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Signature changes in ubiquitin expression in the R6/2 mouse model of Huntington's disease

Nathaniel Safren^{1,2}, Lydia Chang², Kristina M. Dziki², and Mervyn J. Monteiro^{1,2,3,*}

¹Neuroscience Graduate Program, University of Maryland School of Medicine, Baltimore, 20 North Pine Street, Baltimore, MD 21201, School of Medicine, Baltimore, MD 21201

²Center for Biomedical Engineering and Technology, University of Maryland School of Medicine, Baltimore, 20 North Pine Street, Baltimore, MD 21201, School of Medicine, Baltimore, MD 21201

³Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, 20 North Pine Street, Baltimore, MD 21201, School of Medicine, Baltimore, MD 21201

Abstract

Ubiquitin proteins have been implicated in the cause and the pathology of neurodegenerative diseases. In the R6/2 mouse model of Huntington's disease (HD), ubiquitin levels decline during disease progression. Restoration of their levels by transgenic expression of ubiquitin-1 extends survival. Here we provide a comprehensive assessment of the expression and localization of all four ubiquitin proteins in both normal and R6/2-affected mice brains, using antibodies specific for each protein. Ubiquitin-1, 2 and 4 proteins were detected throughout the brain, with increased expression seen in the hippocampus and cerebellum. Ubiquitin-3 expression was not detected. All three ubiquitins expressed in the brain were found in Htt inclusions. Their expression changed during development and disease. Ubiquitin-1 and ubiquitin-2 protein levels decreased from 6 to 18 weeks of mouse development, independent of disease. Ubiquitin-1 and ubiquitin-4 protein levels also changed during HD disease progression. Ubiquitin-4 proteins that are normally expressed in the brain were lost and instead replaced by a novel 115 kDa higher molecular weight immunoreactive band. Taken together, our results demonstrate that all ubiquitin proteins are involved in HD pathology and that distinct changes in the signature of ubiquitin-4 expression could be useful for monitoring end-stage of HD disease.

Keywords

Ubiquitin; Huntington's disease; inclusions; brain; ubiquitin

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*Corresponding author., Mervyn J. Monteiro, Center for Biomedical Engineering and Technology, Room S642, University of Maryland, Baltimore, 20 North Pine Street, Baltimore, MD 21201, Tel: 410-706-8132, Fax: 410-706-8184, monteiro@umaryland.edu.

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Conflicts of interest: none

INTRODUCTION

Huntington's Disease (HD) is a debilitating neurodegenerative disorder caused by a polyglutamine expansion in huntingtin (Htt) protein (1993). There is an inverse correlation between the length of the polyglutamine expansion and age of onset of the disease (Walker, 2007). Longer polyglutamine tracts increase the propensity of mutant Htt protein to aggregate, forming ubiquitin-positive inclusion bodies that are a pathological hallmark of HD (Finkbeiner, 2011). Several reports indicate that Htt inclusions contain ubiquilin, a protein that functions in protein clearance through the proteasome and autophagy pathways (Doi et al., 2004; Mori et al., 2012; Rutherford et al., 2013). Interestingly, in R6/2 mice, which recapitulate many features of HD, ubiquilin proteins are not only present in Htt inclusions, but their levels decline progressively during disease progression (Safren et al., 2014). Restoration of ubiquilin levels by transgenic overexpression of ubiquilin-1 extends survival of R6/2 mice suggesting the decline in ubiquilin levels affects disease (Safren et al., 2014).

Both humans and mouse contain four ubiquilin genes (UBQLN1 to 4), each encoding a separate protein. The four proteins share an N-terminal ubiquitin-like domain (UBL) and C-terminal ubiquitin-associated domain (UBA), but differ from each other due to insertions and deletions in their central domain (Mah et al., 2000; Wu et al., 1999; Davidson et al., 2000; Wu et al., 2002). The domain structure of the proteins is typical of shuttle factors that bind and deliver polyubiquitinated proteins to the proteasome (Elsasser and Finley, 2005). Indeed ubiquilin proteins not only function in proteasome degradation, but also in autophagy (Kleijnen et al., 2003; Kleijnen et al., 2000; Ko et al., 2004; Lim et al., 2009; N'Diaye et al., 2009; Rothenberg and Monteiro, 2010; Rothenberg et al., 2010).

