Threonine 3 regulates Serine 13/16 phosphorylation in the huntingtin exon 1

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Abstract

An abnormal CAG repeat expansion occurring in the first exon of the huntingtin gene (HTTex1) is the cause of Huntington's disease (HD). In the N-terminal region, the huntingtin protein (HTT) contains 3 phosphorylatable residues - threonine 3 (T3) and serines 13 and 16 (S13/S16) - that, upon phosphorylation, strongly modulate aggregation and toxicity of mutant HTTex1 fragments. Here, we present evidence that phosphorylation of T3 prevents the diphosphorylation of S13/S16, while its dephosphorylation increases the chances of S13/S16 diphosphorylation in mutant HTTex1-expressing cells. This single observation has massive potential relevance for the understanding of HTTex1 structure and function and the treatment of HD.

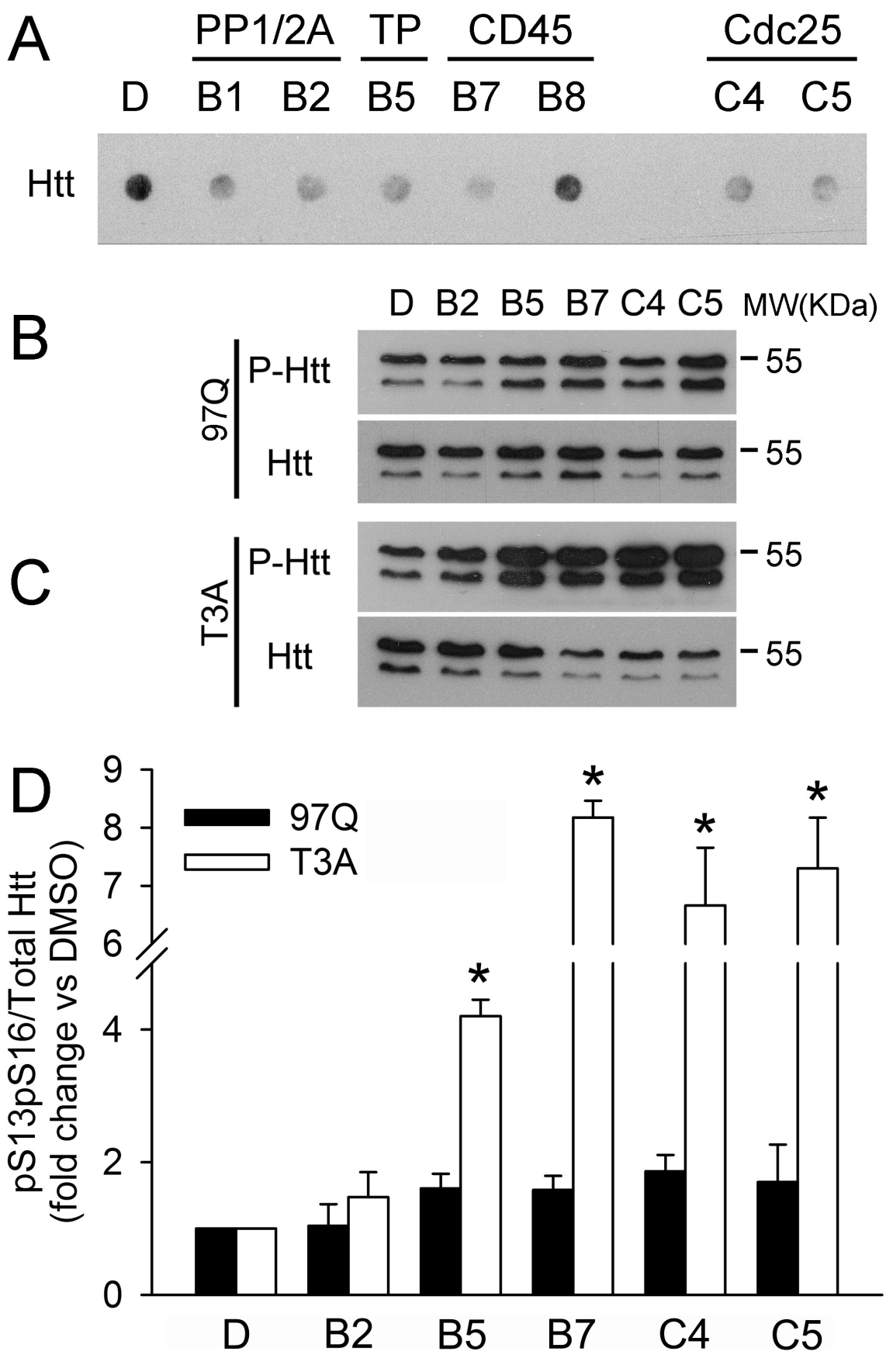
Introduction

Huntington's disease (HD) is a neurodegenerative disorder caused by an abnormal expansion of a CAG repeat located in exon 1 of the *HTT* gene [1]. Expression of polyglutamine-expanded exon 1 (HTTex1) is sufficient to cause the disease in animal models [2] [3]. Interestingly, the polyglutamine tract is flanked in its N-terminus by a short amphipathic alpha-helix composed of 17 amino acids (N17) [4] [5] that control nucleo-cytoplasmic shuttling, membrane binding, aggregation and toxicity of mutant HTT [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18].

Mounting evidence suggests that N17 phosphorylation is associated with reduced HTT accumulation and neurotoxicity [19] [20] [21] [22] [23] [24] [25]. Double phosphomimic substitutions at S13 and S16 reverted histopathology [26]. Single T3, S13 or S16 phosphorylation events are enough to completely block HTT aggregation in cell lines but had cell-, tissue- or developmental stage-dependent effects in *Drosophila* models of HD [23]. Mimicking T3 phosphorylation ameliorates polyQ-mediated toxicity in a *Drosophila* model of HD, but increases the amount of insoluble HTTex1 species [8]. Recent studies on HTTex1 peptides demonstrated that inducing phosphorylation [25] [27] [28], or adding a negative charge (e.g. aspartic acid mutations) [24] to the T3 residue, leads to a decrease in fibril elongation [23]. Furthermore, T3 phosphorylation was found to be reduced in samples from HD models and clinical patients, further supporting a key role for T3 in HD pathogenesis [25].

Objective

In this study- we aimed at elucidating a possible relationship between the phosphorylation state of T3, S13 and S16 in the huntingtin exon 1 fragments.


A
PP1/2A
TP
CD45
Cdc25
D
B1
B2
B5
B7
B8
C4
C5
Htt
B
D B2 B5 B7 C4 C5 MW(KDa)
97Q
P-Htt
Htt
55
55
C
T3A
P-Htt
Htt
55
55
D
pS13pS16/Total Htt
(fold change vs DMSO)
97Q
T3A
9
8
7
6
4
2
0
*
*
*
D
B2
B5
B7
C4
C5

a

Figure Legend

Figure 1. Threonine 3 controls Serine 13/16 phosphorylation in HTTex1.

H4 glioma cells were transfected with Venus-97QHTTex1 BiFC constructs and treated for 24 h with inhibitors against protein phosphatase 1/2A (PP1/2A, B1 and B2), tyrosine phosphatases (TP, B5), CD45 tyrosine phosphatase (B7 and B8) or Cdc25 phosphatase (C4 and C5) or the vehicle (DMSO 0.1% v/v, D). Proteins were extracted in native conditions and submitted to filter traps **(A)** or SDS-PAGE **(B-C)** and probed with anti-N17 (Htt) or anti-phosphoserine 13/16 antibodies (P-Htt).

(A) Representative filter trap showing that the phosphatase inhibitors prevented 97QHTTex1 aggregation, as the signal is directly proportional to the amount of aggregates retained in the filter.

(B) Representative immunobloting after SDS-PAGE of lysates from cells expressing 97QHTTex1 constructs without T3 mutations.

(C) Representative immunobloting after SDS-PAGE of lysates from cells expressing 97QHTTex1 constructs carrying a T3A.

(D) Graphic representation and statistical analysis of the average optic density in immunoblots from 3 independent experiments, with phosphoserine signals normalized versus total HTTex1 signals and versus the control with the vehicle (DMSO). *, significant versus DMSO, $p<0.01$.

