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Unbiased Screen Reveals Ubiquilin-1 and -2 Highly Associated with Huntingtin Inclusions

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Abstract

Recently mutations in ubiquilin-2 were identified in patients with amyotrophic lateral sclerosis (ALS) and ALS/dementia providing direct evidence for the importance of this protein in neurodegenerative diseases. Histological studies have suggested that ubiquilin-1/-2 are associated with various pathological inclusions including Lewy bodies in Parkinson's disease, neurofibrillary tangles in Alzheimer's disease, polyQ inclusions in expansion repeat diseases and various proteinopathies associated with ALS and frontotemporal dementia. Using specific ubiquilin-2 antibodies and a series of transgenic mouse models of proteinopathies associated with neurodegenerative disease, we show that ubiquilin-2 preferentially associates with huntingtin polyQ expansion aggregates compared to α-synuclein, tau and several other types of protein inclusions. These results were confirmed by similar findings for ubiquilin-1 and -2 in human brain tissue sections, where accumulation was observed in huntingtin inclusions, but only infrequently in other types of protein inclusions. In cultured cells, ubiquilin-2 associates with huntingtin/polyQ aggregates, but this is not compromised by disease-causing mutations. Although ubiquilin proteins can function as chaperones to shuttle proteins for degradation, there is persistent co-localization between ubiquilin-2 and polyQ aggregated proteins during disease progression in the N586-82Q-C63 Huntington's disease mouse model. Thus, the co-localization of ubiquilin-2 with the huntingtin aggregates does not appear to facilitate aggregate removal.

Keywords

Huntington's disease; inclusions; ubiquilin; transgenic mice

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1. Introduction

Neurodegenerative diseases are a group of disorders that lead to an insidious erosion in the quality of life and ability to function through the demise of neurons and in some cases glial cells (Forman et al, 2004, Hardy and Orr, 2006, Ross and Poirier, 2004, Taylor et al, 2002). The most common of these disorders is Alzheimer's disease (AD), but they also include Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and many others. The major demographic risk factor for all of these diseases is aging, thus with the aging population they will become even more prevalent and will impose an even greater social and economic burden on society (Forman et al, 2004, Lees et al, 2009, Reitz et al, 2011, Taylor et al, 2002). Most neurodegenerative diseases also present as familial forms due to genetic alteration (Forman et al, 2004, Hardy and Orr, 2006). For example, more than 18 different gene loci have been identified as causes of parkinsonism (Lesage and Brice, 2009, Martin et al, 2011, Westerlund et al, 2010) with a similar number for ALS (Al-Chalabi et al, 2012). Neurodegenerative disorders are also commonly associated with unifying pathological changes such as neuroinflammation, neuronal loss and protein aggregation that may share common primary or secondary pathogenic mechanisms (Beal, 1996, Forman et al, 2004, Ross and Poirier, 2004). Nevertheless, specific neurodegenerative diseases are often associated with a primary or defining pathology. For example, amyloid plaques containing the A peptide and neurofibrillary tangles containing tau are hallmark inclusions of AD, while Lewy bodies comprised of α-synuclein are characteristic of PD (Goedert et al, 1998, Lee et al, 2001, Selkoe, 2001, Waxman and Giasson, 2008a). However, it is becoming clear that there are often two or more distinct proteinacious inclusion pathologies in many neurodegenerative diseases (Beal, 1996, Forman et al, 2004, Geser et al, 2009, Giasson et al, 2002b, Lee et al, 2004). Notably, some of the interactions between specific gene alterations and specific neurodegenerative diseases are not intuitive. For example, recessive mutations in the *GBA* gene encoding the enzyme glucocerebrocidase can cause Gaucher's disease, but the same heterozygous mutations also can be a risk factor for PD and dementia with Lewy bodies (DLB) (Sidransky et al, 2009).

Due to the increasing number of genetic alterations associated with human proteinopathies and awareness of overlap in the different types of protein aggregates involved in neurodegenerative diseases, we surveyed existing transgenic (Tg) mouse models that reproduce aspects of pathology found in human AD, PD, frontotemporal degeneration (FTD), ALS, or HD for the formation of heterogeneous protein aggregation. Because the genetic trigger is defined in each transgenic model, this screen provides an unbiased approach to determine which primary pathologies could ultimately cause a secondary pathology, potentially elucidating an interaction between the primary and secondary proteins. We identified that ubiquilin-2, which was recently associated with ALS and FTD (Deng et al, 2011), is being robustly and uniquely recruited in huntingtin (Htt) inclusions in a mouse model of Huntington's cytoplasmic inclusion pathology and validated these findings in human brains and cell culture studies.

2. Results

Given the increased awareness of the overlap in various proteinaceous inclusions that can occur in a range of neurodegenerative diseases and the increasing number of aggregated proteins recently identified, we performed an immunohistochemical (IHC) screen of a series of Tg mouse models of various neurodegenerative diseases (see Table 1), with robust protein inclusions, with a battery of antibodies to proteins known to aggregate (see Table 2). These studies were aimed at trying to identify novel protein interactions, while also internally controlling for specificity and selectivity of the findings. We used previously well-characterized Tg mouse models of A amyloid: CRND8, Tg2576 and Tg2576 crossed onto

the P264L PS1 knock-in background that enhances amyloid deposition. We also used the following mouse models with robust protein inclusions: α-synuclein (lines M47 and M83), tau (JNPL3 and rTg4510), Htt (line N586-82Q-C63), TDP-43 (line diTDP-$43_{WT,}$ line 5a) and SOD-1 (line 139). We also used progranulin-null mice that demonstrate significant lipofuscinosis. In each case, the tissue sections from all these mice were of an age where they displayed extensive aggregates of their primary proteinopathy. We screened these mice with a battery of antibodies to various proteins including phosphorylated tau, phosphorylated α-synuclein, A phosphorylated TDP-43, ubiquitin, ubiquilin-2, profilin, FUS, Htt and poly-Q. As expected, the primary proteinopathy for each respective mouse model with each respective antibody (eg. anti-phosphorylated tau antibody in tau Tg mouse) were observed. In addition, remarkable anti-ubiquilin-2 immunostaining for Htt inclusions in N586-82Q Tg mouse model of HD was observed (Figure 1). Htt inclusions or ubiquilin-2 aggregates were not observed in the control Tg mouse line N586-23Q-A2 (Figure 1) (Tebbenkamp et al, 2011). No ubiquilin-2 immunostaining for protein inclusions was observed in any of the other Tg mouse models analyzed.

