitination.[6] The high number of PTMs in the N-terminal region of Htt suggest that these modifications might be involved in the regulation of Htt function in health and disease. The close proximity of these different modifications suggests that they might act synergistically, and the cross-talk between different PTMs might constitute an additional molecular switch for regulating the dynamics of Htt function and aggregation. Elucidating the molecular mechanisms that regulate Htt PTMs and the consequences of such modifications for the biochemical, structural, aggregation, and toxic properties of Htt is thus essential for unraveling the molecular basis of the function(s) of this protein in health and disease.

Achieving these goals requires the development of methodologies that enable the site-specific introduction of single or multiple PTMs in Htt and the preparation of these proteins in sufficient amounts for detailed biochemical and biophysical studies. Unfortunately, the enzymes such as kinases, E3 ligase, and proteases, involved in regulating the PTMs within the Nt17 region of Htt remain unknown. Furthermore, many of the enzymes that have been identified lack specificity or show low efficiency for catalyzing these modifications in vitro,[6b] thus precluding the preparation of homogeneously modified forms of Htt. The site-specific introduction of unnatural or modified amino acids into peptides and proteins can be achieved by using a number of different chemical approaches, including solid-phase peptide synthesis (SPPS), native chemical ligation (NCL),[10] and expressed protein ligation (EPL).[11] We recently developed synthetic and semisynthetic strategies for the site-specific introduction of single or multiple PTMs in the C- or N-terminal domains of alpha-synuclein (α-syn).[12] Using these strategies, we generated homogeneously modified (acetylated, ubiquitinylated, and phosphorylated) forms of α-syn in mg quantities. These advances made it possible to investigate the effects of selective phosphorylation at S129 and Y125, N-terminal acetylation, and mono- and polyubiquitination[12b,c] on the structure, aggregation, membrane binding, and subcellular localization of α-syn.



Scheme 1. Schematic depiction showing the amino acid sequence of exon 1 of the Huntingtin protein (Httex1) and our semisynthetic strategy for preparing wt and T3-phosphorylated Httex1-23Q. The green line represents the additional four amino acids (LVPR) remaining after thrombin cleavage and removal of the GST fusion tag at the C terminus of the recombinant fragment Htt10-90(A10C)-23Q.

