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The ubiquitin conjugating enzyme Ube2W regulates solubility of the Huntington's Disease protein, huntingtin

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Abstract

Huntington's Disease (HD) is caused by a CAG repeat expansion that encodes a polyglutamine (polyQ) expansion in the HD disease protein, huntingtin (HTT). PolyQ expansion promotes misfolding and aggregation of mutant HTT (mHTT) within neurons. The cellular pathways, including ubiquitin-dependent processes, by which mHTT is regulated remain incompletely understood. Ube2W is the only ubiquitin conjugating enzyme (E2) known to ubiquitinate substrates at their amino (N)-termini, likely favoring substrates with disordered N-termini. By virtue of its N-terminal polyQ domain, HTT has an intrinsically disordered amino terminus. In studies employing immortalized cells, primary neurons and a knock-in (KI) mouse model of HD, we tested the effect of Ube2W deficiency on mHTT levels, aggregation and neurotoxicity. In cultured cells, deficiency of Ube2W activity markedly decreases mHTT aggregate formation and increases the level of soluble monomers, while reducing mHTT-induced cytotoxicity. Consistent with this result, the absence of Ube2W in *HdhQ200* KI mice significantly increases levels of soluble monomeric mHTT while reducing insoluble oligomeric species. This study sheds light on the potential function of the non-canonical ubiquitin-conjugating enzyme, Ube2W, in this polyQ neurodegenerative disease.

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Keywords

Huntington's disease; Huntingtin; ubiquitination; ubiquitin-conjugating enzyme; Ube2W; protein misfolding; neurodegeneration

Introduction

The accumulation of ubiquitinated proteins is a pathological hallmark shared by many neurodegenerative diseases including Huntington's disease (HD). HD is the most common among nine polyglutamine (polyQ) diseases, caused by CAG repeat expansions that encode a polyQ stretch in the disease proteins. In HD, when the polyQ length exceeds a threshold close to 40 glutamines, the HD protein huntingtin (HTT) becomes pathogenic (1). HD patients typically develop involuntary movements such as chorea and dystonia, neuropsychiatric symptoms and cognitive deficits. Pathologically, HD displays preferential degeneration of the striatum, with the medium spiny neurons expressing dopamine D2 receptors, DARPP-32 and enkephalin being particularly vulnerable(2–5).

In HTT, the N-terminal 17 amino acids and adjacent polyQ stretch are largely unstructured and disordered, contributing to the relative “disorderedness” of this domain of the protein(6). Mutant HTT (mHTT) becomes insoluble and forms aggregates known as inclusion bodies in neuronal nuclei, perinuclear regions and neurites(7–9). These inclusion bodies contain N-terminal fragments of HTT, ubiquitin, proteasomal components and numerous other proteins(8). The presence of ubiquitin and proteasomal subunits in HD inclusions supports the importance of ubiquitin-dependent pathways in HD. However, the precise roles of ubiquitination pathways in HD are not fully understood. Studies have shown that numerous components of ubiquitin-dependent systems can contribute to and alter HTT degradation, aggregation and cytotoxicity(10–12). K11, K48 and K63-linked polyubiquitin-modified proteins have been identified in HD inclusions, suggesting a regulatory role for the proteasomal and/or autophagy systems in inclusion formation(13, 14). Furthermore, ubiquitin-like molecules such as SUMO have been implicated as regulators of HTT aggregation(15, 16).

Ubiquitin (Ub) conjugation requires the sequential action of enzymes to target ubiquitin to substrates: Ub activating enzyme (E1), Ub conjugating enzyme (E2) and Ub ligase (E3). Given the diversity in Ub-chain lengths, linkages and substrate attachment sites, dramatically different kinds of ubiquitination can occur. The diversity of ubiquitination patterns is primarily achieved by different combinations of E2/E3 pairs. Recently, Ube2W was identified as the only E2 that initiates ubiquitination at the α -amino group of the N-termini of proteins, preferably recognizing substrates with disordered N-termini (17–19). Ube2W can function with various ubiquitin ligases including the C terminus of Hsc-70-interacting protein (CHIP) and the BRCA1/BARD1 complex to mono-ubiquitinate select substrates at their amino-termini(17, 18, 20–22). *Ube2W* null mice show an incompletely penetrant multi-organ defect and post-natal lethality, suggesting an important function of N-terminal ubiquitination by Ube2W(23).

The relatively disordered nature of the N-terminal domain of HTT predicts it to be a potential candidate target for Ube2W. In this study, we employ a range of model systems to study the effect of Ube2W on HTT protein levels, aggregation and neurotoxicity.

Materials and Methods

Animals

Ube2W germline KO mice were generated as described in our previous publication(23). *Ube2W* neuronal KO mice were generated by crossing *Ube2W*^{Flox/Flox} mice with Nestin promoter driven Cre transgenic mice from Jackson Laboratory (B6.Cg-Tg(Nescre)1kln/J), in which Cre is expressed in central nervous systems. All mice in this study were maintained on a pure C57BL/6 genetic background, housed in cages with a maximum number of five animals and maintained in a standard 12-hour light/dark cycle with food and water *ad libitum*. Genotyping was performed using DNA isolated from tail biopsy at the time of weaning, otherwise indicated. Genotype was determined by PCR amplification of a fragment of gene-of-interest. For crosses to a mouse model of HD, we utilized male HD-KI Q₂₀₀ (*Hdh*Q₂₀₀) mouse model expressing murine Htt with ~200 CAG repeats (CAG sizing is verified by Laragen) (24, 25). Mice were euthanized at the specified ages, anesthetized with ketamine/xylazine, and perfused transcardially with phosphate-buffered saline.

PCR primers

For *Ube2W* KO genotyping: 5' AAAGGAAGAGCCCAGTATGGACCCT3' and 5' AGAGTCCCTGCAGCTATTAC3'; Cre genotyping: 5' GTCCAATTTACTGACCGTACACC3', 5' GTTATTTCGGATCATCAGCTACACC3', 5' CTAGGCCACAGAATTGAAAGATCT3' and 5' GTAGGTGGAAATTCTAGCATCATCC; Flox genotyping: 5' AAAGGAAGAGCCCAGTATGGACCCT3' and 5' TGTGTTTTGTTTTAATCTTTCTGGCC3'; Hdh genotyping: 5' CCCATTCATTGCCTTGCTG3' and 5' GCGGCTGAGGGGGTTGA3'; Hdh qRT-PCR: 5' TTGTGTTAGATGGTGCCGAT3' and 5' GTTGAAGGGCCAGAGAAGAG3'.

Transfection and immunofluorescence imaging

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM, supplemented with 10% FBS, 100U/ml penicillin/streptomycin. Transfections were carried out with lipofectamine 2000 (Invitrogen) as described previously(25). GFP fluorescence was visualized with an Olympus IX-71 fluorescence microscope.

Plasmids

pcDNA3.1-Htt^{ex1}Q₁₀₃-GFP and pGW1-mApple have been reported previously(25–27); pCMV6-Ube2W plasmid was obtained from Origene (RC204985), C91A and W144E mutations were introduced using Quikchange Lightning Site-Directed Mutagenesis (#210518, Agilent Technologies) and sequence-verified; pGW1-Htt^{ex1}-(Q₁₇ or Q₇₂)-EGFP plasmids were kindly provided by Dr. Steven Finkbeiner(28).