Ubiquilin-2 is a member of a family of 4 proteins (Lee and Brown, 2012). Ubiquilin-1 and -2 have a high degree of homology and many antibodies that has been generated against these proteins can react with both of these proteins (Brettschneider et al, 2012). Ubiquilin-3 is exclusively expressed in the testes (Davidson et al, 2000), whereas ubiquilin-4, also known as ataxin-1-interacting protein, has the least homology (Davidson et al, 2000). In order to pursue these studies further we first needed to validate the specificity of the commercially available ubiquilin-1 and -2 antibodies (6431 and U7258) that were used in our primary screen, as well as additional antibodies (#74 and 6H9) that were generated. Using immunoblotting analysis with recombinant proteins or extracts for cells overexpressing ubiquilin-1 or -2, we demonstrated that antibody 6431 is specific for ubiquilin-2 (Figure 2). Antibody 6H9 was highly selective, but not completely specific, for ubiquilin-2. Antibody U7258 was highly selective for ubiquilin-1; however, this antibody does not recognize murine ubiquilin-1. Antibody #74 reacted equally with ubiquilin-1 and -2.

We then proceeded to characterize the spatial and temporal sequence for the accumulation of ubiquilin-2 in Htt in N586-82Q-C63 mice. In these mice, few Htt aggregates can be observed at 2 month of age, but become abundant at 4 months of age and continue to accumulate up to 8–9 months, at which time the mice develop severe dyskinesia and have to be sacrificed (Tebbenkamp et al, 2011). Immunostaining for ubiquilin-2 was performed in cohorts of 1,2,4,6 and 8 month old N586-82Q-C63 Tg mice. The presence of ubiquilin-2 immunoreactivity within Htt inclusions of N598-82Q-C63 remarkably paralleled the timing and the spatial distribution of Htt inclusions such that they almost perfectly overlapped both temporally and spatially [i.e. highly abundant in the cortex, striatum, granular cells of the cerebellum and the hippocampus (Tebbenkamp et al, 2011)] (Figure 3). These same findings also were observed with anti-ubiquilin-1/2 antibody #74 (data not shown). We were not able to assess the effects of Htt inclusions on ubiquilin-1 distribution in N586-82Q-C63 Tg mice, since antibody U7258 does not recognize murine ubiquilin-1. We obtained other commercially available ubiquilin-1 antibodies, but were unable to demonstrate that they specifically react with murine ubiquilin-1.

To assess if ubiquilin-2 was also recruited in Htt inclusions in human brain, we performed IHC and double immunofluorescence analysis on HD brains. Similar to the finding in Tg mice, we found that a significant proportion of Lewy body-like Htt inclusions in 2 HD patients were also labeled with ubiquilin-2 antibodies (Figure 3) and, as previously reported (Mori et al, 2012), some Htt inclusions in HD patients were also stained for ubiquilin-1 (data not shown). Several studies of human brains have indicated that ubiquilin-1 and -2 are found

in many different types of inclusions including neurofibrillary tangles in AD, Lewy bodies in PD, glial cytoplasmic inclusions in multiple system atrophy (MSA) and many different types of inclusions in patients with polyQ disease (Mori et al, 2012). These findings seemed at odds with our screen of a collection of mouse models of neurodegenerative diseases, since our findings indicated that tau and α-synuclein inclusions were negative for ubiquilin-2 staining. Therefore, we used our ubiquilin-1 and -2 antibodies to investigate the presence of these proteins within pathological aggregates in a series of human brains with neurodegenerative diseases. In 8 cases with PD and 5 cases with MSA we did not observe any ubiquilin-1 or -2 inclusions. In one additional case of PD, only scant ubiquilin-2 dystrophic neurite aggregates were observed and Lewy bodies were not labeled. Limited ubiquilin-2 inclusions were also observed in 1 out of 3 DLB cases and no ubiquilin-1 aggregates were observed. Out of 13 cases with AD with Lewy bodies (AD/DBL), 3 presented with scant ubiquilin-1 aggregates while 5 depicted rare ubiquilin-2 inclusions and the remaining cases displayed no ubiquilin-1 or -2 aggregates. Of 9 AD cases analyzed, 5 displayed no ubiquilin-1 or -2 aggregates, 3 presented with occasional ubiquilin-1 and -2 aggregates, while one case showed moderate levels of both ubiquilin-1 and 2 inclusions especially in the hippocampus and amygdala (Figure 4). In this case, a subset of ubiquilin-1 and -2 aggregates co-localized within α-synuclein and tau inclusions, but biquilin-1 and -2 also often co-localized (Figure 5). None of these AD cases had ubiquilin-2 inclusions in the cerebellar granular layer, as has been reported for patients with ALS/frontotemporal dementia with *C9ORF72*-hexanucleotide repeat expansion (Brettschneider et al, 2012), and repeat primed PCR analysis of their genomic DNA, carried out as previously described (Dejesus-Hernandez et al, 2011), showed that their *C9ORF72*- repeat lengths were within the normal range.

Given the apparent specificity of ubiquilin-2 for Htt inclusions, we investigated whether ubiquilin-1 and -2 could be recruited within Htt aggregates in cultured cells and if recently identified ubiquilin-2 disease-causing mutations (Deng et al, 2011) altered this property. We expressed a fragment of Htt with a polyQ expansion using the plasmid Htt N118-82Q/pcDNA 3.1 to model intracellular Htt inclusion formation. In this model, Htt inclusions form in a subset of the transfected cells. We observed that both ubiquilin-1 and -2 were efficiently recruited into the Htt aggregates as 84.8 +/−8.4 % and 62.1+/− 4.3 % of Htt inclusions co-labeled for ubiquilin-1 and -2 respectively, in cells that overexpressed both ubiquilin and Htt. As noted by others, ubiquilin-1 and ubiquilin-2 also formed cytoplasmic puncta when expressed in cells and these have previously been shown to be at least partially due to localization with autophagesome (Heir et al, 2006, Rothenberg et al, 2010), furthermore, using an antibody to TIA-1, we also showed that these are not stress granules, that can morphologically appear very similar, and have been implicated in neurodegenerative diseases (Wolozin, 2012) (Supplementary Figure 1). We also show that as reported previously these puncta occur when ubiquilin-1 and -2 are expressed alone (Supplementary Figure 1). The P497H, P497S and P506T ubiquilin-2 mutations did not impair the ability of ubiquilin-2 to be recruited to Htt inclusions relative to the wild-type protein; expressed as a percentage of Htt inclusions that co-localized with ubiquilin-2 in cells that over-expressed both proteins: wild-type (62.1 +/− 4.3 %), P497H (70.3 +/− 4.9 %), P497S (63.9 +/− 6.7%) and P506T (66.7+/− 4.2%).