Herein, we describe a semisynthetic strategy for the site-specific introduction of PTMs in the Nt17 domain of Httex1 by using EPL (Scheme 1). This strategy was subsequently used to produce Httex1 without large protein tags and to determine the effect of phosphorylation at threonine 3 (T3) on the structure and aggregation of Httex1-23Q. Our strategy is based on the NCL of a synthetic Nbz-peptide fragment[13] (Nbz = N-acyl-benzimidazolinone) containing the desired PTM (Htt2-9-Nbz), and a recombinantly expressed C-terminal fragment in which the alanine residue at position 10 was replaced with a cysteine residue (Htt10-90(A10C)GST-23Q) to mediate NCL. The conversion of the cysteine residue back to alanine was achieved under desulfurization[14] conditions (0.3 M tris(2-carboxyethyl)phosphine (TCEP), 840 mM *tert*-butyl mercaptan and 5.4 mM of the azo initiator VA-044 at 37 °C for 2 h). The expression and purification of the C-terminal fragment bearing an N-terminal cysteine residue presented a series of challenges requiring extensive studies and the optimization of several expression and purification parameters, including the expression time and temperature, bacterial strains, and chromatographic purification conditions. To achieve this, we designed a vector expressing the construct Htt10-90(A10C)GST-23Q, a truncated Httex1-23Q with an N-terminal cysteine, fused to GST at the C-terminus of the protein (**1**; Figure S1 in the Supporting Information). To facilitate the removal of GST after purification of the proteins, we introduced a thrombin cleavage site that leaves the shortest possible sequence, with cleavage after arginine (LVPR′G). Expression in *E. coli* resulted in the generation of Htt10-90(A10C)GST-23Q with an N-terminal cysteine residue. The protein was then purified by using a GST affinity column and fragment **1** was obtained with > 90 % purity as confirmed by SDS-PAGE, Western Blot (WB), MALDI-TOF MS and UPLC analyses (Figure S2), and with a yield of 45 mg/2 L of bacterial culture. The impurities, visible on the gel, represent truncated side-products, which are commonly generated and observed during the expression of different recombinant GST-Htt fragments (Figure S2 A).[15] Significant efforts were made to optimize the thrombin cleavage conditions because of the occurrence of additional cleavage events and the generation of side-products during the cleavage reaction (See Figures S3 and S5). Removing the dithiothreitol (DTT) and reducing the reaction time minimized the formation of miscleavages and side products, and efficient GST cleavage was achieved within 4 h (0.8 units of thrombin per nmol of protein in 50 mM Tris buffer, pH 8). The reaction was monitored by using UPLC and SDS-PAGE (Figure S1 B). The desired product **2** eluted earlier than cleaved GST and was easily separated from any remaining starting material by using an optimized cation-exchange chromatography purification protocol (Figure S4). This procedure yielded 10 mg/2 L of culture of highly pure fragment **2** with the expected mass of 9525 Da (Figure S1 C). This mass includes a 26 Da thiazolidine adduct as previously reported,[12,16] which was removed by treating fragment **2** with methoxylamine. Two synthetic peptides corresponding to Htt2-9-Nbz (peptide **3**) and Htt2-9-pT3-Nbz (peptide **4**) were synthesized (Figure 1 A), purified, and ligated separately to fragment **2** to generate Httex1-23Q (**7**, wt) and Httex1-23Q phosphorylated at T3 (**8**, pT3; Figure 1 A, B). The NCL reactions were performed under denaturing conditions (6 M guanidine hydrochloride, 0.2 M phosphate buffer pH 7.4, and 20 mM TCEP). A total of 15 mg of fragment **2** (1.5 μmol) was reacted with 1.5 mole eq of Nbz-peptide and the product formation was monitored by UPLC (Figure 1 A) and mass spectrometry. The NCL proceeded readily with approximately 90 % ligation achieved after 1–2 h, and additional Nbz-peptide (0.5 eq) was added to drive the reaction to completion. Under these conditions, complete conversion of fragment **2** was achieved after 3–4 h, with more than 95 % yield of the desired product. The ligated products **5** and **6** were directly desulfurized without isolation (3 M guanidine hydrochloride, 0.3 M TCEP, 0.84 M *tert*-butyl mercaptan and 5.4 mM VA-044 in 0.2 M phosphate buffer pH 7.4). Complete desulfurization as evidenced by the loss of 32 mass units from each protein was achieved after 2 h. One-pot desulfurization is possible owing to the absence in the ligation reaction of thiols, which are detrimental for the desulfurization reaction.[17]

The final semisynthetic proteins were directly purified through reverse-phase (RP)-HPLC to yield 5.2 mg of **7** (0.5 μmol, 33 %) and 6.8 mg **8** (0.65 μmol, 43 %). MALDI-TOF MS, UPLC and SDS-PAGE analyses (Figure 1 B) revealed a single peak/band with masses corresponding to that of wt or pT3 Httex1-23Q. WB analyses of these samples showed that wt and pT3 Httex1-23Q were detectable with anti-htt1-82 and anti-pT3 antibodies, respectively (Figure 1 B).

Having established the purity and chemical integrity of the semisynthetic wt and pT3 Httex1-23Q, we focused on characterizing and comparing the secondary structure, aggregation propensity, and membrane binding properties of the two proteins by using circular dichroism (CD) spectroscopy, a thioflavin T (ThT) binding assay, atomic force microscopy (AFM), and transmission electron microscopy (TEM). In solution (10 mM Tris, 75 mM NaCl, pH 7.4), both wt and pT3 Httex1-23Q exhibited CD spectra with minima at ~203 nm,