Western blotting

Protein lysates from HEK293 cells were generated by lysis in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Complete-mini; Roche Diagnostics, Indianapolis, IN), followed by sonication and centrifugation at 16,000 rpm for 5 mins at 4 °C. The supernatants were collected for analysis. For transfection experiments, transfected cells were lysed with 0.5% Triton X-100 lysis buffer containing 150 mM NaCl, 20 mM Tris/HCl, pH 8.0, 5 mM EDTA and complete Mini Protease Inhibitor tablets. Insoluble pellet from centrifugation was further lysed with 2X Laemmli buffer. Total protein concentration was determined using the BCA method (Pierce, Rockford, IL) and samples were stored at -80 °C. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and corresponding polyvinylidene difluoride membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-Ube2W (1:1,000; 15920-1-AP; Protein Tech Group), mouse anti-GAPDH (1:10,000; MAB374; EMD Millipore), rabbit anti- α tubulin (1:10,000; #2144; Cell Signaling Technology), rabbit anti-GFP (1:1,000; sc-8334; Santa Cruz Biotechnology), mouse anti-polyglutamine (1:2,000; MAB1574; EMD Millipore), mouse anti-Huntingtin (1:250, MAB2166, EMD Millipore), rabbit anti-Hsp40 (1:1,000; #4868, Cell Signaling Technology), rabbit anti-Hsp60 (1:1,000; ab46798, abcam), mouse anti-Hsp70 (1:1,000; SPA-810, Enzo Life Sciences) mouse anti-Hsp90 (1:1,000; SAP-830, Enzo Life Sciences), anti-ubiquitin (1:2,000; z0458, Dako), and rabbit anti-p62 (1:1000; 5114S, Cell Signaling Technology). Bound primary antibodies were visualized by incubation with a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:10,000; Jackson Immuno Research Laboratories, West Grove, PA) followed by treatment with the ECL-plus reagent (Western Lighting; PerkinElmer, Waltham, MA) or Pico/Dura Western Blotting Detection System (Pierce) and exposure to films for images.

³⁵S-Methionine labeling and immunoprecipitation

24h after transfection, cells were cultured in depletion media for 30 mins and labeled with 100 μ Ci of ³⁵S-Met (Perkin Elmer) in Cys/Met-free DMEM. After 20 min of labeling, cells were rapidly rinsed with ice-cold PBS then lysed in RIPA buffer (pH 7.4) containing protease inhibitors (Roche). Lysates were immunoprecipitated overnight with anti-GFP, boiled in SDS sample buffer with 0.1 M DTT, and analyzed by 10% Tris-Acetate SDS-PAGE followed by autoradiography.

Histological analysis

All tissues were fixed for 96hrs at 4 °C in 4% Formaldehyde solution in PBS, transferred to 30% sucrose in PBS, processed, and sectioned. For immunohistochemical staining, tissue underwent antigen retrieval in boiling citrate-based buffer (0.01 mol/L citric acid, pH 6.8) for 20 mins at 80 °C. Endogenous peroxidases were quenched with 3% H₂O₂, followed by blocking in 5% goat serum and incubation with primary antibodies (anti-Huntingtin 1:250, sc8767, Santa Cruz Biotechnology). Bound antibodies were detected with the Vector M.O.M. peroxidase (Vector Laboratories, Burlington, CA), using SigmaFast diaminobenzidine as a peroxidase substrate (Jackson ImmunoResearch, West Grove, PA).

Primary neuron culture and transfection

Cortical neurons were dissected from P0 WT or *Ube2WKO* pups and cultured at 0.6×10^6 cells per mL for 4 d in vitro before transfection, as described previously(29). Transfection of primary neurons was performed using Lipofectamine 2000 (Invitrogen). A total of 0.2 μ g DNA (total) and 0.5 μ L Lipofectamine 2000 were used per well in 96-well plates. Cells were incubated with Lipofectamine/DNA complexes for 20 min at 37 °C before rinsing and changing to normal culture media. Rest of the transfection was performed according to manufacture recommendation.

Automated fluorescence microscopy

Cultured primary mouse neurons were imaged by automated fluorescent microscopy (26–28, 30). The system consists of an inverted microscope (Nikon Eclipse Ti) equipped with the Perfect Focus System (Nikon), a high-numerical aperture 20 \times objective lens, a digital camera, a Lambda XL lamp (Sutter) and an ASI MS2000 stage to automatically control the platform. Neurons were imaged inside a thermo chamber, in which temperature and CO₂ concentration was maintained at 37°C and 5% respectively.

Image analysis

25 raw images were taken from each well of primary neurons. Raw images were stitched together to represent larger areas. According to the sequence and alignment, stitched images were stacked together to represent time-elapse change of fluorescence. Neuronal survival was calculated by a software algorithm developed in ImageJ (26). The time of death for each neuron was recorded as the last time a neuron was confirmed to be alive. Intensities of transfected proteins were determined automatically by segmentation of neuronal cell bodies in ImageJ and measurement of mean pixel intensity within each region of interest. Statistical analyses and cumulative hazard plots were generated using the survival package within R (26).

RNA extraction and qRT-PCR

RNA was extracted from forebrain of mice using TRIzol® (Life Technologies) and further purified using the RNeasy kit with on-column DNase I digestion (Qiagen). For qPCR, 1 μ g of RNA was reverse-transcribed using iScript™. 0.5 μ l was used with the SYBR® GreenMasterMix and each reaction was performed in triplicate. qRT-PCR was performed on the Bio-Rad iCycler with MyIQ single color real-time PCR detection system module with the following parameters: 95°C at 3 min, (95°C 10 s, 55°C 30 s) \times 40, 95°C 1 min, 55°C 1 min. The fold change in transcript level was calculated using the $\Delta\Delta$ Ct method(31). Gapdh was used as control. The primers used for qRT-PCR are listed above. For figure 6A and 6B, qRT-PCR was performed with TaqMan® Gene Expression Master Mix (#4369016, Life technologies). qRT-PCR was performed on the Applied Biosystems 7500 real-time PCR system and the remainder of the transfection protocol was per the manufacturer's suggestions. Primers used for TaqMan qRT-PCR are from Life technologies (Drd2: Mm00438545_m1, Ppp1r1b: Mm00454892_m1 and Actb: Mm00607939_s1).

Results

Ube2W increases HTT inclusion formation in cultured cells

Htt^{ex1}Q₁₀₃-GFP overexpression in HEK293 cells results in nuclear inclusion formation (Fig 1A). Using fluorescence microscopy, we visualized the formation of GFP-positive HTT inclusions when Ube2W or functional mutants of Ube2W were expressed with Htt^{ex1}Q₁₀₃-GFP. Mutating Ube2W's enzymatic active site cysteine, C91, to alanine eliminates the ability of Ube2W to transfer Ub to substrates while still allowing Ube2W to bind substrates(17, 20). In contrast, amino acid W144 near the C-terminus of Ube2W is critically important for substrate binding, and mutating this residue to glutamic acid eliminates substrate binding(17). Both the C91 and the W144 mutants are predicted to disrupt Ube2W-mediated ubiquitination. When WT Ube2W is coexpressed with Htt^{ex1}Q₁₀₃, inclusion number and size significantly increases, whereas coexpression of either Ube2W mutant markedly decreases Htt^{ex1}Q₁₀₃ inclusion number and average size (Fig 1B-D). Cell number were quantified, revealing that neither WT Ube2W nor either mutant alters cell viability.