Results & Discussion

Single phosphorylation events at T3, S13 or S16 are enough to completely abolish HTTex1 aggregation in living mammalian cells, and several phosphatase inhibitors are able to prevent HTTex1 aggregation [23] (Fig. 1A). In order to know if these inhibitors prevent HTTex1 aggregation by means of its phosphorylation at serines 13 and 16, we tested their effect on serine phosphorylation by means of immunoblotting with an anti-phosphoserine 13/16 antibody (Fig. 1B) (see Suppl. Info. for full names). The immunoblots show two bands corresponding to 97QHTTex1-Venus1 and 97QHTTex1-Venus2 constructs, as we carried out these experiments in a bimolecular fluorescence complementation (BiFC) model of HD (see Methods). Although 97QHTTex1-expressing cells treated with B5, B7, C4, and C5 inhibitors showed a trend towards increased levels of serine phosphorylation, no statistically significant differences were detected (Fig. 1C). However, when the phosphatase inhibitors were applied to T3A phosphoresistant constructs (Fig. 1D), a striking increase was observed in HTTex1 serine phosphorylation, with the exception of the protein phosphatase 1/2A inhibitor (B2). We were not able to detect S13/S16 diphosphorylation in T3D constructs, either in the presence or absence of phosphatase inhibitors (data not shown). Taken together, these results suggest that T3 phosphorylation state controls serine phosphorylation in HTTex1. We cannot rule out the possibility that the structural changes induced by mutations in T3 could facilitate or prevent access to the phosphoserine antibody to its epitope. However, this is still a very relevant observation for the HD field, as N17 phosphorylation has attracted great interest for the putative treatment of HD. Serine phosphorylation has been shown to prevent HTT aggregation and toxicity in cell and animal models of the disease [19] [21] [23] [26], and current evidence also indicates that T3 could be relevant to HD pathology [8] [23] [24] [25] [27] [28] [29]. Recent work on synthetic peptides bearing the first 19 amino acids of HTTex1 suggests that cross-talk between phosphorylation at T3 and S13 and/or S16 might regulate the alpha-helical conformation of N17 *in vitro* [30].

Conclusions

Our results strongly support that there is significant interaction between T3 and S13/S16 residues. Further research should be carried out to elucidate this interesting relationship.

Alternative Explanations

Additional Information

Methods

The HTTex1 bimolecular fluorescence complementation system was previously described [23] [31]. Briefly, two constructs for the expression of Venus 1 (1-157 amino acids) and Venus 2 (158-236 amino acids) fused to HTTex1 were transfected into human H4 neuroglioma cells (ATCC HTB-148, LGC Standards, Barcelona, Spain) and treated with the different phosphatase inhibitors or the vehicle (final concentration DMSO 0.1% v/v) for 24 h (Table I) (Enzo Life Sciences, Lausen, Switzerland). Filter trap assays and immunoblotting were carried out as previously described [23]. Total anti-N17 and anti-phosphoserine 13/16 antibodies were kindly provided by Dr. Ray Truant (McMaster University, ON, Canada) [20] [21]. Data were plotted and analyzed by means of Sigmaplot software (Systat Software, Inc., San Jose, CA, USA). Data are shown as the average of 3 independent experiments and error bars represent the standard deviation. Data were analyzed by means of a one-way ANOVA followed by the Holm-Sidak test of means. Significance was accepted at $p<0.01$.

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Ethics Statement

Not applicable.

Citations

[1] Adam Rosenblatt et al. "Age, CAG repeat length, and clinical progression in Huntington's disease". In: *Movement Disorders* 27.2 (2012), pp. 272–276. DOI: 10.1002/mds.24024. URL: https://doi.org/10.1002/mds.24024.

[2] Laura Mangiarini et al. "Exon 1 of the HD Gene with an Expanded CAG Repeat Is Sufficient to Cause a Progressive Neurological Phenotype in Transgenic Mice". In: *Cell* 87.3 (1996), pp. 493–506. DOI: 10.1016/s0092-8674(00)81369-0. URL: https://doi.org/10.1016/s0092-8674(00)81369-0.

[3] Gillian P. Bates, Laura Mangiarini, and Stephen W. Davies. "Transgenic Mice in the Study of Polyglutamine Repeat Expansion Diseases". In: *Brain Pathology* 8.4 (1998), pp. 699–714. DOI: 10.1111/j.1750-3639.1998.tb00196.x. URL: https://doi.org/10.1111/j.1750-3639.1998.tb00196.x.

[4] Giulia Rossetti et al. "Conformations of the Huntingtin N-term in aqueous solution from atomistic simulations". In: *FEBS Letters* 585.19 (2011), pp. 3086–3089. DOI: 10.1016/j.febslet.2011.08.036. URL: https://doi.org/10.1016/j.febslet.2011.08.036.

[5] Matthias Michalek et al. "Membrane Interactions of the Amphipathic Amino Terminus of Huntingtin". In: *Biochemistry* 52.5 (2013), pp. 847–858. DOI: 10.1021/bi301325q. URL: https://doi.org/10.1021/bi301325q.