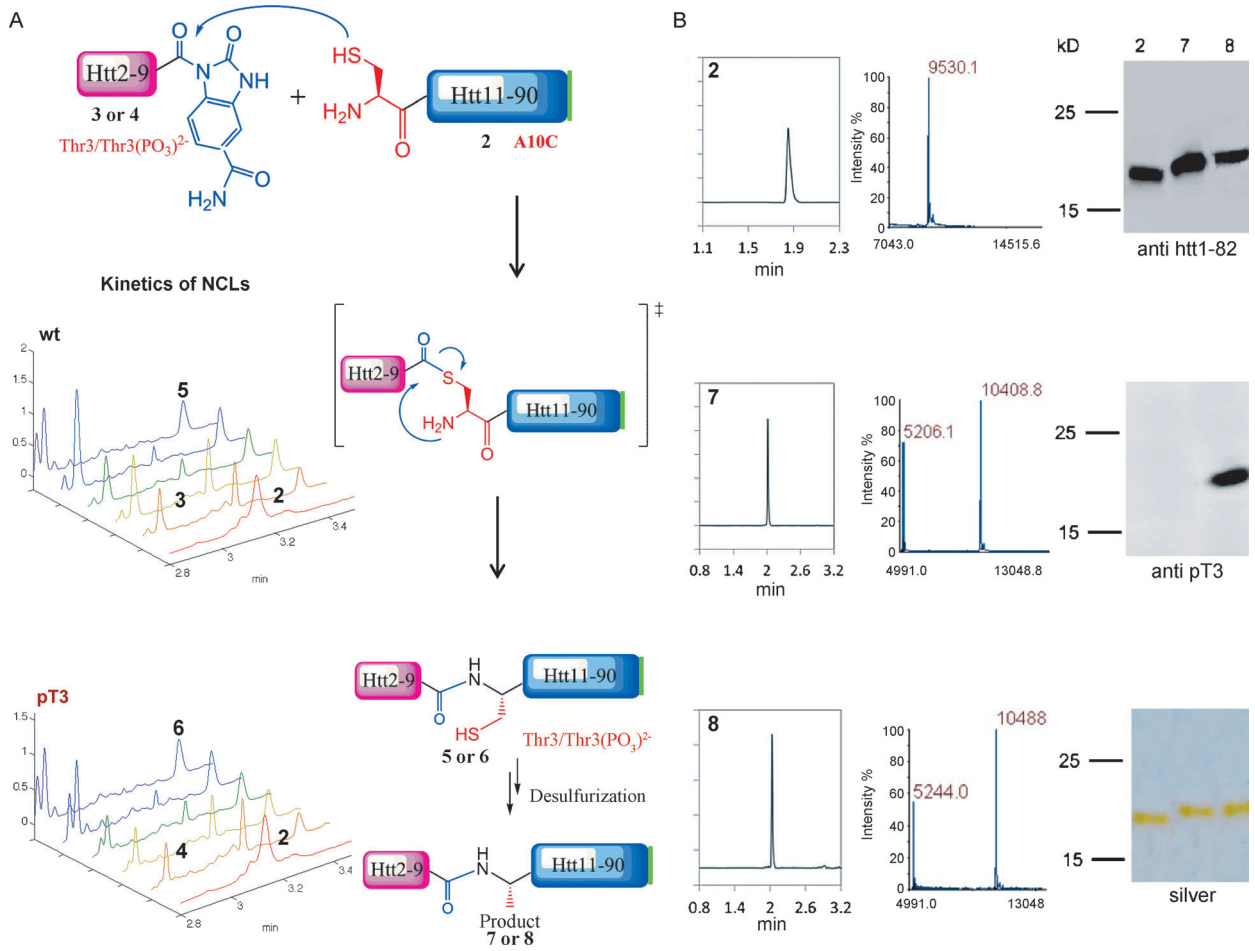


Figure 1. A) Schematic depiction of the semisynthesis of wt (**7**) and pT3 (**8**) Httex1-23Q. The native chemical ligation reactions were monitored by UPLC. B) The purity of fragment **2** and the final products **7** and **8** was assessed by UPLC, MALDI-TOF, silver staining, and western blotting with antibodies against the polyQ repeat region (anti htt1-82 MAB5492, Millipore) and a phospho-T3-specific antibody.

a result consistent with predominantly disordered structures (Figure 2A).