Genetic mutations in UBQLN1, 2 and 4 genes have all been linked to different neurodegenerative diseases (Deng et al., 2011; Fahed et al., 2014; Gonzalez-Perez et al., 2012; Yan et al., 2013). It is possible that the mutations in each ubiquilin gene cause a different spectrum of disease due to variability in the expression of the genes throughout the nervous system. However, the distribution of each ubiquilin protein in the brain is not known. Here we used antibodies specific for each of the four ubiquilins to determine their expression patterns in mouse brain. We also used the antibodies to determine whether all ubiquilins colocalize with Htt inclusion bodies in R6/2 mice, as this was unknown. We further examined whether expression of each ubiquilin changes with disease progression.

RESULTS

Characterization of antibodies that discriminate each of the four ubiquilin proteins in mouse

In order to assess the profile and distribution of ubiquilin expression in normal and HD-afflicted mouse brains we screened ubiquilin antibodies from commercial sources and the ones we had generated to identify those that were specific for each of the four ubiquilin gene products expressed in mammals. To establish their specificity, each of the four different ubiquilin isoforms was expressed as a GFP-fusion protein in mouse NB2a neuroblastoma cells and HeLa cells (Fig 1). Protein lysates from the transfected cells, and the mock-

transfected control, were probed with the antibodies to see which, and how many GFP-ubiquilin-fusion proteins, were recognized by the ubiquilin antibodies. For these tests, cDNAs encoding the entire open reading of human ubiquilin isoforms 1 to 4 were expressed as they each share high homology with their corresponding mouse isoforms. We also expressed mouse ubiquilin-1 for similar purposes for the reasons described below. An anti-GFP immunoblot confirmed successful expression of all the fusion proteins (Fig 1A and B). These fusion proteins were slightly different in size, consistent with known differences in the lengths of the predicted ubiquilin polypeptides.

Through this screening procedure we identified antibodies that specifically recognized each of the four different ubiquilin isoforms, with no cross-reaction with the other ubiquilin isoforms (Fig 1 A–I). Antibodies UMY75, UMY78 and ARP57-355 were found to be specific for ubiquilin-2, ubiquilin-3 and ubiquilin-4, respectively (Fig 1E, G, H and I). Because the peptides used to raise these antibodies (Table 1) are common between human and mouse we are confident that the antibodies recognize mouse ubiquilins as well. Further confirmation of their specificity was achieved through siRNA knockdown of the proteins (Fig 2). A commercial antibody, PA1-759, which specifically recognized mouse ubiquilin-1 (Fig 1F), but not human ubiquilin-1 (Fig 1E), or the other ubiquilins was further identified. Apart from the GFP-fusion proteins, the antibodies also reacted with other bands in the lysates. It is likely that some of these bands represent reaction with the endogenous ubiquilin proteins, or their cleaved products. The ubiquilin-1 antibody reacted with prominent 70 kDa and 35 kDa bands. The former is consistent with the size of endogenous mouse ubiquilin-1 polypeptide and the latter we presume is a cleaved fragment of it. Consistent with the conclusion that these bands are ubiquilin-1 products, both bands were responsive to ubiquilin-1 siRNA knockdown (Fig 2A, B). Similar bands were detected with the ubiquilin-2 antibody (Fig 1G). A 70 kDa band is present in these blots, which we believe to be endogenous ubiquilin-2. This band is particularly prominent in NSC-34 mouse motor neuron cells when blotting with the affinity-purified UMY75 antibody (Fig 2C). Knockdown with ubiquilin-2 siRNA confirmed the band is ubiquilin-2 specific (Fig 2C, D). The ubiquilin-3 antibody did not react with any specific band in NB2a lysates apart from a weakly reactive 100 kDa band that we presume is non-specific due to the variability of its detection. The ubiquilin-4 antibody reacted with a 115 kDa band. We believe the protein is related to ubiquilin-4 as the intensity of the band was reduced following siRNA knockdown of ubiquilin-4 (Fig 2E, F). Besides these antibodies we also probed the lysates with the commercial ubiquilin monoclonal antibody from Invitrogen #37-7700, which we have used in several of our studies (Massey et al., 2005; Ford and Monteiro, 2006; Lim et al., 2009; Safren et al., 2014). The antibody was found to react with both mouse and human ubiquilin-1 and 2 proteins, but not ubiquilin-3 (Fig 1C and D). The antibody reacted with human ubiquilin-4 (Fig 1C and D), but not with mouse ubiquilin-4. Note, recognition of a 115 kDa band in human HeLa lysates and lack of detection in mouse NB2a lysates. The 115 kDa band in NB2a cells is however, recognized by the isoform specific ubiquilin-4 antibody (Fig 1I). As expected, the monoclonal antibody otherwise reacted to different degrees with the full spectrum of bands that were detected with antibodies made to the individual proteins, suggesting that they are all ubiquilin-related. Taken together the blots demonstrate that we possess antibodies specific for each ubiquilin isoform.