3. Discussion

Ubiquilins are bifunctional proteins having both an ubiquitin-like (UBL) domain and a carboxy ubiquitin-associated (UBA) domain, which binds to ubiquitinated proteins (Ko et al, 2004, Lee and Brown, 2012, Zhang et al, 2008). The amino-terminal UBL domain associates with the ubiquitin-interacting motif of the S5a subunit of the proteasomal 19S cap (Walters et al, 2002), therefore ubiquilins can act as proteasomal shuttling linkers (Kleijnen

et al, 2000). Consistent with this function, they also are involved in recruiting targets for proteasome-mediated degradation via ER- associated protein degradation (ERAD)(Lim et al, 2009). Ubiquilins are also associated with autophagosomes and regulate the degradation of proteins by autophagy, perhaps also by recruiting ubiquitinated proteins to these cellular degradation organelles (Rothenberg et al, 2010). Therefore, ubiquilins are multi-function proteins involved in selective protein degradation required to maintain cellular homeostasis.

Ubiquilin-1 and ubiquilin-2, also known as protein linking integrin-associated protein and cytoskeleton (PLIC) -1and-2, have previously been reported to bind to several polyQ proteins including Htt and ataxin-3 (Doi et al, 2004, Heir et al, 2006). Ubiquilin-4 was also shown to interact with ataxin-1, another polyQ protein (Davidson et al, 2000). Functional studies in cell culture and *C. elgans* models of polyQ aggregation indicated that ubiquilin-1 expression could reduce polyQ aggregation and toxicity while promoting degradation (Rothenberg et al, 2010, Wang et al, 2006, Wang and Monteiro, 2007).

Ubiquilin-1 has also previously been shown to interact with and affect the levels of presenilin-1 and -2, the enzymatic subunit of the γ-secretase enzyme, involved in the processing of the amyloid precursor protein to yield the amyloid-β peptide that is the major component of senile plaques in AD (Mah et al, 2000). Ubiquilin-1 can also directly interact with the amyloid precursor protein and modulate its processing by acting as a chaperone (El et al, 2012, Stieren et al, 2011). Initial IHC studies also suggested that ubiquilin-1 was a major component of neurofibrillary tangles in AD and Lewy bodies in PD (Mah et al, 2000). More recently it was reported that ubiquilin-1 and ubiquilin-2 were present in polyQ inclusions from various diseases (eg. HD and spinal cerebral ataxia1–3) as well as Lewy bodies in PD and DLB and glial cytoplasmic inclusions in MSA (Mori et al, 2012). In contrast to these studies, we did not detect ubiquilin-1 or ubiquilin-2 aggregation or accumulation within pathological inclusions in the majority of PD, DLB or MSA brains that we analyzed with specific ubiquilin-1 and -2 antibodies. In a subset of PD, DLB and AD cases, scant or rare ubiquilin-1 or ubiquilin-2 inclusions were observed and only 1 case presented with moderate levels of ubiquilin-1 or ubiquilin-2 aggregates, predominantly in the hippocampus and amygdala.

A distinct pattern of ubiquilin pathology was reported in cases with ALS and frontotemporal lobar degeneration-TDP43 with the *C9ORF72* hexanucleotide (GGGGCC) repeat expansion (Brettschneider et al, 2012). In particular ubiquilin cytoplasmic inclusions were uniquely observed in the cerebellar granular layer. Although our AD cases with ubiquilin-1 or ubiquilin-2 pathology did not have cerebellar pathology, we screened them for *C9ORF72* hexanucleotide repeat expansion and found that they did not have the expansion. In our analyses of this subset of unique cases, we found that ubiquilin-1 or ubiquilin-2 accumulations often co-localized with α-synuclein and tau inclusions. Other studies in human brain have shown that a subset of ubiquilin aggregates can be found concurrently in the same cells that have TDP-43 inclusions (Brettschneider et al, 2012, Deng et al, 2011) and in some instances ubiquilins can also co-localize with other proteins such as FUS, optineurin, and superoxide dismutase-1 that are involved in ALS (Deng et al, 2011), but the overall frequency of these occurrences was not reported. It is not known if ubiquilins are found in inclusions because they associate with misfolded proteins and/or they can self-aggregate to form inclusions. In addition, it is unclear if the accumulation of ubiquilins within pathological inclusions would be due to direct interactions with the specific proteins or with the ubiquitinated form of the proteins. Our data would suggest that tau, α-synuclein, amyloid, TDP-43 aggregation, and lipofuscin cannot directly or efficiently trigger the aggregation of either ubiquilin, while Htt aggregation robustly triggers ubiquilin aggregation. Furthermore, if the appearance of the ubiquilin pathology in the Htt mice was driven solely by its interaction with ubiquitinated protein, then it also would be expected to

see its accumulation in the ubiquitinated inclusions of progranulin null mice and α-synuclein and tau Tg mice (Ahmed et al, 2010, Lewis et al, 2000, Sampathu et al, 2003).

Collectively, our screen using Tg mouse models of neurodegenerative disease and human brain specimens demonstrate that ubiquilin-2 is more likely to associate with polyQ inclusions and only infrequently associate with inclusions comprised of α-synuclein or tau. The functional consequences of these interactions are not clear, but it is likely that accumulation of ubiquilins in polyQ inclusions may be due to attempts to degrade aggregated protein either by the proteasome or by autophagy. Some cellular studies have shown that the ubiquilin expression can at least transiently reduce polyQ protein aggregation, but as shown by other findings in Tg mice, this attempt is eventually not successful as N586-82Q-C63 Tg mice continue to degenerate despite intense association of ubiquilin-2 in the inclusions. It is even possible that this association between ubiquilin-2 and aggregated Htt contributes to neuronal demise by depleting the functional protein pool of ubiquilin-2 and the interaction with ubiquilin-2 may even enhance protein aggregation by linking the recruitment of other misfolded, ubiquitinated proteins, but further studies will be needed to assess these possible pathogenic mechanisms.