Biochemically, mHTT exists in at least three distinct states in cells: soluble monomers, soluble oligomers and insoluble aggregates(32, 33). Accordingly, lysates from transfected cells were separated into soluble and insoluble fractions based on solubility in the nonionic detergent Triton-X100. Coexpression of Ube2W-W144E significantly increases soluble monomeric mHTT whereas WT Ube2W and Ube2W-C91A do not lead to a statistically significant change in soluble mHTT monomers (Fig 2A and B). In the insoluble fraction mHTT partitions as two species discernible by gel electrophoresis: high molecular weight (HMW) mHTT in the stacking gel and monomeric mHTT in the resolving gel. Both Ube2W mutants significantly reduced HMW mHTT in the insoluble fraction whereas WT-Ube2W did not (Fig 2A and C). The same trend can be observed for monomeric mHTT in the insoluble fraction, possibly due to the solubilization of HMW HTT species.

The effect of Ube2W on HTT most likely occurs post-translationally. But as an E2 that ubiquitinates N-termini, Ube2W could act cotranslationally by interacting with the nascent N-terminal polypeptide as it exits the ribosome and thus alter the rate of HTT protein synthesis itself. To study whether Ube2W affects HTT synthesis, we measured HTT translation using ³⁵S-methionine pulse-labeling. HEK293 cells co-expressing Htt^{ex1}Q₁₀₃ with WT Ube2W or Ube2W-C91A were pulse-labeled 20 min with ³⁵S-methionine to label all newly synthesized proteins, then immediately lysed. Anti-GFP immunoprecipitation (IP) captured the soluble Htt^{ex1}Q₁₀₃-GFP present in cell lysates and newly synthesized radiolabeled HTT was visualized by autoradiography. The rate of Htt^{ex1}Q₁₀₃ translation appeared similar regardless of which form of Ube2W was co-expressed (Fig 2D and E). Efforts to determine HTT half-life using pulse-chase labeling were not successful, most likely due to HTT's tendency to aggregate and become inaccessible to gel autoradiography over the long chase times needed.

Ube2W deficiency results in decreased mHTT inclusion formation and reduced neurotoxicity

To investigate the consequences of Ube2W deficiency in vivo, we generated primary neuronal cultures from WT or conditional *Ube2W* KO mice in which Nestin-Cre expression was used to delete Ube2W in the nervous system. To assess the potential relationship between Ube2W and Htt^{ex1}Q₇₂-mediated neurotoxicity, we measured single-cell fluorescence and cell survival by automated fluorescence microscopy (AFM) (26–28, 30, 33). Using longitudinal AFM, we plotted the risk of death for neurons expressing EGFP alone (as a control), normal (nonpathogenic) repeat Htt^{ex1}Q₁₇-EGFP, or expanded (mutant) repeat Htt^{ex1}Q₇₂-EGFP, and assessed for differences in neuronal survival among conditions with Cox proportional hazards analysis. Qualitative measures used to estimate cell death in these assays (loss of fluorescence or disruption of cell integrity) are equally sensitive as conventional measures such as staining for apoptotic markers (33)(Fig 3A).

GFP-expressing neurons lacking Ube2W showed significantly increased rates of death compared to GFP-expressing WT neurons, suggesting that Ube2W plays a role in neuronal health, at least in the cell culture environment employed here. The expression of Htt^{ex1}-EGFP, whether normal or expanded repeat, increased neuronal cell death. As expected, Htt^{ex1}Q-EGFP was more toxic than Htt^{ex1}Q₁₇-EGFP in WT neurons, revealing a glutamine repeat length-dependent toxic effect.

Despite the apparent role of Ube2W in cultured neuronal health, the toxicity of mutant Htt^{ex1}Q₇₂-EGFP was significantly reduced by the absence of Ube2W (comparison of solid versus dashed red lines in figure 3B). In contrast, the presence or absence of Ube2W did not have a significant effect on the survival of neurons expressing nonpathogenic Htt^{ex1}Q₁₇-EGFP (comparison of solid versus dashed blue lines in figure 3B).

To assess the effect of Ube2W on Htt^{ex1} expression levels, we measured the fluorescence signal separately in each of hundreds of EGFP- or Htt^{ex1}Q_n-EGFP-expressing neurons 24 hours after transfection, a time when Htt^{ex1}Q₇₂-EGFP inclusion formation is minimal. In neurons expressing EGFP alone, fluorescence did not differ between WT and *Ube2W* KO neurons (Fig 3C). In contrast, Ube2W KO significantly decreased the fluorescence intensity of both Htt^{ex1}Q-EGFP and Htt^{ex1}Q₇₂-EGFP (Fig 3D, E). In comparison to Ube2W WT neurons, Ube2W KO neurons displayed ~20% fewer Htt^{ex1}Q₇₂-EGFP inclusions (Fig 3F). The increased population of inclusion-negative neurons in the absence of Ube2W continued to show diffuse mHTT signal to the endpoint of the experiment (216 hr).

Absence of Ube2W increases soluble, monomeric mutant Htt in a knock-in mouse model of HD

The above results in neurons transiently expressing an expanded Htt fragment support the view that Ube2W regulates mutant Htt levels, aggregation and toxicity. To further study Ube2W's effect on full-length mutant Htt in vivo, we crossed *Ube2W* germline KO mice to heterozygous *Hdh*Q₂₀₀ KI mice(34, 35). This HD mouse model displays cytoplasmic aggregation foci by 9 weeks of age, followed by neuronal intranuclear inclusion by 20 weeks of age (34, 35). At 32 weeks of age, frontal brain lysates including the cortex and striatum

(known vulnerable regions in HD) were homogenized in RIPA buffer, separated by gel electrophoresis and visualized by anti-Htt immunoblot. Strikingly, soluble mutant HttQ₂₀₀ monomers were increased approximately 5-fold in the absence of Ube2W (Fig 4A and B). A similar increase in soluble HttQ₂₀₀ was also observed in 46 week-old in *Ube2W*KO/*HdhQ200* KI mice (data not shown). Moreover, in *HdhQ200* KI mice the absence of Ube2W also resulted in an approximately 3-fold increase in WT (i.e. nonexpanded) Htt monomers (Fig 4A and B). By contrast, in *Ube2W*KO mice, WT Htt levels were not significantly altered, suggesting that the observed increase in WT Htt in *HdhQ200* mice lacking Ube2W occurs only in the presence of mutant HttQ₂₀₀.