Expression patterns of ubiquilin proteins in mouse brain

To determine the expression patterns of the different ubiquilin proteins in mouse brain we stained sagittal sections cut through a 4-month-old female C57BL/6J mouse brain with each of the ubiquilin antibodies (Fig 3A). Consistent with a previous report showing that the ubiquilin-3 transcript is only expressed in the testis (Conklin et al., 2000), we did not find any ubiquilin-3 protein staining in the brain (Fig 3A). By contrast both the ubiquilin-1 and ubiquilin-2 antibodies displayed relatively uniform staining throughout the brain, although ubiquilin-1 staining, and to a lesser extent ubiquilin-2 staining, was higher in the granule cell layer of the dentate gyrus as well as the CA1, CA2 and CA3 regions of the hippocampus (Fig 3B). Ubiquilin-4 staining was also widely expressed throughout the brain, but staining was particularly prominent in the granular layers of the hippocampus and cerebellum (Fig 3A, B and C), where expression is most enriched in Purkinje cells. Brain sections that were stained with the monoclonal ubiquilin antibody, which reacts with multiple ubiquilin isoforms, produced a pattern that was somewhat similar to the combined staining patterns produced by the individual antibodies.

Ubiquilin-1, 2, and 4 proteins colocalize with huntingtin inclusions

We next performed double immunofluorescence staining of brain sections of R6/2 transgenic mice that had developed HD symptoms (Mangiarini et al., 1996) to determine whether huntingtin inclusions that form in the brains of these mice contain all four ubiquilins. The brains used were of mice that were between 15 and 18 weeks of age, at the endpoint of disease, when Htt inclusions are abundant (Davies et al., 1997). The double immunofluorescence staining revealed colocalization of ubiquilin-1, ubiquilin-2 and ubiquilin-4 proteins with Htt inclusions throughout the brain, including the striatum, frontal cortex and hippocampus (Fig 4A and B). As expected, ubiquilin-3 staining was absent in these brains too (data not shown). The results demonstrate that Htt inclusions contain all three ubiquilins expressed in the brain.

Age and genotype dependent changes in ubiquilin protein expression

Previously we reported that the amount of soluble ubiquilin protein decreases dramatically in the brains of R6/2 mice at endpoint of disease (Safren et al., 2014). This observation, along with previous data showing that restoration of ubiquilin levels by transgenic overexpression of ubiquilin-1 can extend life span in these mice, suggests that the reduction of ubiquilin levels has important implications for disease. In that study, the monoclonal Invitrogen (INV) 37–7700 ubiquilin antibody was used to demonstrate the effect. Here we were able to reproduce this effect. A Two-Way Anova revealed an interaction of age and genotype ($F_{1,8} = 56.80, p = .003$). Since INV 37–7700 recognizes ubiquilins-1 and 2, it was unclear whether the decline in ubiquilin levels was due to a concomitant reduction in one or all of the ubiquilin isoforms. Using the isoform-specific antibodies we were able to elucidate this (Fig 5). Interestingly, ubiquilin-1 protein expression decreased with age, regardless of genotype (Main effect of age $F_{1,8} = 49.57, p = .0001$) (Fig 5B and H). This would suggest that ubiquilin-1's expression is developmentally regulated to be higher in juveniles than adults. Ubiquilin-1 protein expression decreased even further in R6/2 animals at endpoint of disease. Ubiquilin-2 expression was similar between WT and R6/2 mice at both 6 weeks and