4. Experimental Procedures

4.1 Tg mouse models of neurodegenerative disease

All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

The following previously described mouse models of neurodegenerative pathology were used: To model amyloidosis, we utilized two independent transgenic lines (TgCRND8 and Tg2576) as well as the Tg2576 mice on a mutant PS1 background. TgCRND8 mice overexpress a double mutant (K670N/M671L and V717F) form of human amyloid precursor protein 695 amino acid isoform leading to age-dependent cognitive deficits and Aβ amyloid pathology (Chishti et al, 2001). Tg2576 mice express human amyloid precursor protein with the Swedish double mutation K670N/M671L and develop amyloid plaques (Hsiao et al, 1996), which is enhanced when Tg2576 mice are crossed onto a P264L PS1 knock-in background that enhances amyloid deposition (Flood et al, 2002, Siman et al, 2000). To model tauopathy, we utilized the rTg4510 and the JNPL3 Tg mouse models that express P301L (0N4R) human tau. The bigenic rTg4510 model uses a CamKII α-dependent tetracycline transactivator transgene (Mayford et al, 1996) to drive the conditional expression of mutant human tau and resultant mature tauopathy primarily in the forebrain between 4–5.5 month depending on forebrain structure (Santacruz et al, 2005). The JNPL3 mouse model utilizes the mouse prion promoter (Borchelt et al, 1996) to drive human tau expression in the spinal cord and multiple regions of the brain (Lewis et al, 2000). JNPL3 mice develop tauopathy focused in the spinal cord and the hindbrain, resulting in progressive motor dysfunction. To model α-synucleinopathy, we used the M83 and M47 Tg mouse models that express A53T and E46K human α-synuclein, respectively, driven by the mouse prion protein promoter. These mice develop age-dependent severe motor impairments leading to death associated with the widespread neuronal formation of α-synuclein amyloidogenic inclusions (Emmer et al, 2011, Giasson et al, 2002a). To model Huntington's pathology, we utilized the N586-82Q-C63 mouse model expressing 586 amino acids N-terminus fragment of Htt containing 82 glutamine repeats that develop robust cytoplasmic inclusions of Htt (Tebbenkamp et al, 2011). The Tg mouse line N586-23Q-A2 expressing 586 amino acids N-terminus fragment of Htt containing 23 glutamine repeats was also included as a control (Tebbenkamp et al, 2011). To model TDP-43 proteinopathies, we used the diTDP-43_{WT} Tg mouse line that conditionally express human TDP-43 (Cannon

et al, 2012). We also used the mutant SOD-1 mouse model (line 139) that accumulates significant protein aggregates observed in human ALS caused by mutant SOD1 (Wang et al, 2002). Progranulin null mice that have robust accumulation of lipofuscin were also investigated (Ahmed et al, 2010).

4.2 Antibodies

pSer129 is a mouse monoclonal antibody that specifically recognizes phosphorylated α-synuclein at S129 (Waxman and Giasson, 2008b). AT8 (Thermo-fisher) is specific towards phosphorylation sites S202 and T205 in tau (Goedert et al, 1995). PHF1 (generously provided by Dr. Peter Davies, Albert Einstein University, New York, NY) is specific towards phosphorylation sites S396 and S404 in tau (Otvos, Jr. et al, 1994). Anti-Htt antibody 2B4 (Millipore) and anti-polyglutamine track antibody 1C2 (Thermo-fisher) were used to detect Htt aggregates. Anti-total A antibody 33.1.1 was previously characterized (Kim et al, 2007). Anti-profilin-1antibodies P7624 and P7749 were obtained from Sigma-Aldrich (St. Louis, MO) and C56B8 from Cell Signaling Technology Inc (Danvers, MA). Mouse monoclonal anti-ubiquitin antibody MAB1510 and anti-actin antibody (clone C4) were purchased from Millipore (Billerica, MA). Rabbit anti-ubiquilin-1 antibody U7258 and rabbit anti-ubiquilin-2 antibody 6431 were purchased from Sigma. Rabbit anti-ubiquilin-1/2 polyclonal antibody #74 was generated using recombinant ubiquilin-1 as an antigen and mouse monoclonal anti-ubiquilin-2 antibody 6H9 was generated using recombinant ubiquilin-2 as an antigen. Goat anti-TIA-1 (C-20) antibody was purchased from Santa Cruz Biotechnologies.

4.3 Immunohistochemistry

Mice were humanely euthanized either with cervical dislocation or sacrificed with CO_2 euthanization and brains and spinal cords were fixed by immersion in PBS-buffered formalin. In some cases, mice were perfused with PBS/heparin and brains and spinal cords were immersion fixed in 70% ethanol/150 mM NaCl. Human brain specimens were also fixed by immersion in PBS-buffered formalin. Mouse and human brain specimens were dehydrated using an ascending series (70–100%) of ethanol solutions followed by xylene and paraffin infiltration. The specimens were mounted in paraffin blocks and cut into 8 μm sections mounted on glass slides. Mouse and human paraffin-embedded sections were deparaffinized in xylene and rehydrated by immersion in a descending series of ethanol solution (100-70%). In some cases, sections underwent antigen retrieval in a steam bath for 30 minutes or treatment with 70% formic acid (1–5 minutes). Endogenous peroxidase activity was quenched by incubation in an 80% methanol/2% H_20_2 solution for 10 minutes. Sections were incubated with primary antibodies in 0.1M Tris pH 7.6/2% FBS overnight at 4°C and subsequently incubated with biotintylated anti-rabbit or anti-mouse antibodies (Vector) for one hour. To detect signal, a standard peroxidase ABC system (Vector) was used with a DAB reagent kit (KPL). Sections were counterstained with hematoxylin, dehydrated by an ascending series of ethanols and xylene, and cover slipped with Cytoseal (Thermo Scientific).

4.4 Immunofluorescence staining of mouse and human tissue

Sections were deparaffinized in xylene and rehydrated by immersion in a descending series of ethanols. Sections underwent antigen retrieval in a steam bath for 30 minutes. Sections were incubated with primary antibodies in 0.1M Tris pH 7.6/5% non-fat milk with azide overnight and subsequently incubated with secondary antibodies conjugated to either Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen) for 1 hour. Sections were post-fixed with 10% phosphate buffered formalin, immersed in amino-black to quench lipofuscin auto-fluorescence, and counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Pierce). Sections were mounted and cover slipped with Fluoromount G (Southern Biotech). Pictures

were obtained using an Olympus BX51 fluorescent microscope with FITC, Texas Red, and DAPI filters. To visualize co-localization, images from each filter were layered using Photoshop software.

4.5 Recombinant ubiquilin-1 and -2 and expression of ubiquilin-1 and -2 in cultured cells

The cDNAs for full-length human ubiquilin-1 and ubiquilin-2 were purchased from Open Biosystems. They were subcloned into the *Eco*RI/*Sac*I and *Spe*I/*Not*I restriction sites, respectively, of the mammalian expression plasmid pAAV1, where expression is driven by the CMV early enhancer/chicken β actin (CAG) promoter.

Human embryonic kidney derived QBI293 cells were maintained using Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. For the formation of intracellular Htt inclusions, cells were transfected with the mammalian expression vector Htt N118-82Q/pcDNA 3.1.

Recombinant human ubiquilin-1 and -2 were expressed in *E. coli* as N-terminal His-tagged proteins and purified by Ni-exchange chromatography as a service provided by Genescript (Piscataway, NJ).

4.6 Western blot analysis

Protein samples were resolved by SDS-PAGE on 10% gels followed by electrophoretic transfer onto nitrocellulose membranes. Membranes were blocked in Tris buffered saline (TBS) with 5% dry milk, and incubated overnight with primary antibodies diluted in TBS/5% dry milk. Protein bands were detected by incubating with goat anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugated antibodies (Jackson ImmunoResearch Laboratories) followed by chemiluminescent reagents (NEN, Boston, MA) and using a FluorChem E and M Imager (Proteinsimple, San Jose, California).