In these experiments, in which RIPA buffer was the lysis buffer and samples were analyzed by SDS-PAGE, no Htt signal was observed in the stacking gel corresponding to detergent-resistant HMW Htt species. To better visualize non-monomeric Htt species, we used a milder detergent to fractionate brain lysate into soluble and insoluble fractions (15, 36) (see method section). Absence of Ube2W significantly decreased the level of HMW Htt signal, presumably representing aggregated Htt, in both detergent soluble and insoluble fractions (Fig 4C and D).

HttQ₂₀₀ inclusion bodies in striatum are not altered by Ube2W deficiency

Htt-containing inclusions within neurons are a pathological hallmark of HD. Due to its hyperexpansion, HttQ₂₀₀ forms widespread neuronal inclusions in neurons of the striatum, cortex, hippocampus and cerebellum in *HdhQ200* KI mice, beginning by ~20 weeks of age (35). To determine whether the marked increase in HttQ₂₀₀ monomers correlated with a change in size or number of inclusion bodies, we performed immunostaining with an N-terminal Htt antibody on brain sections from 32 and 46 week old mice (Fig 5A and B). The striatum of *HdhQ200* KI mice showed an equally robust presence of inclusions in the presence or absence of Ube2W, with no significant difference noted in inclusion number or size at 32 or 46 weeks of age (Fig 5C and D). Both in the presence and absence of Ube2W, Htt inclusions immunostained positively for ubiquitin (data not shown). These results suggest that Ube2W preferentially affects levels of soluble and intermediate species of HttQ₂₀₀ that are detectable by gel electrophoresis and western blotting, without altering the level of insoluble, aggregated disease protein that accumulates over time within inclusions.

Ube2W deficiency does not alter transcript levels of Htt or striatal markers in *HdhQ200* mice

Medium spiny neurons of the striatum are selectively vulnerable in HD and are susceptible in *HdhQ200* mice. Expression levels of two striatal neuronal markers, Dopamine D2 receptors (*Drd2*) and DARPP-32 (*Ppp1r1b*), decline dramatically over the course of disease in human HD patients and animal models (24, 35, 37–39). Transcript levels of *Drd2* and *Ppp1r1b* are sensitive measurements of HD pathology in mice (5). Indeed, at 46 weeks of age transcript levels for both markers are markedly decreased in *HttQ200* het KI mice (Fig 6A and B). Ube2W deficiency had little effect upon *Drd2* and *Ppp1r1b* transcript levels in *HdhQ200* mice (Fig 6A and B). Thus, the presence or absence of Ube2W does not result in detectable changes to HttQ₂₀₀-mediated striatal neuron dysfunction, assessed by these striatal neuronal markers.

Transcriptional analysis also allowed us to establish that the observed changes in soluble levels of mutant Htt in the absence of Ube2W are not simply due to an effect on Htt expression. By qRT-PCR, we investigated whether Ube2W affects WT or mutant *Hdh* transcript levels. Ube2W deficiency did not alter levels of the *Hdh* transcript (Fig 6C), arguing that Ube2W instead affects Htt levels co-translationally or, more likely, post-translationally.

Levels of key components of protein quality control are unchanged in *Ube2W* KO mice

Given Ube2w's role in ubiquitin pathways, we investigated whether global ubiquitination in *HttQ200* het KI mice is altered by loss of Ube2W. Levels of ubiquitin-positive HMW species in mouse brain lysate did not significantly differ in the presence or absence of Ube2W (Figure 7A). Molecular chaperones and autophagy are important regulators of mutant polyQ protein processing generally, and of HTT processing, accumulation and HD pathology specifically. Accordingly, we sought to determine whether the Ube2W-mediated increase in HttQ₂₀₀ soluble monomers reflects a change in key components of the neuronal protein quality control machinery. The heat shock protein (hsp) family of molecular chaperones has been implicated in maintenance of protein homeostasis. We measured expression levels of four key hsp family members (hsp40, hsp60, hsp70 and hsp90) as well as the autophagy marker P62. Of these, only hsp70 showed a decrease in *Ube2W*^{-/-}, *HdhQ200* mice (Fig 7).

A decrease in hsp70 has been reported in various HD mouse models (24, 40) but whether it correlates with HD progression is not known. Collectively, these results suggest that Ube2W deficiency does not lead to the observed increase in soluble HttQ₂₀₀ monomers simply by changing levels of key components of protein quality control.

Discussion

We undertook this study because the disordered nature of the mHTT N-terminus suggested it as a target for Ube2W, an E2 that uniquely ubiquitinates N-termini and may preferentially act on disordered N-termini. Ubiquitin pathways actively regulate mHTT levels, IB formation and disease progression in model systems (13, 15, 16, 41–45), but the role of Ube2W in HD has not been addressed. Here, we utilized a range of model systems to show that Ube2W deficiency increases soluble mHTT levels while reducing aggregation and neurotoxicity in primary cortical neurons. Our results further support the importance of ubiquitin pathways in HD and shed light on a possible function of Ube2W in modulating HD pathogenesis.

We observed a significant increase in soluble mHTT monomers when Ube2W is functionally inactive or eliminated, accompanied by increased neuronal survival in cultured cells (Fig 1, 2 and 3). mHTT can exist in at least three distinct states in cells: soluble monomers, soluble oligomers and insoluble aggregates(32, 33, 46). mHTT oligomers that precede large scale aggregate formation are considered to be more toxic than monomers or aggregates(32, 47–50). For example, using FRET confocal microscopy, researchers showed that neuronal cells containing oligomers die faster than those with either monomers or inclusions(32). And in drosophila, polyphenol epigallocatechin-gallate reduces oligomeric species by promoting inclusion formation with a corresponding protective effect towards

mHTT neurotoxicity(48). In contrast to toxic oligomers, mHTT insoluble aggregates may be neuroprotective, possibly in part by recruiting and neutralizing toxic oligomers(29, 32, 33, 51, 52). In the current study, the increase in mHTT monomers and reduced neurotoxicity in the absence of Ube2W could result from reduced mHTT oligomerization or increased levels of readily soluble mHTT complexes that may lack the toxicity of more insoluble oligomers and would electrophorese as monomers on denaturing gels.

What is the mechanism by which Ube2W alters solubility of mHTT? We offer several hypotheses: 1. N-terminal ubiquitination of HTT stabilizes the protein, ultimately leading to increased HTT aggregation; 2. N-terminal ubiquitination of HTT alters the probability of other post-translational modification(s) to the protein, thus indirectly promoting HTT aggregation; 3. Ube2W acts indirectly on HTT by ubiquitinating one or more proteins that regulate HTT, such as SUMO-2, a documented Ube2W substrate that is known to increase mHTT aggregation(15, 36); and, least likely, 4. Ube2W acts directly or indirectly on HTT through a mechanism unlinked to its ubiquitinating function (53, 54). Further studies are required to determine whether indeed Ube2W directly ubiquitinates HTT or acts in a more indirect manner to alter behavior of the disease protein.