16–18 weeks, although a slight reduction in its expression as well as ubiquilin-1 was noticed in R6/2 animals (Fig 5C and H). Instead, the most pronounced change between WT and R6/2 animals was seen for ubiquilin-4, during endpoint of disease (Fig 5E and H). In the brains of WT and 6 week-old R6/2 mice, the ubiquilin-4 antibody recognized bands at 70, 35 and 18 kDa that were of similar intensity (Fig 5F). However, in endpoint R6/2 mice, the bands were considerably diminished compared to WT animals, and instead a new band at 115 kDa appeared. The 70 kDa product was reduced by 87% (Bonferroni posttest $p < .001$), and the 35 kDa band by 45% (Bonferroni posttest $p < .05$). There are two reported variants of murine ubiquilin-4. The major variant is 596 amino acids long with a predicted molecular mass of 66 kDa, which likely corresponds to the 70 kDa band seen on the blots (Davidson et al., 2000; Li et al., 2008). The second variant is 543 amino acids long with a predicted mass of 60 kDa, which might correspond to the weaker 60 kDa band seen in the blots. The smaller <35 kDa bands probably represent breakdown products of the larger 60 and 70 kDa polypeptides as their two profiles changes is similar accord during disease. The new 115 kDa band detected by the ubiquilin-4 antibody in endpoint animals appears distinct from a slightly smaller size ~100 kDa band that is detected by the INV 37–7700 monoclonal antibody, because their patterns are different (compare Fig 5A with Fig 5E and F). We do not know the source of the 100 kDa band that is detected by the monoclonal ubiquilin antibody, but presume it is possibly a protein(s) that has sequence homology to the central region of ubiquilin-1 to which the monoclonal antibody was raised. Although unknown this protein(s) also declines during disease progression in HD mice, just like ubiquilin. The 115 kDa band that is induced in endpoint R6/2 mice and detected by the ubiquilin-4 antibody remains unaccounted, as it does not corresponds to any reported variants of UBQLN4. Therefore, it is possible it is a non-specific band that is induced at endpoint of HD that happens to cross-react with the ubiquilin-4 antibody. However, the same size band, as well as the 35 kDa band is also present in NB2a mouse neuroblastoma lysates, and both are subject to knockdown using ubiquilin-4 specific siRNA. Therefore we can conclude they are bona fide ubiquilin-4 products. Unfortunately, we do not know whether the 115 kDa band is a novel spliced variant of ubiquilin-4 that is induced during endpoint of disease or a post-translationally modified form of ubiquilin-4.

DISCUSSION

Here we establish that all ubiquilin proteins expressed in brain colocalize with huntingtin inclusion bodies. This provides further support for the idea that ubiquilin proteins are key players in HD pathology. It is likely that the ubiquilin proteins are recruited to the aggregates because of ubiquitination of the expanded misfolded Htt protein. Ubiquilins possess a UBA domain, which is known to have high affinity for ubiquitin moieties (Raasi et al., 2005). Consistent with this idea, we found ubiquilin proteins coimmunoprecipitate more efficiently with expanded Htt proteins that are highly polyubiquitinated than with Htt proteins with a shorter CAG tract and polyubiquitinated to a lesser degree (Wang and Monteiro, 2007). Additionally, the C-terminal UBA domain of ubiquilin-4 was found to bind ataxin-1 protein with an expanded polyglutamine repeat, consistent with the involvement of the UBA domain in binding misfolded ubiquitinated proteins with polyglutamine expansions (Davidson et al., 2000). However, it remains possible that

ubiquilins are also recruited to the Htt inclusion because of its chaperonelike activity. The protein possesses chaperone-binding motifs and bind Hsp70-like proteins that are known to be present in Htt inclusions (Kaye et al., 2000; Hay et al., 2004; Sakahira et al., 2002). Of course, these two possibilities are not mutually exclusive. One question that remains is whether ubiquilins are actively disaggregating inclusion bodies, or rather just being sequestered in them. We have previously demonstrated that ubiquilin overexpression leads to a reduction in inclusion bodies (Safren et al., 2014; Wang et al., 2006; Wang and Monteiro, 2007), however, it is unclear whether ubiquilin is reducing the formation of inclusions or enhancing their clearance once they have already formed.