[6] Randy Singh Atwal et al. "Huntingtin has a membrane association signal that can modulate huntingtin aggregation, nuclear entry and toxicity". In: *Human Molecular Genetics* 16.21 (2007), pp. 2600–2615. DOI: 10.1093/hmg/ddm217. URL: https://doi.org/10.1093/hmg/ddm217.

[7] Erica Rockabrand et al. "The first 17 amino acids of Huntingtin modulate its sub-cellular localization, aggregation and effects on calcium homeostasis". In: *Human Molecular Genetics* 16.1 (2007), pp. 61–77. DOI: 10.1093/hmg/ddl440. URL: https://doi.org/10.1093/hmg/ddl440.

[8] Charity T. Aiken et al. "Phosphorylation of Threonine 3: IMPLICATIONS FOR HUNTINGTIN AGGREGATION AND NEUROTOXICITY". In: *The Journal of Biological Chemistry* 284.43 (2009), pp. 29427–29436. DOI: 10.1074/jbc.m109.013193. URL: https://doi.org/10.1074/jbc.m109.013193.

[9] Kimberly B. Kegel et al. "Huntingtin Associates with Acidic Phospholipids at the Plasma Membrane". In: *The Journal of Biological Chemistry* 280.43 (2005), pp. 36464–36473. DOI: 10.1074/jbc.m503672200. URL: https://doi.org/10.1074/jbc.m503672200.

[10] Jonathan Cornett et al. "Polyglutamine expansion of huntingtin impairs its nuclear export". In: *Nature Genetics* 37.2 (2005), pp. 198–204. DOI: 10.1038/ng1503. URL: https://doi.org/10.1038/ng1503.

[11] Ashwani K Thakur et al. "Polyglutamine disruption of the huntingtin exon 1 N terminus triggers a complex aggregation mechanism". In: *Nature Structural & Molecular Biology* 16.4 (2009), pp. 380–389. DOI: 10.1038/nsmb.1570. URL: https://doi.org/10.1038/nsmb.1570.

[12] Stephen Tam et al. "The chaperonin TRiC blocks a huntingtin sequence element that promotes the conformational switch to aggregation". In: *Nature Structural & Molecular Biology* 16.12 (2009), pp. 1279–1285. DOI: 10.1038/nsmb.1700. URL: https://doi.org/10.1038/nsmb.1700.

[13] Ferdinando Fiumara et al. "Essential Role of Coiled Coils for Aggregation and Activity of Q/N-Rich Prions and PolyQ Proteins". In: *Cell* 143.7 (2010), pp. 1121–1135. DOI: 10.1016/j.cell.2010.11.042. URL: https://doi.org/10.1016/j.cell.2010.11.042.

[14] Zhiqiang Zheng et al. "An N-terminal Nuclear Export Signal Regulates Trafficking and Aggregation of Huntingtin (Htt) Protein Exon 1". In: *The Journal of Biological Chemistry* 288.9 (2013), pp. 6063–6071. DOI: 10.1074/jbc.m112.413575. URL: https://doi.org/10.1074/jbc.m112.413575.

[15] Murali Jayaraman et al. "Slow Amyloid Nucleation via α-Helix-Rich Oligomeric Intermediates in Short Polyglutamine-Containing Huntingtin Fragments". In: *Journal of Molecular Biology* 415.5 (2012), pp. 881–899. DOI: 10.1016/j.jmb.2011.12.010. URL: https://doi.org/10.1016/j.jmb.2011.12.010.

[16] Kathleen A. Burke et al. "Huntingtin disrupts lipid bilayers in a polyQ-length dependent manner". In: *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1828.8 (2013), pp. 1953–1961. DOI: 10.1016/j.bbamem.2013.04.025. URL: https://doi.org/10.1016/j.bbamem.2013.04.025.

[17] Kathleen A. Burke et al. "The Interaction of Polyglutamine Peptides with Lipid Membranes Is Regulated by Flanking Sequences Associated with Huntingtin". In: *The Journal of Biological Chemistry* 288.21 (2013), pp. 14993–15005. DOI: 10.1074/jbc.m112.446237. URL: https://doi.org/10.1074/jbc.m112.446237.

[18] Matthew B. Veldman et al. "The N17 domain mitigates nuclear toxicity in a novel zebrafish Huntington's disease model". In: *Molecular Neurodegeneration* 10.1 (2015), p. 67. DOI: 10.1186/s13024-015-0063-2. URL: https://doi.org/10.1186/s13024-015-0063-2.