The biological role of Ube2W-mediated N-terminal ubiquitination remains poorly understood. Researchers have identified at least 13 proteins that can be N-terminally ubiquitinated(18, 55–63). All but two known substrates of Ube2W (ataxin-3 and LMP2A) are targeted for proteasomal degradation by N-terminal ubiquitination via K48-linked polyubiquitination. We previously showed an accumulation of relatively disordered proteins in *Ube2W*KO mouse testis, an organ in which Ube2W is highly expressed, suggesting that Ube2W-mediated N-terminal ubiquitination functions as a degradation signal(23). While these lines of evidence favor the view that Ube2W-mediated N-terminal ubiquitination can target proteins for degradation, Ube2W strictly monoubiquitinates substrates and does not itself specify subsequent Ub chain linkage(17, 18, 20, 21, 64). And, of course, cellular functions of ubiquitination are not limited to degradation pathways. For example, working together with Ube2N/Ube2V2, Ube2W is able to facilitate the formation of K63-linked ubiquitin chains on TRIM5 α and TRIM21, which regulates their reverse transcription activity rather than their degradation (21, 22). The potential functions of N-terminal ubiquitination in non-degradative pathways cannot be ruled out as yet.

Besides direct N-terminal ubiquitination by Ube2W, alternative mechanisms of action, such as an effect on SUMOylation, could underlie the alteration in HTT monomers we observed. SUMO-2 itself can be N-terminally monoubiquitinated by Ube2W in vitro(19), though the biological function of this signal is still unknown. Conceivably, Ube2W could ubiquitinate SUMO-2 that is already conjugated to HTT. SUMO-2 modification of Htt^{ex1} decreases mHTT solubility, favoring mHTT accumulation and decreasing monomer levels(15); conceivably, Ube2W-mediated ubiquitination of SUMO attached to mHTT underlies this decreased solubility of mHTT. Similar to our finding here with Ube2W mutants, knockdown of an E3 SUMO ligase that enhances SUMO modification of HTT significantly decreases insoluble mHTT species(15). Further mechanistic studies will need to be carried out to study the biologic function of Ube2W on SUMOylation, ubiquitination and mHTT processing and its relationship to mHTT solubility and aggregation.

Our analysis of key protein quality control components in the chaperone and autophagy pathways revealed a reduction only in Hsp70 levels in HD KI mice lacking Ube2W. We would not expect this reduction in Hsp70 to lead to increased levels of soluble, mutant Htt as observed in HD KI mice lacking Ube2W. Thus, despite the fact that Hsp70 has been implicated in Htt handling and toxicity in numerous studies (see, for examples, 65, 66), the isolated reduction of Hsp70 suggests that the effects of Ube2W on mutant Htt are not principally an indirect effect on protein quality control pathways.

Our observation that eliminating Ube2W increased the solubility of full length mutant Htt in HD KI mice complemented our results in transfected cells and cultured neurons expressing an N-terminal mutant Htt fragment. Collectively, these results suggest Ube2W regulates mutant Htt levels and aggregation. Unexpectedly, however, we did not observe an effect of Ube2W on Htt inclusions in the brains of HD KI mice; inclusions appear to be equally abundant in HD KI mice whether or not Ube2W is present. This discrepancy may reflect the substantial differences between cells transiently overexpressing an Htt fragment and neurons continually expressing physiological concentrations of the full length disease protein. It also implies that the slow development of Htt inclusion formation in KI mice and human HD is complex, and that Ube2W is only one of many factors that influence the process.

Our results in cultured neurons suggest Ube2W also modulates Htt toxicity. An important limitation of our study, however, is that we cannot translate this finding of an effect on toxicity to the mouse model. The relationship between disease protein aggregation and toxicity remains an unsettled question in many neurodegenerative diseases, including HD. Our focus here was on the biochemical relationship of Ube2W to Htt, not on the broader question of whether modulating Htt aggregation or solubility affects toxicity in vivo. Our studies were not powered to carry out behavioral phenotypic measurements, and such measurements were not performed on mice in this study. Our findings provide a framework for a future, larger study that can determine the role of Ube2W in modulating Htt toxicity in vivo.

Acknowledgments

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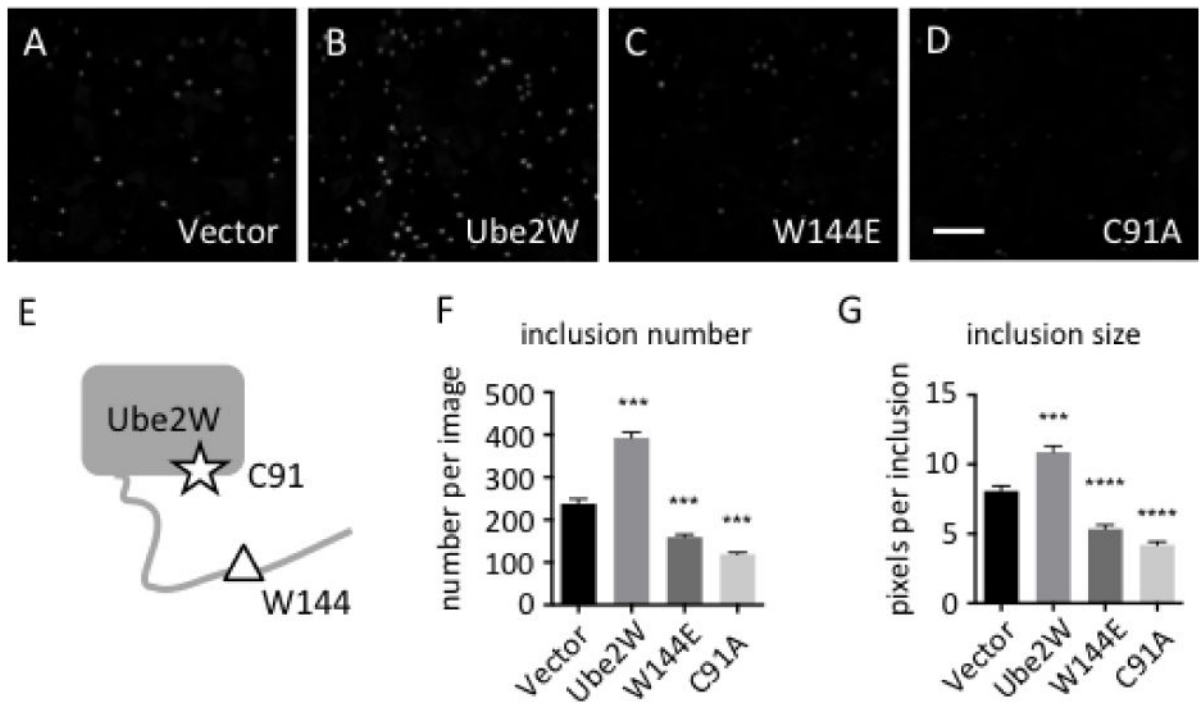
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**FIGURE 1.**