Relative to other brain regions, we observed enrichment of ubiquilin-1 in granule cells of the hippocampus as well as enrichment of ubiquilin-4 in granule cells and Purkinje cells of the cerebellum. The increase in ubiquilin-4 protein that we detected in the cerebellum matches the high abundance of ubiquilin-4 transcripts found in the same cell types in an earlier study (ubiquilin-4 was called A1U) (Davidson et al., 2000). Those investigators identified ubiquilin-4 by its interaction with ataxin-1, the protein responsible for causing spinocerebellar ataxia type 1 (SCA1). Similar to HD, SCA1 is a polyglutamine expansion disease. It results from an expansion in ataxin-1 (Orr et al., 1993). Purkinje cells are the primary site of SCA1 pathology (Zoghbi, 1995). As such, the enrichment of ubiquilin-4 protein in the cerebellum strengthens the link between ubiquilin-4 and its possible involvement in SCA1 pathology. The increased expression of ubiquilin-1 in the hippocampus is noteworthy because alterations in ubiquilin-1 expression, or its variants, have been linked to increased incidence of Alzheimer's disease (AD) (Bertram et al., 2005; Viswanathan et al., 2011; Stieren et al., 2011). Also ubiquilin-1 interacts and modulates expression of presenilins, which are frequently mutated in AD (Ford and Monteiro, 2007; Ganguly et al., 2008; Mah et al., 2000; Massey et al., 2004; Massey et al., 2005). The hippocampus is crucial for memory and it is a primary site of damage in AD. Perhaps then, alteration in ubiquilin-1 expression increase the incidence of Alzheimer's disease because they leave hippocampal neurons particularly vulnerable to proteotoxic stress that occurs during aging. Mutations in ubiquilin-2 cause ALS with dementia, and pathology is seen in multiple brain regions (Deng et al., 2011; Fahed et al., 2014; Williams et al., 2012). Therefore, the widespread expression of ubiquilin-2, should not be surprising. Future studies will look to see if ubiquilin-2 is enriched in the ventral horn of the spinal cord.

The differential expression of ubiquilin-4, along with its colocalization with inclusion bodies implicates the protein in HD pathology. The dramatic reduction of ubiquilin-4 proteins that are normally seen in wild-type animals, and replacement by a novel band at 115 kDa in endpoint R6/2 mice is particularly noteworthy. It is possible that this band represents an isoform that is stress responsive and/or which is a hallmark of endpoint of HD. The appearance of this novel form might be a useful biomarker for assessing the severity of HD in this and other models of HD. Future experiments will explore this possibility.

EXPERIMENTAL PROCEDURE

Antibodies

Rabbit antibodies were produced that specifically recognize ubiquilin-2 (UMY-75) and ubiquilin-3 (UMY-78). Peptides were synthesized containing sequences with low homology between the ubiquilin isoforms. These include ubiquilin-2: RPSRGPAAA, ubiquilin-3: RSLRPDGMNP peptides. The peptides were conjugated to Inject maleimide activated mCKLH (Thermo Scientific, Rockford, IL) and sent to Cocalico Biologicals, Inc. (Reamstown, PA) who injected them into rabbits to produce antibody sera. Affinity-purified ubiquilin-2 and ubiquilin-3 antibodies were prepared by passing the sera over columns containing GST-ubiquilin fusion proteins encompassing the appropriate peptide sequences used for the antibody injections. We used the following commercially available antibodies as well: Affinity Bioreagents (Golden, CO) anti-ubiquilin-1 Polyclonal PA1-759, Invitrogen (Carlsbad, CA) monoclonal ubiquilin antibody 37-7700, monoclonal EM48 (Millipore, Billerica, MA) and Aviva Biosystems (San Diego, CA) anti-ubiquilin-4 ARP57355.

Animals

Ovary transplanted female R6/2 mice (strain 006494) from Jackson Labs (Bar Harbor, Maine) were crossed with male C57BL/6J. At postnatal day 21 mice were weaned and tail snipped. Tail samples were used to genotype mice as previously described (Hockly et al., 2003) and subsequently were sent to Laragen (Los Angeles, CA) to measure CAG repeat length. The mean number of CAG repeats in R6/2 mice used was 125 with a range between 122 to 128 repeats. Upon reaching advanced pathology, animals were evaluated using previously reported euthanization criteria (Hockly et al., 2003). Mice that were euthanized after meeting euthanization criteria are referred to as endpoint R6/2 mice. All the animal work was conducted following the University of Maryland IACUC guidelines.

Immunohistochemistry

25 μ m thick cryosections were cut from mouse brain as previously described (Safren et al., 2014). UMY75, UMY78, Invitrogen 37-7700, and Aviva Bioscience Ubiquilin-4 were used at a dilution of 1:100. AlexaFluor (Invitrogen) 488 donkey-anti-mouse (A21202) and 594 donkey-anti-rabbit (A21207) secondary antibodies were used at a 1:500 dilution.