4.7 Double Immunofluorescence Analysis of Cultured Cells

For double-immunofluorescence, cells were plated on glass slides coated with poly-d-Lys. Following transfection, cells were rinsed with PBS and fixed in 4% paraformaldehyde for 10 minutes, followed by PBS/0.1% Triton X-100 for 10 min. Following washes with PBS, coverslips were blocked with PBS containing 5% FBS and incubated with primary antibodies diluted into blocking solution for 1–2 h at room temperature. After PBS washes, coverslips were incubated in secondary antibodies conjugated to Alexa488 or Alexa594 for 1 h. Nuclei were counterstained with DAPI and coverslips were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL). Double-immunofluorescence pictures were captured on an Olympus BX51 fluorescence microscope mounted with a DP71 digital camera (Olympus, Center Valley, PA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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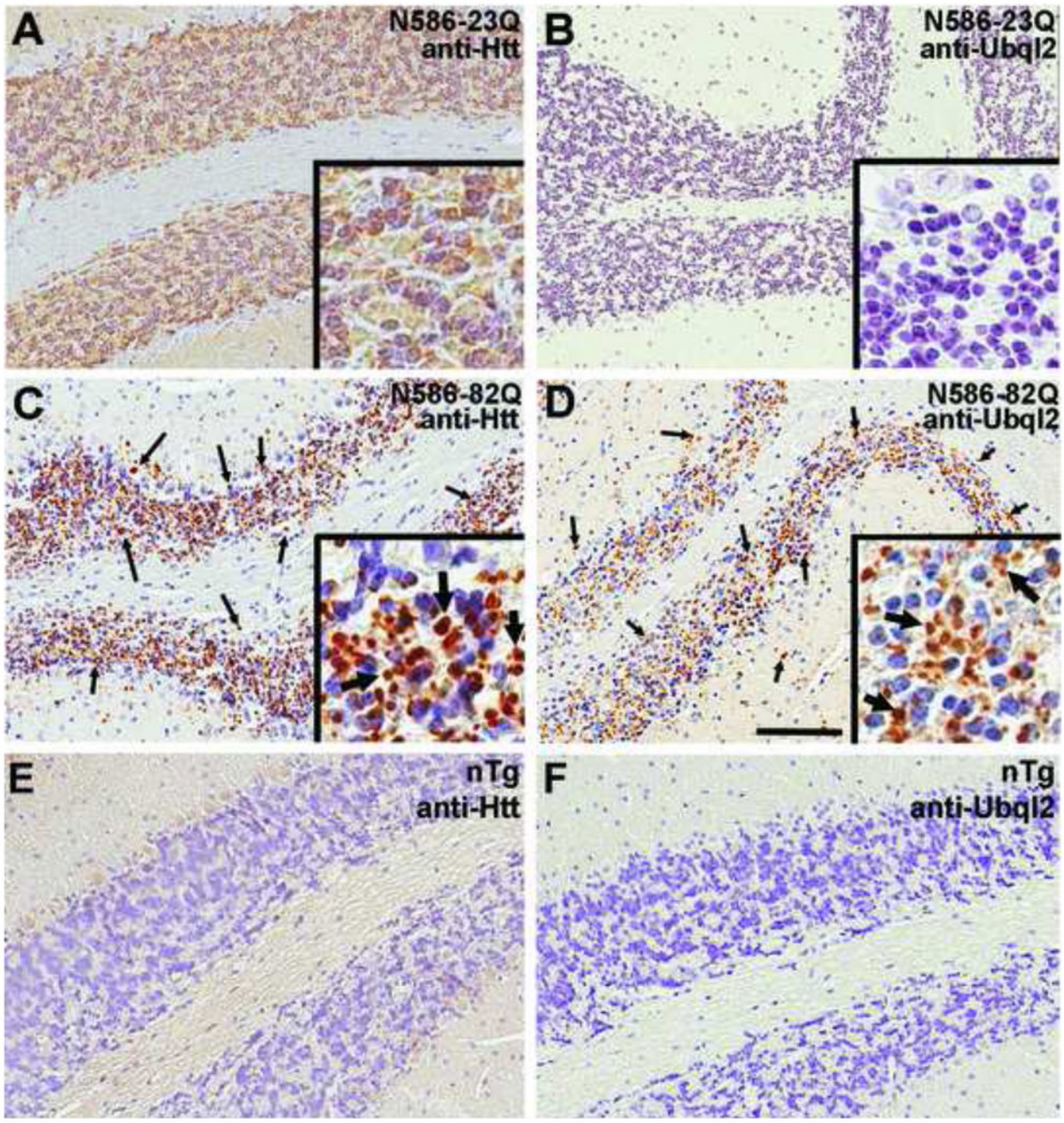

Figure 1. IHC analyses of Htt and ubiquilin-2 aggregates in N586-82Q mice

Immunohistochemistry with anti-Htt antibody 2B4 (**A, C, E**) and anti-ubiquilin-2 antibody 6431 (**B, D, F**) in the cerebellum of 6 month old N586-23Q (**A, B**), N586-82Q (**C, D**) Tg and non-Tg (nTg) mice. Htt has a general cytoplasmic distribution in N586-23Q mice, while abundant Htt inclusions that accumulate in the cerebellar granule and molecular cell layer of 6 month old N586-82Q mice extensively recruit ubiquilin-2. Arrows depict some of the Htt inclusions that accumulate ubiquilin-2. Sections were counterstained with hematoxylin. Bar = 100 μm; inset 25 μm.