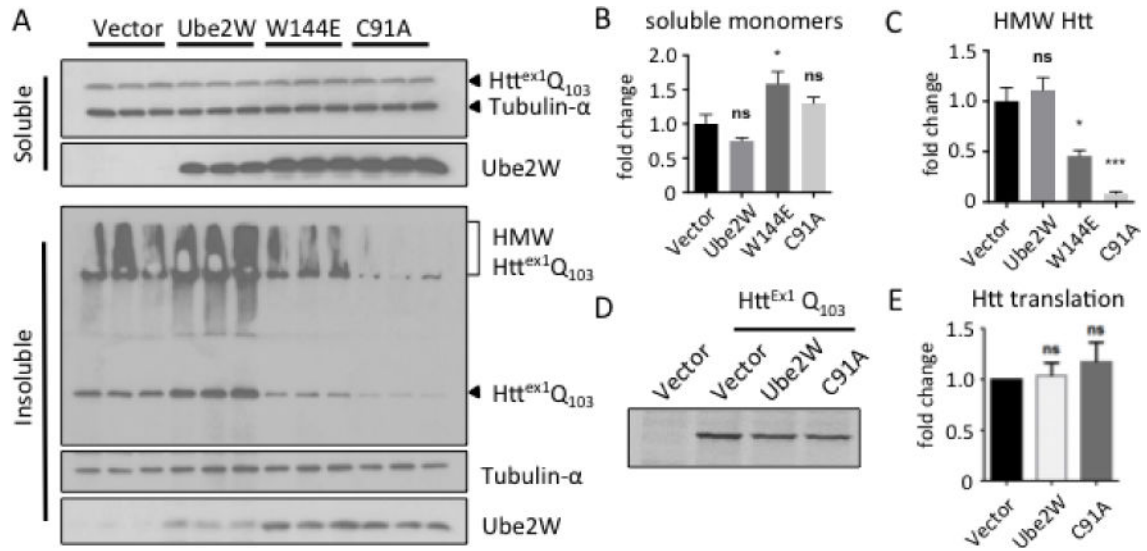
Ube2W alters Htt^{ex1}Q₁₀₃-GFP inclusion formation in HEK293 cells

A-D. In HEK293 cells, Htt^{ex1}Q₁₀₃ inclusions were visualized by GFP fluorescence 48 hrs after transfection.

Htt^{ex1}Q₁₀₃-GFP was co-expressed with vector alone (A), Ube2W (B), Ube2W-W144E (C) or Ube2W-C91A (D). Scale bar=30 μm.

E. Schematic of Ube2W structure illustrates relative positions of C91 (the active site cysteine) and W144 (needed for substrate binding).

F,G. Htt^{ex1}Q₁₀₃ inclusion number (F) and size (G) from all four groups were plotted. Graphs show means ± SEM; ***, p<0.001; ****, p<0.0001. n=8 images(F), n>34 inclusions (G).

**FIGURE 2.**

Ube2W alters solubility of Htt^{ex1}Q₁₀₃

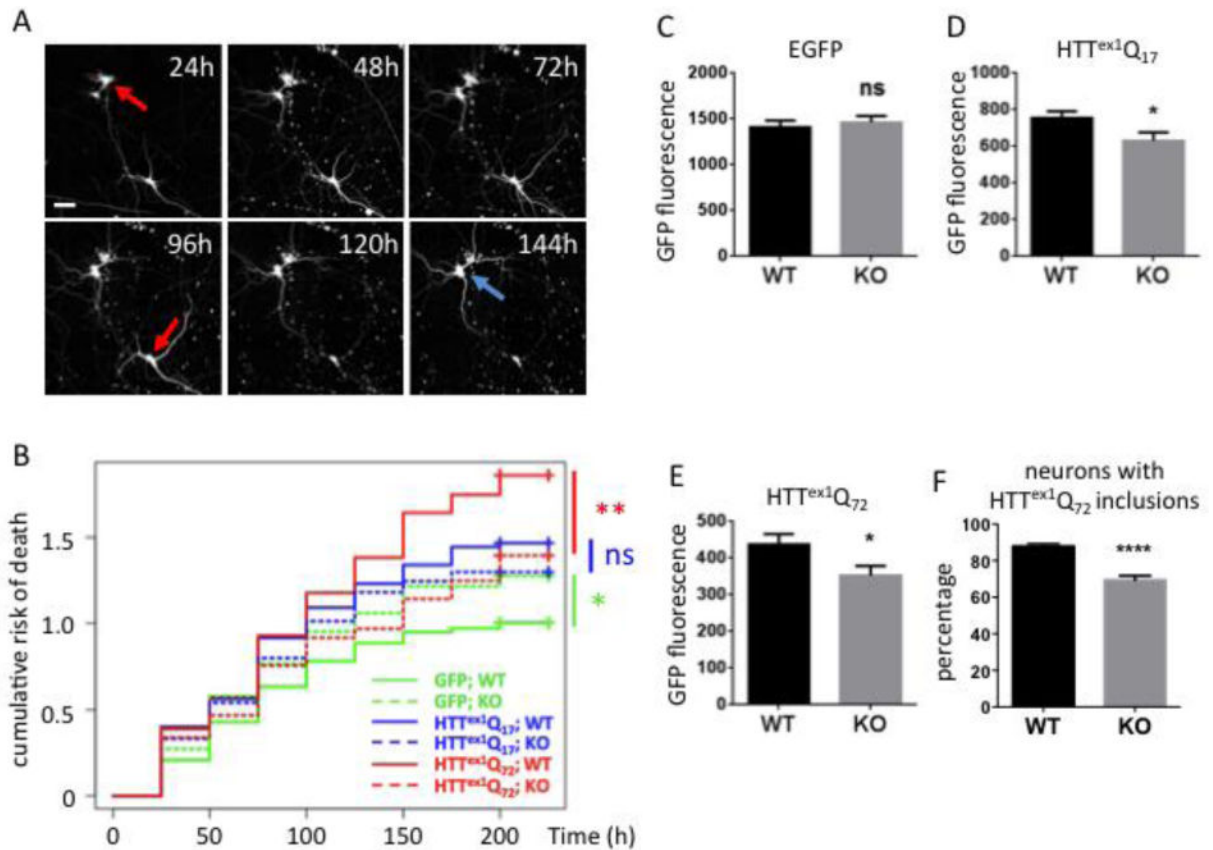
A. Western Blot of lysates from HEK293 cells transfected with Htt^{ex1}Q₁₀₃ and Ube2W or its mutants, Ube2W-W144E or Ube2W-C91A. Upper panel is the TritonX-100 soluble fraction and lower panel is the TritonX-100 insoluble fraction. Immunoblots were performed with anti-GFP (visualizing Htt^{ex1}Q₁₀₃), anti-tubulin-α, and anti-Ube2W. (15 μg total protein/lane.) Arrowheads indicate monomeric Htt^{ex1}Q₁₀₃, Ube2W or tubulin, and bracket indicates high molecular weight (HMW) Htt^{ex1}Q₁₀₃ in stacking gel.

B. Quantification of soluble HTT monomers in upper panel of Fig 2A. Graphs show means +/- SEM; ns, not significant; *, p<0.05. n=3.

C. Quantification of HMW HTT species in lower panel of Fig 2A. Graphs show means +/- SEM; ns, not significant; *, p<0.05; ***, p<0.001. n=3.