Previous studies have shown that Nt17 assumes an α-helical conformation in the Httex1 protein fused to MBP,[18] and forms transient helical structure when the Nt17 peptide is studied in isolation in the presence of alcohols such as trifluoroethanol (TFE).[19] Nt17 has also been implicated in the membrane binding and subcellular localization of Httex1.[4c,9] To determine whether phosphorylation at T3 influences the helical propensity of the protein, we compared the CD spectra of wt and pT3 Httex1-23Q under conditions that favor the helical conformation and mimic the membrane environment,[20] that is, in the presence of increasing amounts of TFE (Figure 2B and S6) or lipids (neutral lipid POPC or a negative charged POPC/POPS lipid mixture, Figure S7). The addition of increasing amounts of TFE resulted in a slight red shift of the minima from 203 nm to 208 nm and the appearance of a shoulder at 220 nm, thus suggesting the induction of partial α-helical structure at the N terminus. The CD spectra suggest that pT3 exhibits a slightly increased helical propensity as evidenced by the presence of a shoulder at 220 nm in the absence of TFE. Neither wt nor pT3 Httex1-23Q exhibited changes in their CD spectra in the presence of different concentrations of neutral lipids (POPC) or a negatively charged lipid mixture (POPC/POPS 3:1; Figure S7).

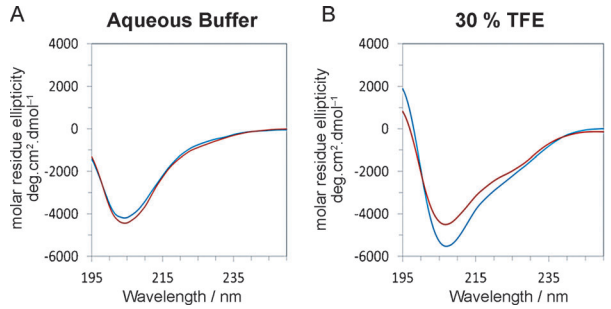


Figure 2. Far-UV CD of wt (blue) and pT3 (red) Httex1-23Q in aqueous buffer (A) and in the presence of 30% of TFE (B). Samples in aqueous buffer were used for aggregation studies to a final concentration of 50 μM, while a concentration of 30 μM was used for the TFE treatments.

To assess the effect of phosphorylation at T3 on the aggregation properties of Httex1-23Q, we first employed AFM, which allows the visualization and characterization of early aggregation events and the quantitative assessment of

the size and morphological distribution of the aggregates. Wild-type and pT3 Httex1-23Q were incubated separately at 37 °C without agitation and aggregation was monitored by AFM over 14 days. The samples were filtered through 100 kDa molecular-weight cutoff filters to remove any preformed aggregates prior to incubation at 37 °C. Figure 3 A shows AFM images of wt and pT3 Httex1-23Q after 1 h, 7 days and 14 days of incubation at 37 °C. Within the first few hours of incubation, wt Httex1-23Q formed oligomers with a typical height of 1 nm and a broad distribution of diameters ranging from 1–100 nm (Figure 3 C), whereas pT3 Httex1-23Q samples showed oligomers with an average height of approximately 1 nm and diameters of less than 20 nm (Figure 3 C). After 7 days, mainly fibrils, comprising two populations with average heights of 4 nm or 6 nm, were observed for the wt Httex1-23Q (Figure 3 A,B). By contrast, no fibrillar structures could be detected in the pT3 Httex1-23Q samples by AFM or TEM. Instead, only oligomers with diameters ranging from 20 to 120 nm were observed (Figure 3 D). After 14 days, wt Httex1-23Q showed extensive fibril formation (Figure 3 A), whereas pT3 Httex1-23Q had formed predominantly elongated curvilinear structures with an average height of about 2 nm (Figure 3 A,B), in addition to small amounts of mature fibrils with an average height of about 5 nm (Figure 3 B). These results demonstrate that wt Httex1-23Q aggregates and forms fibrils more rapidly than pT3 Httex-23Q and reveal that wt Httex1 containing polyQ repeats below the pathogenic threshold exhibit high oligomerization propensity as evidenced by the rapid formation of oligomers by wt Httex1-23Q. Similar structures and oligomerization/fibrillization kinetics were observed when the aggregation of the two proteins was monitored by using TEM (Figure S9). Furthermore, the inhibitory effect of phosphorylation at T3 was also confirmed by monitoring the loss of monomeric protein by using size exclusion chromatography (Figure S10). Altogether, these data demonstrate that phosphorylation at T3 slows but does not prevent the aggregation of Httex1-23Q.