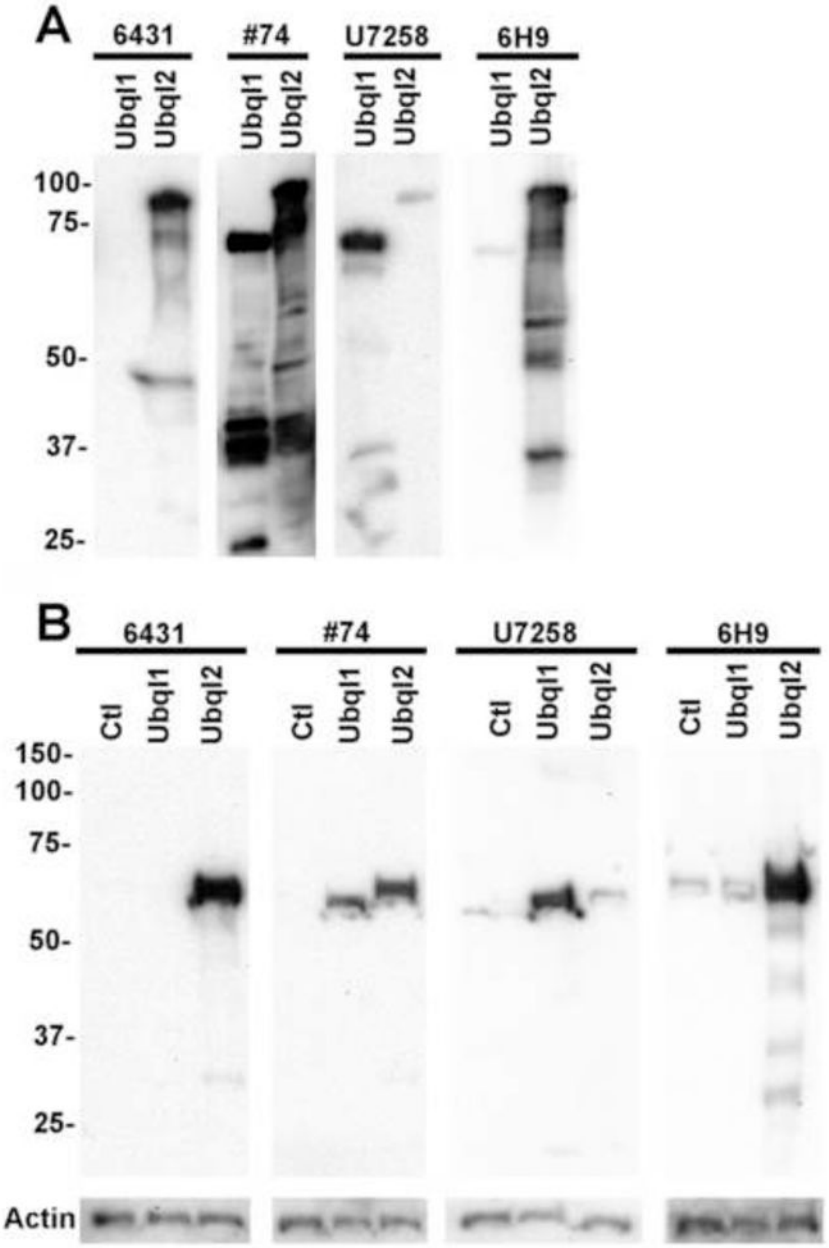

Figure 2. Immunoblotting analysis of the specificity of ubiquilin-1 and ubiquilin-2 antibodies

(**A**) 100 ng of recombinant human ubiquilin-1 (Ubql1) or ubiquilin-2 (Ubql2) or (**B**) 5 μg of cell extract from untransfected QBI293 cells (ctl) or cells transfected to over-express ubiquilin-1 or ubiquilin-2 were resolved on separate lanes of SDS-polyacrylamide gels. The ubiquilin antibodies identified above each panel were used for immunoblotting analysis. The mobility of molecular mass of protein standards are labeled on the left. For cell extracts, actin is shown as a loading control.

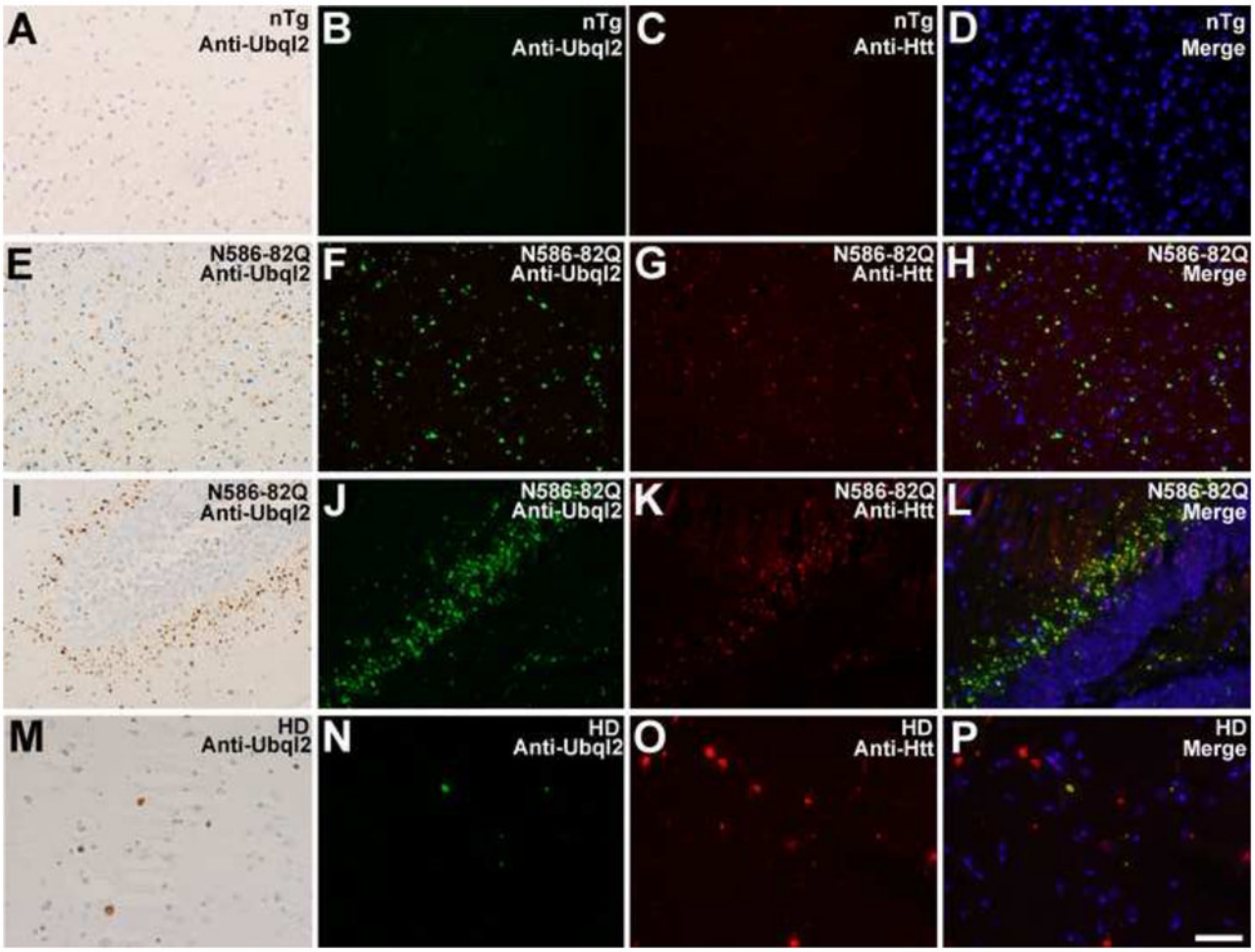

Figure 3. Accumulation of ubiquilin-2 in Htt inclusions of N586-82Q Tg mice and human HD brain

Immunostaining in an 8 month-old non-transgenic (nTg) mouse (**A–D**), 8 month-old N586-82Q-C63 mouse (**E–L**) or an HD patient (**M–P**). Sections were stained anti-ubiquilin-2 antibody 6431 (**A, B, E, F, I, J, M** and **N**) or anti-Htt antibody 2B4 (**C, G**, **K** and **O**). Overlays of double immunofluorescence with DAPI staining shown in **D, H**, **L** and **P**. The cortex is shown in **A–H**, the hippocampus in **I–L** and the globus pallius in **M–P**. Bar = 50 μM.