Immunoblotting

Brain lysates were prepared and immunoblotted as previously described (Safren et al., 2014; Xiao and Monteiro, 1994).

Cell Culture

NB2a neuroblastoma cells were maintained in Dubelco's Modified Eagle Media (DMEM) with 4.5g/L glucose, L-glutamine and sodium pyruvate (Cellgro, Manassas VA) supplemented with 10% fetal bovine serum and penicillin streptomycin. NSC-34 and HeLa cells were cultured in the same media without sodium pyruvate. Cells were transiently transfected with 7 μ g of plasmid DNA using Lipofectamine LTX (Life Technologies, Carlsbad CA). Lysates were collected 24 hours following transfection and then

immunoblotted. In order to knockdown ubiquilin proteins, Accell SMARTpool siRNA (GE Healthcare Dharmacon, Pittsburg, PA) was transfected into NB2a and s34 cells using DharmaFect1 (GE Healthcare Dharmacon). The protocol provided by the manufacturer was used to achieve successful transfection.

Statistical Analysis

Two-way Analysis of Variance (ANOVA) was performed using GraphPad Prism 5 software (La Jolla, CA). The Bonferroni post-hoc test was used to determine significant differences between individual groups.

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Highlights

- Generation of antibodies specific for each of the four ubiquilin proteins in mouse.
- Expression and localization of the four ubiquilin proteins in mouse brain.
- Demonstrate huntingtin inclusions contain all ubiquilin proteins expressed in brain.
- Demonstrate changes in ubiquilin protein expression during development.
- Demonstration of distinct changes in ubiquilin-4 expression in R6/2 mice during HD.

FIGURE 1. Specificity of ubiquitin antibodies

Lysates from HeLa and NB2A mouse neuroblastoma cells transfected with GFP-ubiquitin cDNAs. Successful expression of human ubiquitin-1 (hUBQ1), 2, 3 and 4 fusion proteins in (A) NB2A cells and (B) HeLa cells. (C,D) The Invitrogen 37–7700 antibody recognizes both mouse and human ubiquitin 1 and 2, as well as human ubiquitin-4. (D) In HeLa cell lysates this antibody also recognizes endogenous ubiquitin-4 (marked with an arrow). (E) The PA1 Ubiquitin-1 antibody fails to recognize human ubiquitin-1. (F) Lysates from NB2A cells transfected with a construct encoding GFP-mouse-ubiquitin-1 (mUBQ1) indicates the PA1 ubiquitin-1 antibody does specifically recognize mouse ubiquitin-1. Endogenous ubiquitin-1 runs as two distinct bands, one at 70 kDa and one at 35 kDa. (G) UMY75 specifically recognizes the transfected ubiquitin-2 product. The arrow highlights a band predicted to be the endogenous ubiquitin-2 protein. (H) UMY78 specifically recognizes ubiquitin-3. (I) ARP57-355 specifically binds ubiquitin-4.

FIGURE 2. siRNA knockdown of ubiquilins 1, 2, 4 further validates the specificity of the PA1 (UBQLN1), UMY75 (UBQLN2), and ARP57-355 (UBQLN-4) antibodies

(A,B) NSC-34 cells (mouse motor neuron cell line) were either mock transfected or transfected with an siRNA scramble, 20 nM mouse UBQLN1 (Thermo Scientific Accell 56085) smartpool siRNA, denoted as A, or 20 nM mouse UBQLN1 (Thermo Scientific ON-TARGETplus L-041012-01-0005) smartpool siRNA, denoted as B. Lysates were collected at 24, 48, and 72 hrs post transfection and blotted with the PA1 antibody. After 72 h smartpool B produces a robust knockdown of both the 70 kDa and 35 kDa bands. (C,D) NSC-34 cells were either mock transfected, or transfected with either scramble siRNA, or 20 nM mouse ubiquilin-2 siRNA (ON-TARGETplus SMARTpool L-042279-01-0005). Lysates were collected at 24, 48, and 72 hrs (Day 1, 2 and 3) posttransfection and blotted with an affinity purified UMY75 antibody. At both 48 and 72 hrs after transfection there is an almost full knockdown of ubiquilin-2. (E,F) NB2a were transfected with different amounts of ubiquilin-4 siRNA (0, 20 and 40 nM mouse ubiquilin-4 siRNA: ON-TARGETplus SMARTpool A-049080-16) that are specific for ubiquilin-4. Lysates were prepared from the cultures after 24, 48 and 72 hrs after transfection and equal amounts of protein were probed with the ubiquilin 4 antibody, or actin to monitor protein loading. Both, the 115 and 35 kDa ubiquilin-4 immunoreactive bands decreased progressively over time in the cultures transfected with the ubiquilin-4 specific siRNAs strongly suggesting they are indeed ubiquilin-4 proteins.