Several studies have demonstrated a strong correlation between the length of the polyQ repeat and the propensity of different proteins and model systems to aggregate and form inclusions in vitro and in vivo. Our AFM and TEM measurements demonstrate clearly that, contrary to previous reports,[21] Httex1 containing polyQ repeats below the pathogenic threshold (23Q) can still aggregate and form spherical and short fibril-like structures. Interestingly, wt and pT3 Httex1-23Q aggregates weakly bind to the amyloid-specific dye thioflavin T. One possible explanation for why the aggregation of Httex1 with nonpathogenic polyQ repeats has not been observed to date is that in previous studies, the aggregation properties of Httex1 were assessed by using fusion proteins in which peptides (6XHis, polylysine, HA, Myc, or Flag tags)[22] or proteins [e.g., glutathione S-transferases (GST) or maltose binding protein (MBP)][15,21] were fused to the N and/or C termini of Httex1. Although the fusion of GST or MBP enhances the solubility of mutant Httex1, the presence of such large protein tags could significantly alter the rate and pathway of Httex1 aggregation and change the structural properties of the Httex1 aggregates.

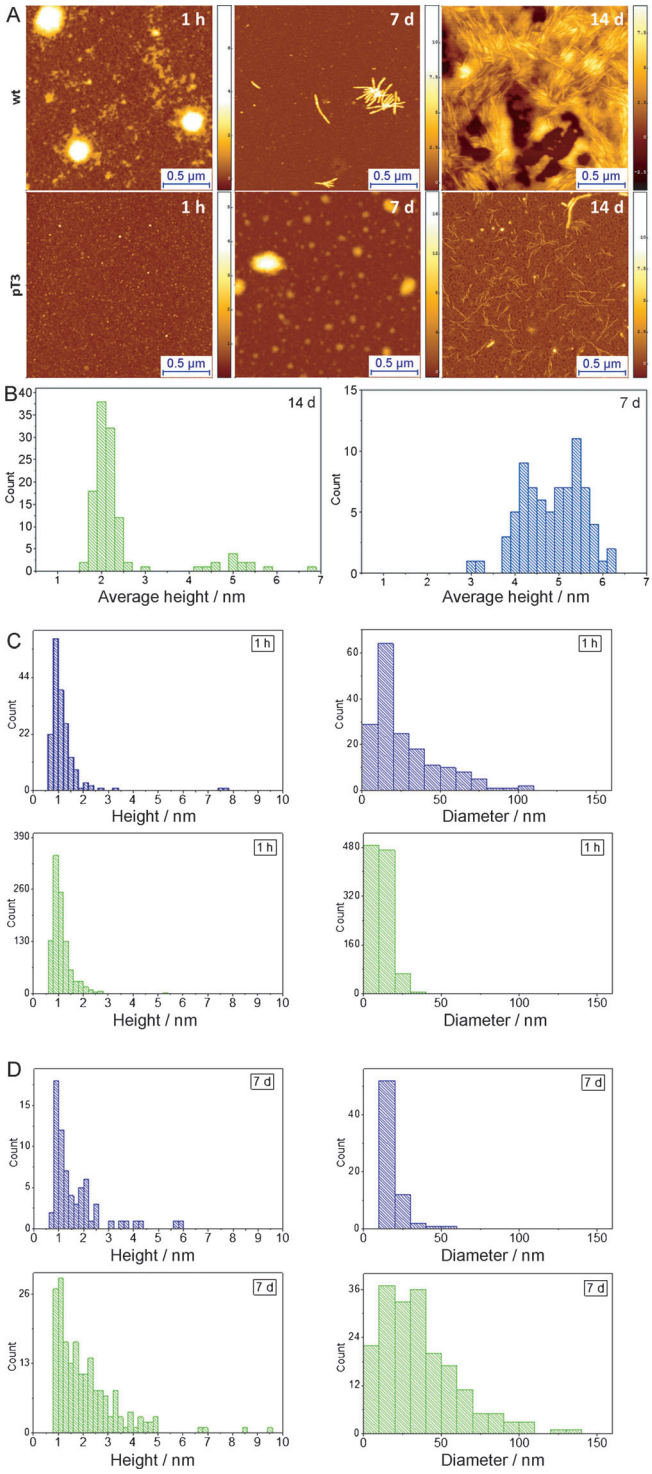


Figure 3. A) AFM images of wt and pT3 Httex1-23Q aggregates at 1 h, 7 days, and 14 days. Samples were prepared at a concentration of 50 μM in 10 mM Tris, 75 mM NaCl, pH 7.4 and statically incubated at 37 °C. B) Average height distribution of wt (blue) and pT3 (green) Httex1-23Q aggregates after 7 and 14 days, respectively. The plotted values correspond to the average height along the length of each fibril measured. Height and diameter distribution of wt (blue) and pT3 (green) Httex1-23Q oligomers formed after 1 h (C) and 7 days (D).

In the majority of the published work, the biochemical and aggregation properties of Httex1 were assessed after cleavage but not removal of these proteins tags in situ and, despite

their being present at equimolar levels in solution, the effect of the cleaved protein tags on the aggregation process was not assessed or accounted for. These limitations highlight the advantages of our semisynthetic strategies, which enable more accurate characterization of the structure and of the effect of polyQ-length and PTMs on the aggregation propensity and aggregate morphology of Httex1-23Q. In the absence of large protein tags, any changes we observe in the structure or aggregation properties of Httex1 are mainly due to changes in the intrinsic sequence and structure of the protein. Although our current semisynthetic strategy still results in the presence of an additional four amino acids (ca. 0.4 kDa) in the C terminus of the protein, this represents a very small change to the sequence of the protein compared to that introduced by the protein tags used in all the reported recombinant forms of Httex1, which range in MW from 12–42 kDa. Preliminary studies from our group show that the recombinant fragment Htt10-90(A10C)-23Q without any additional amino acids (thrombin cleavage site) can be prepared by using an intein-based strategy.