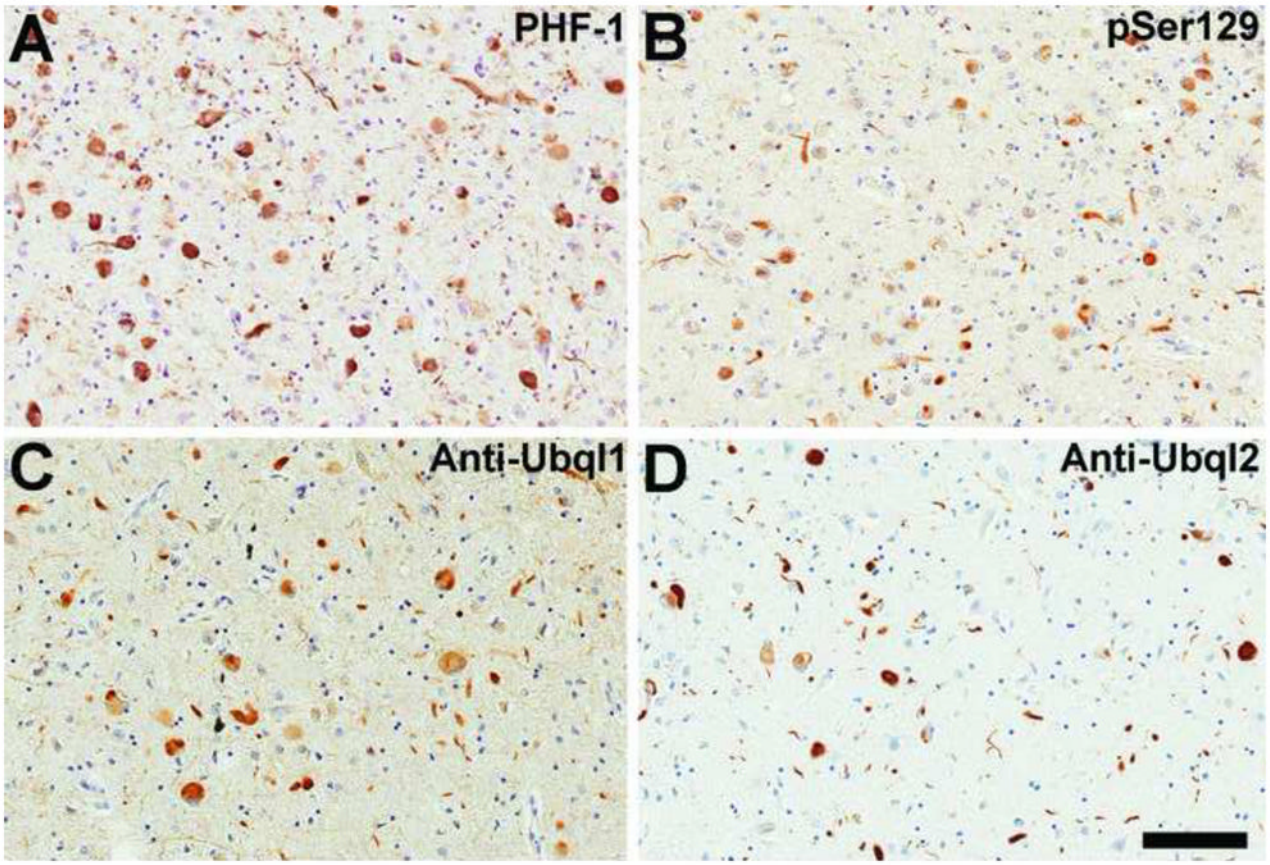

Figure 4. IHC demonstrating significant ubiquilin-1 and ubiquilin-2 pathology in the amygdala of an Alzheimer's disease patient

(**A**) Immuno-staining with anti-phospho-tau antibody PHF-1, (**B**) anti-pSer129 α-synuclein antibody, (**C**), anti-ubiquilin-1 antibody U7258, and (**D**) anti-ubiquilin-2 antibody 6431 in the amygdala. Sections were counterstained with hemotoxylin. Antibodies labeled numerous globus and curvilinear perikaryal neuron inclusions, swollen dystrophic neurites and thread-like accumulations. Bar = 100 μm.