[19] Leslie Michels Thompson et al. "IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome". In: *The Journal of Cell Biology* 187.7 (2009), pp. 1083–1099. DOI: 10.1083/jcb.200909067. URL: https://doi.org/10.1083/jcb.200909067.

[20] Randy Singh Atwal et al. "Kinase inhibitors modulate huntingtin cell localization and toxicity". In: *Nature Chemical Biology* 7.7 (2011), pp. 453–460. DOI: 10.1038/nchembio.582. URL: https://doi.org/10.1038/nchembio.582.

[21] Alba Di Pardo et al. "Ganglioside GM1 induces phosphorylation of mutant huntingtin and restores normal motor behavior in Huntington disease mice". In: *Proceedings of the National Academy of Sciences* 109.9 (2012), pp. 3528–3533. DOI: 10.1073/pnas.1114502109. URL: https://doi.org/10.1073/pnas.1114502109.

[22] Rakesh Mishra et al. "Serine Phosphorylation Suppresses Huntingtin Amyloid Accumulation by Altering Protein Aggregation Properties". In: *Journal of Molecular Biology* 424.1-2 (2012), pp. 1–14. DOI: 10.1016/j.jmb.2012.09.011. URL: https://doi.org/10.1016/j.jmb.2012.09.011.

[23] Joana Branco-Santos et al. "Protein phosphatase 1 regulates huntingtin exon 1 aggregation and toxicity". In: *Human Molecular Genetics* 26.19 (2017), pp. 3763–3775. DOI: 10.1093/hmg/ddx260. URL: https://doi.org/10.1093/hmg/ddx260.

[24] Anass Chiki et al. "Mutant Exon1 Huntingtin Aggregation is Regulated by T3 Phosphorylation-Induced Structural Changes and Crosstalk between T3 Phosphorylation and Acetylation at K6". In: *Angewandte Chemie International Edition* 56.19 (2017), pp. 5202–5207. DOI: 10.1002/anie.201611750. URL: https://doi.org/10.1002/anie.201611750.

[25] Cristina Cariulo et al. "Phosphorylation of huntingtin at residue T3 is decreased in Huntington's disease and modulates mutant huntingtin protein conformation". In: *Proceedings of the National Academy of Sciences* 114.50 (2017), E10809–E10818. DOI: 10.1073/pnas.1705372114. URL: https://doi.org/10.1073/pnas.1705372114.

[26] Xiaofeng Gu et al. “Serines 13 and 16 Are Critical Determinants of Full-Length Human Mutant Huntingtin Induced Disease Pathogenesis in HD Mice”. In: *Neuron* 64.6 (2009), pp. 828–840. DOI: 10.1016/j.neuron.2009.11.020. URL: https://doi.org/10.1016/j.neuron.2009.11.020.

[27] Annalisa Ansaloni et al. “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”. In: *Angewandte Chemie International Edition* 53.7 (2014), pp. 1928–1933. DOI: 10.1002/anie.201307510. URL: https://doi.org/10.1002/anie.201307510.

[28] James R. Arndt et al. “Huntingtin N-Terminal Monomeric and Multimeric Structures Destabilized by Covalent Modification of Heteroatomic Residues”. In: *Biochemistry* 54.28 (2015), pp. 4285–4296. DOI: 10.1021/acs.biochem.5b00478. URL: https://doi.org/10.1021/acs.biochem.5b00478.

[29] Maria Blaire Bustamante et al. “Detection of huntingtin exon 1 phosphorylation by Phos-Tag SDS-PAGE: Predominant phosphorylation on threonine 3 and regulation by IKKβ”. In: *Biochemical and Biophysical Research Communications* 463.4 (2015), pp. 1317–1322. DOI: 10.1016/j.bbrc.2015.06.116. URL: https://doi.org/10.1016/j.bbrc.2015.06.116.

[30] Sean M. DeGuire et al. “N-terminal Huntingtin (Htt) phosphorylation is a molecular switch regulating Htt aggregation, helical conformation, internalization, and nuclear targeting”. In: *The Journal of Biological Chemistry* 293.48 (2018), pp. 18540–18558. DOI: 10.1074/jbc.ra118.004621. URL: https://doi.org/10.1074/jbc.ra118.004621.

[31] Federico Herrera et al. “Visualization of cell-to-cell transmission of mutant huntingtin oligomers”. In: *PLOS Currents Huntington Disease* 3 (2011), RRN1210. DOI: 10.1371/currents.rrn1210. URL: https://doi.org/10.1371/currents.rrn1210.