In conclusion, we describe herein the first one-pot semisynthetic strategy for the generation of untagged wt and phosphorylated (pT3) variants of exon 1 of the Huntingtin protein, Httex1-23Q. The general semisynthetic strategy described here can be easily extended to generate homogeneously phosphorylated, acetylated, or ubiquitinylated forms of Httex1-23Q in mg quantities by following the same approaches we recently used to generate similarly modified forms of α-syn.[12,23] Furthermore, preliminary studies from our group show that this approach can be used to prepare mutant forms of Httex1 containing expanded polyQ repeats (42Q; Figures S11 and S12), although the yields remain very low, mainly as a result of the high aggregation propensity of the mutant proteins (Figure S13). The availability of homogeneously and site-specifically modified forms of Httex1 should facilitate more detailed studies on the structural properties of Httex1 and the role of posttranslational modifications in regulating its structure, function, and interactions with other proteins. In addition, these reagents are desperately needed for the development of quantitative assays to determine whether the levels of specific Nt17 posttranslational modifications correlate with Htt toxicity and/or with disease progression and severity in HD. We focused on phosphorylation at T3 because, despite being among the most commonly observed Nt17 PTMs, this modification remains under-studied compared with phosphorylation at S13 and/or S16. Our AFM and TEM studies show for the first time that phosphorylation at T3 significantly slows the oligomerization and fibril formation of Httex1-23Q. In addition, we demonstrate that untagged Httex1 containing polyQ repeats below the pathogenic threshold can still aggregate and form fibrils. These findings suggest that crossing the polyQ pathogenic threshold confers additional toxic properties on Httex1 aggregates. Moreover, our results highlight the critical importance of assessing the effects of fusion proteins when investigating complex and sensitive processes such as protein aggregation and amyloid formation. More detailed characterization of the structure and aggregation properties of untagged Httex1 proteins containing polyQ repeats of different lengths should provide novel insight into the role of polyQ-repeat length in the pathogenesis of HD and the molecular and structural basis of Htt aggregation and toxicity.

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