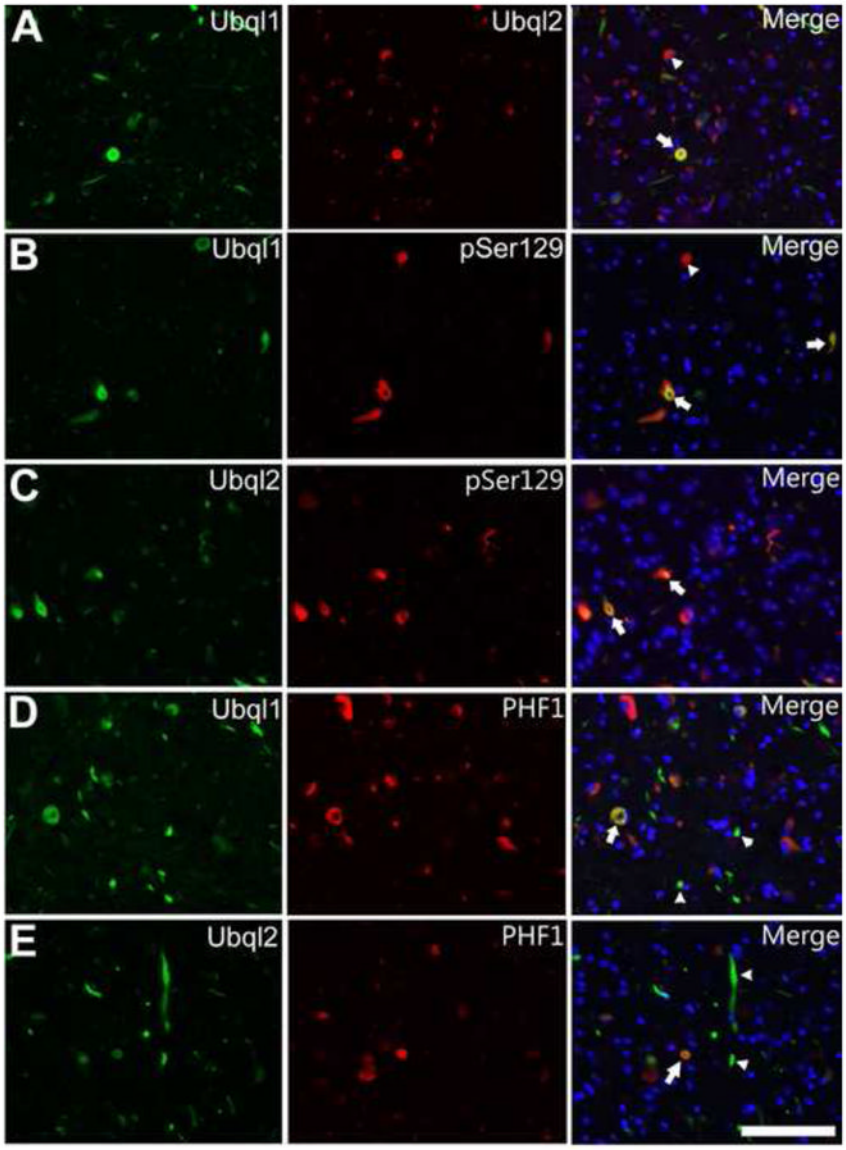

Figure 5. Co-localization of ubiquilin-1 and -2 with each other, pSer129 or PHF1 in the amygdala of an AD patient

Representative double immunofluorescence staining between (**A**) anti-ubiquilin-1 antibody U7258 (green) and anti-ubiquilin-2 antibody 6H9 (red), (**B**) anti-ubiquilin-1 antibody U7258 (green) and anti-pSer129 α-synuclein antibody (red), (**C**) anti-ubiquilin-2 antibody 6431 (green) and anti-pSer129 α-synuclein antibody (red), (**D**) anti-ubiquilin-1 antibody U7258 (green) and anti-phospho-tau antibody PHF-1 (red), and (**E**) anti-ubiquilin-2 antibody 6431 (green) and anti-phospho-tau antibody PHF-1 (red). Sections were counterstained with DAPI and overlays are shown on the right column. Arrows depict examples of co-localization, while arrowheads indicate inclusions without co-localization. Bar = 100 μm.

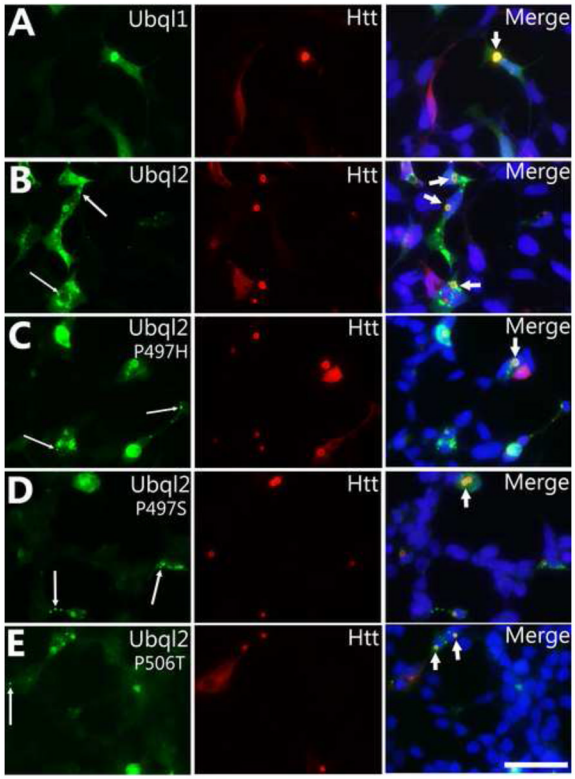

Figure 6. Immunofluorescence analysis of the co-localization of wild-type ubiquilin-1/-2 and ubiquilin-2 mutants with huntingtin inclusions in cultured cells

QBI293 cells were transfected with constructs expressing N118Htt-Q82 and wild-type ubiquilin-1 (**A**), wild-type ubiquilin-2 (**B**) or ubiquilin-2 mutants P497H (**C**), P497S (**D**) P506T (**E**). Fixed cells were stained with anti-ubiquilin-1 antibody U7258 (**A**; green) or anti-ubiquilin-2 antibody 6431 (**B–E**; green) and anti-Htt antibody 2B4 (**A–E**; red). Cells were counter stained with DAPI and the merge images are shown on the right column. Thicker solid arrows indicate Htt inclusions with ubiquilin accumulation. Thin arrows point to ubiquilin puncta staining pattern that is mainly due to association with autophagosomes. Bar = 50μm.

Table 1

Summary of the mouse lines used.

Line	Transgene	Reference
TgCRND8	APP (K670N/M671L and V717F)	Chishti et al, 2001
Tg2576	APP (K670N/M671L)	Hsiao et al, 1996
Tg2576 x P264L PS1 knock-in	APP (K670N/M671L) x P264L PS1 knock-in	(Flood et al, 2002, Siman et al, 2000
M47	SNCA (E46K)	Emmer et al, 2011
M83	SNCA (A53T)	Giasson et al, 2002a
JNPL3	MAPT (P301L)	Borchelt et al, 1996; Lewis et al, 2000
rTg4510	MAPT (P301L)	Mayford et al, 1996; Santacruz et al, 2005
N586-82Q-C63	HTT (82 Gln repeat)	Tebbenkamp et al, 2011
N586-23Q-A2	HTT (23 Gln repeat)	Tebbenkamp et al, 2011
diTDP-43WT, line 5a	TARDBP	Cannon et al, 2012
Line 139	SOD1 (H46R/H48Q)	Wang et al, 2002
PGRN-null	N/A	Ahmed et al, 2010

Table 2

Summary of antibodies used.

Antibody	Target protein	Source
AT8	Tau phosphorylated at S202/T205	Thermo-Fisher
PHF1	Tau phosphorylated at S396/S404	Provided by Dr Peter Davies
pSer129	α-synuclein phosphorylated at S129	Generated by Waxman and Giasson, 2008b
33.1.1	β-amyloid	Kim et al, 2007
	Phosphorylated Ser409/410 TDP-43	CosmoBioUSA
MAB1510	Ubiquitin	Millipore
U7258	Ubiquilin-1 (human)	Sigma-Aldrich
6431	Ubiquilin-2	Sigma-Aldrich
#74	Ubiquilin-1/-2	Generated
6H9	Ubiquilin-2	Generated
P7624	Profilin-1	Sigma-Aldrich
P7749	Profilin-1	Sigma-Aldrich
C56B8	Profilin-1	Cell Signaling Technology Inc
AV40278	Fus	Sigma-Aldrich
2B4	Huntingtin	Millipore
1C2	Poly-glutamine	Thermo-Fisher
C-20	TIA-1	Santa Cruz Biotechnologies
Clone C4	Actin	Millipore

-Ubiquilin-1/2 preferentially associated with aggregates of huntingtin/polyQ expansion.

-Ubiquilin-2 disease-causing mutations do not compromised association with huntingtin.

-Ubiquilin-2 does not appear to effectively remove huntingtin aggregates.