D. Autoradiograph of a representative ³⁵S-methionine pulse-chase experiment followed by GFP-IP and gel electrophoresis.

E. Quantification of autoradiograph in Fig 2D. Graphs show means +/- SEM; ns, not significant. n=3.

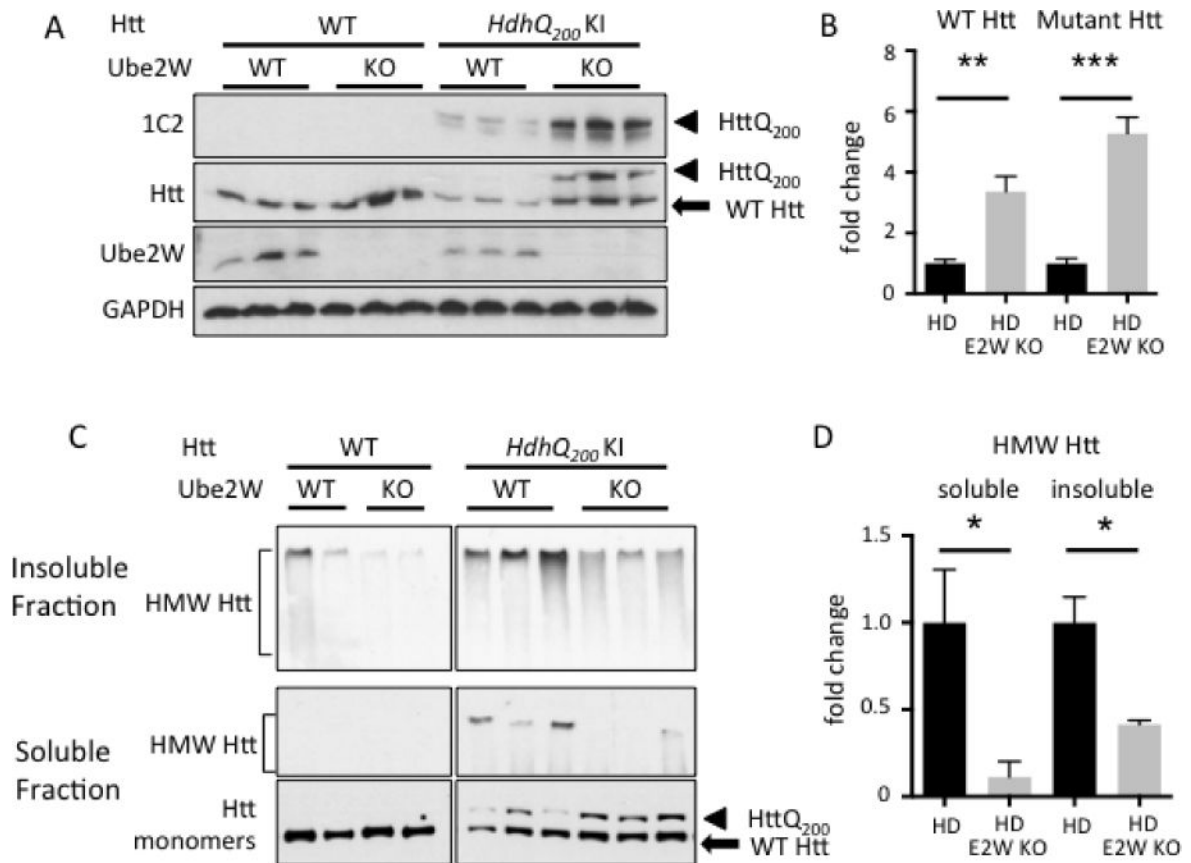
**FIGURE 3.**

Ube2W deficiency results in decreased Htt^{ex1}Q₇₂ inclusion formation and increased neuronal survival

A. Representative images from automated fluorescence microscopy. Primary cortical neurons from WT or *Ube2W* KO mice were transfected with mApple and Htt^{ex1}Q₇₂-EGFP, and survival was determined by repeated imaging at regular intervals. The last time at which the cell was noted to be alive (red arrows) was used as the time of death. Cells that survive the entire length of the experiment (blue arrow) were censored (analyzed as living cell at the end of experiment). Scale bar=25 μm.

B. Cumulative risk of death over time for WT and *Ube2W* KO neurons transfected with EGFP, Htt^{ex1}Q₁₇-EGFP and Htt^{ex1}Q₇₂-EGFP. Results were pooled from 16 wells per condition, with experiments performed in duplicate. ns, not significant; *, p<0.05; **, p<0.01. n>250.

C-F. Quantification of EGFP signals (C-E) or inclusion formation (F) from experiments as in panel A, 48 hours after transfection. Graphs show means ± SEM; ns, not significant; *, p<0.05; ****, p<0.0001. n>232 for all analyses.

**FIGURE 4.**

Ube2W deficiency increases soluble Htt levels in *HdhQ200* KI mice

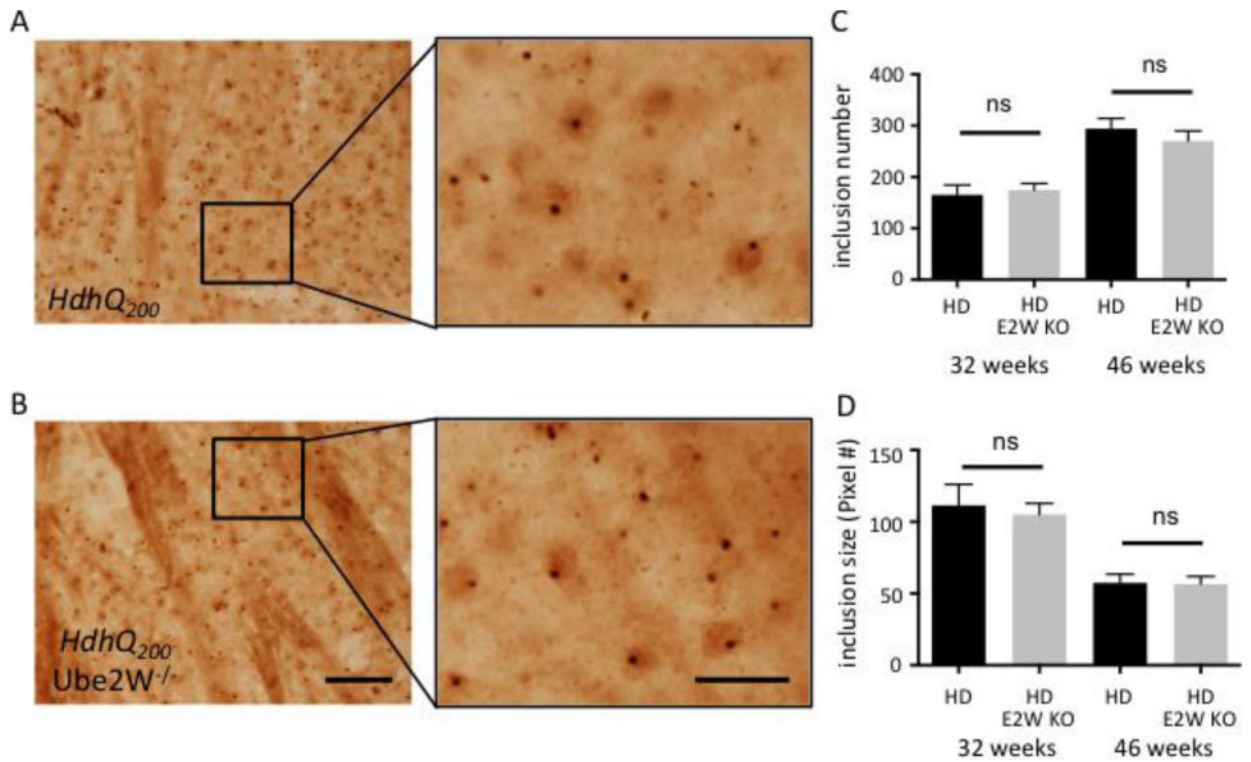
A. Western Blot of brain lysates from WT, *Ube2W* KO, *HdhQ200* KI, or *HdhQ200* KI/*Ube2W* KO mice. Immunoblot were performed with anti-polyQ (1C2), anti-Htt, anti-Ube2W and anti-GAPDH antibodies. (40 µg total protein/lane.)