FIGURE 3. Expression profiles of ubiquilin proteins in mouse brain

25 μm thick sagittal brain sections were cut from a 4 month-old female C57BL/6J mouse.

(A) Immunostaining with each ubiquilin antibody reveals mostly uniform expression of ubiquilin-1 and 2, which are both enriched somewhat in the hippocampus. Ubiquilin-2 has high expression in the dentate gyrus, while ubiquilin-1 is elevated in CA3 and CA1 as well. Particularly high expression of ubiquilin-4 is observed in the hippocampus and cerebellum. Magnified views of the hippocampus (B) and cerebellum (C) illustrate these findings in greater detail. Ubiquilin-3 staining was not detected in the brain. Bars: 1000 μm in A, 200 μm in B, and 250 μm in C.

FIGURE 4. Ubiquilin 1,2, and 4 co-localize with Htt inclusions in endpoint R6/2 mice
(A) Double Immunofluorescence staining of a frontal cortex brain section stained with EM48 (green) and ubiquilin-2 (red) showing colocalization of ubiquilin-2 with Htt inclusion bodies (yellow: right panel). **(B)** Immunoreactivity of PA1 murine ubiquilin-1 antibody. Co-localization with nuclear inclusions is observed in the (a) striatum, (b) frontal cortex, and (c) hippocampus. Similar colocalization was observed with the ubiquilin-2 specific antibody UMY75 (c,d,e) and the ubiquilin-4 specific antibody ARP57-355 (g,h,i). Bars: 10 µm in both A and B.

FIGURE 5. Changes in ubiquitin expression in HD pathology

Immunoblots of brain lysates from 6 week old and endpoint R6/2 mice along with aged matched wild-type controls. **(A)** Expression changes for ubiquitin-1 and 2 using the Invitrogen 37-7700 antibody. **(B)** Age-dependant decrease in ubiquitin-1 expression. Ubiquitin-1 levels decline even further in end point animals. **(C)** The expression of ubiquitin-2 does not change regardless of age or genotype. **(D)** Ubiquitin-3 protein is not expressed in the brain. **(E,F)** Ubiquitin-4 expression changes as a result of HD pathology. In endpoint R6/2 mice there is a dramatic reduction in both the 70 kDa and 35 kDa bands seen in earlier time-point R6/2 mice and wild type (WT) control animals. This coincides with the appearance of a 115 kDa immunoreactive band. **(G)** Quantification of ubiquitin expression relative to WT ubiquitin expression at the different time intervals shown in panels A to F. All values are normalized for actin loading. (*) Denotes a statistically significant difference defined as a $p < .05$ result from a Bonferroni post-hoc test comparing 6 week old and ~18wk old mice of the same genotype. (#) Indicates a significant difference defined as a $p < .05$ result from a Bonferroni post-hoc test comparing WT and R6/2 mice of the same age.

Table 1

PROTEIN	PRODUCT #	PEPTIDE SEQUENCE	SPECIES RECOGNIZED
Ubiquilin-1	Affinity Bioreagents PA1-759	amino acids: 2–18 AESAESGGPPGAQDSAA	mouse
Ubiquilin-1	UMY74	amino acids: 501–510 NGSNATPSEN	human
Ubiquilin-2	UMY75	amino acids: 12–20 RPSRGPAAA	mouse, human
Ubiquilin-3	UMY78	amino acids: 484–493 RSLRPDGMNP	mouse, human
Ubiquilin-4	Aviva Biosystems ARP 57–355	amino acids:287–336 TDIQEPMFSAAREQFGNNPFSSLAGNS DSSSSQPLRTENREPLPNWSPS	mouse, human
Ubiquilin 1,2,4	Invitrogen 37–7700	amino acids 113–540	mouse, human

Antibodies used in the study and their specificity.