B. Quantification of Blot against Htt in panel A.

C. Western Blot of soluble and insoluble fraction of mouse brain lysates from WT, *Ube2W* KO, *HdhQ200* heterozygous (het) KI, *HdhQ200* het KI with *Ube2W* KO mice. Blotted with anti-Htt antibody.

D. Quantification of HMW Htt blotted in panel C.

Arrowheads represent mutant HttQ₂₀₀, arrow represents WT Htt, and bracket indicates high molecular weight (HMW) Htt in stacking gel. Graphs show means \pm SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. n=3.

**FIGURE 5.**

Absence of Ube2W does not alter HttQ₂₀₀ inclusion levels in *HdhQ₂₀₀* KI mice

A and B. Htt immunostaining of striatum showing HttQ₂₀₀ intranuclear inclusions in 46 week old *HdhQ₂₀₀* KI (A) and *HdhQ₂₀₀* KI/*Ube2W* KO mice (B). Magnified regions from insets are shown in the right panels. Scale bar, left panels=200 μm; Scale bar, right panels=50 μm.

C and D. HttQ₂₀₀ inclusion number (C) and size (D) from 32 and 46 week old mice were plotted. Graphs show means ± SEM; ns, not significant. n=10 images (C), n>10 images (D).

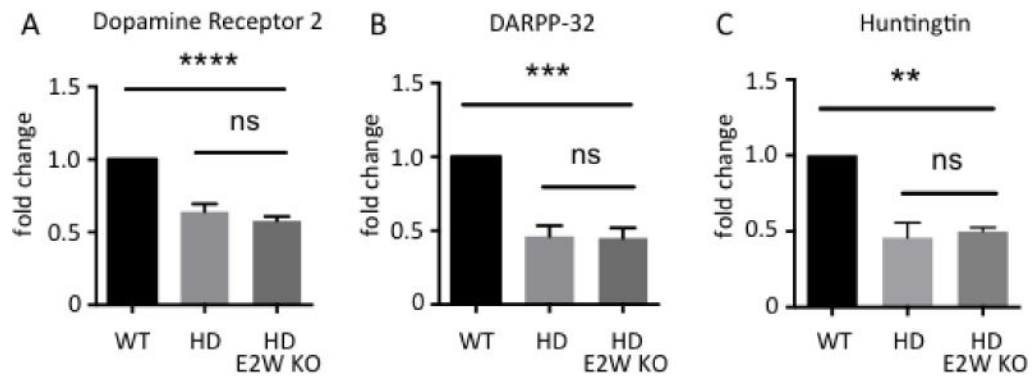
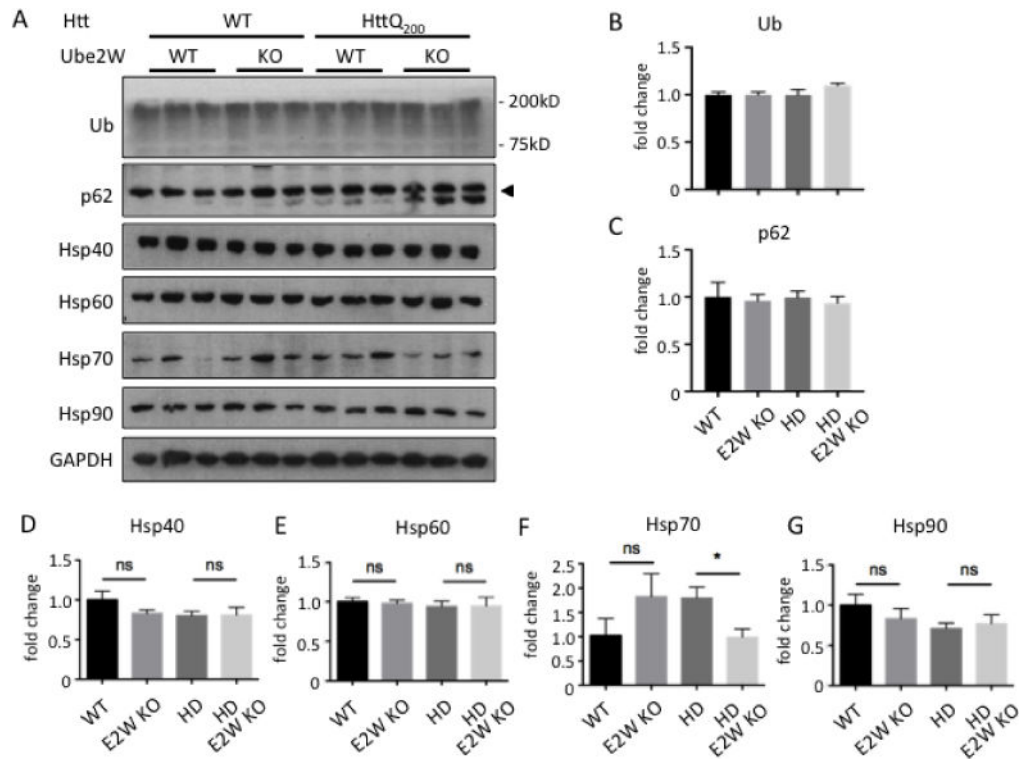


FIGURE 6.

Ube2W deficiency does not alter transcript levels of striatal neuronal markers in *HdhQ200* KI mice. Dopamine receptor 2 (*Drd2*) (A), DARPP-32 (*Ppp1r1b*) (B) and Huntingtin (*Hdh*) (C) transcript levels are measured by qRT-PCR amplification. RNA was extracted from WT, *HdhQ200* KI, and *HdhQ200* het KI/*Ube2W* KO mice forebrain. Results are normalized to β -actin transcript levels. Results were quantified and plotted as graphs. Graphs show means \pm SEM; ns, not significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. $n = 4$.

**FIGURE 7.**

Levels of key components of protein quality control are unchanged in *Ube2W* KO mice.

A. Western Blot of mouse forebrain lysates from WT, *Ube2W* KO, *HdhQ₂₀₀* het KI, *HdhQ₂₀₀* het KI with *Ube2W* KO mice. Blotted with anti-ubiquitin, anti-p62, anti-Hsp40, anti-Hsp60, anti-Hsp70 and anti-Hsp90 antibodies. (40 μ g total protein loaded per lane.)

B–G. Quantification of the indicated proteins from Western blots in panel A. Graphs show means \pm SEM; ns, not significant; *, $p < 0.05$; $n = 3$.