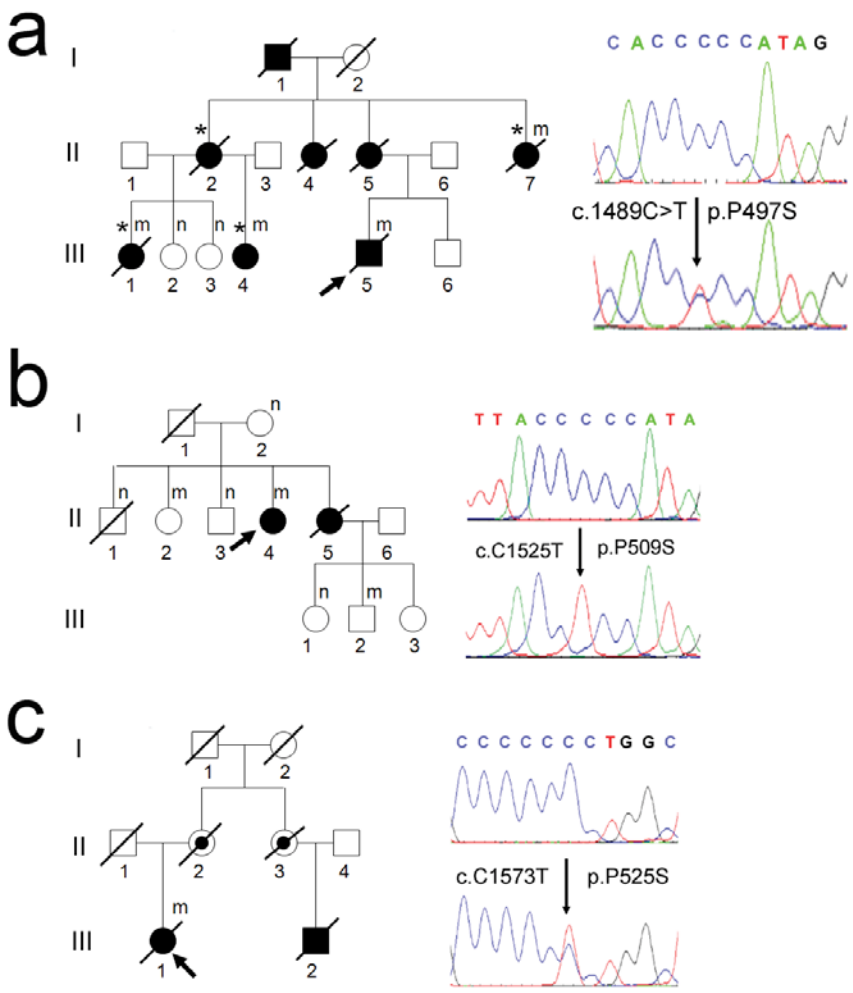
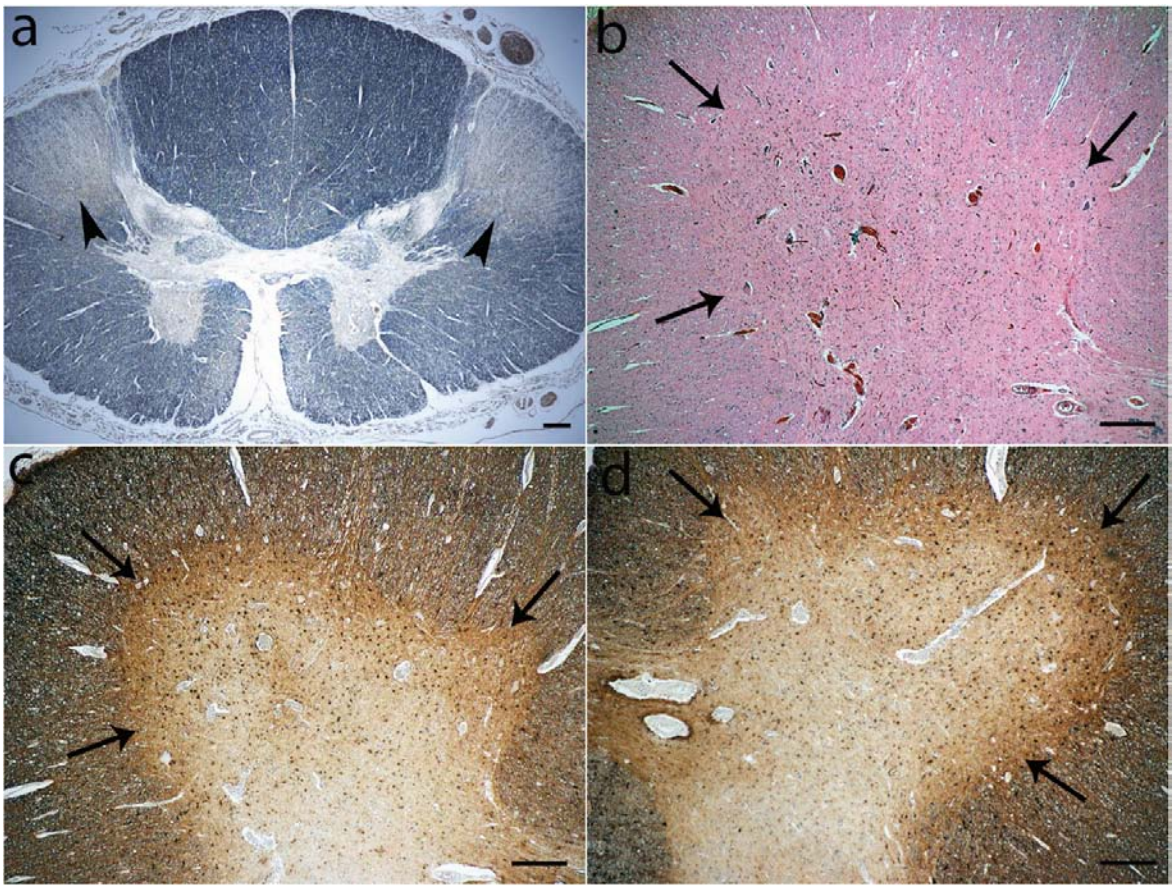
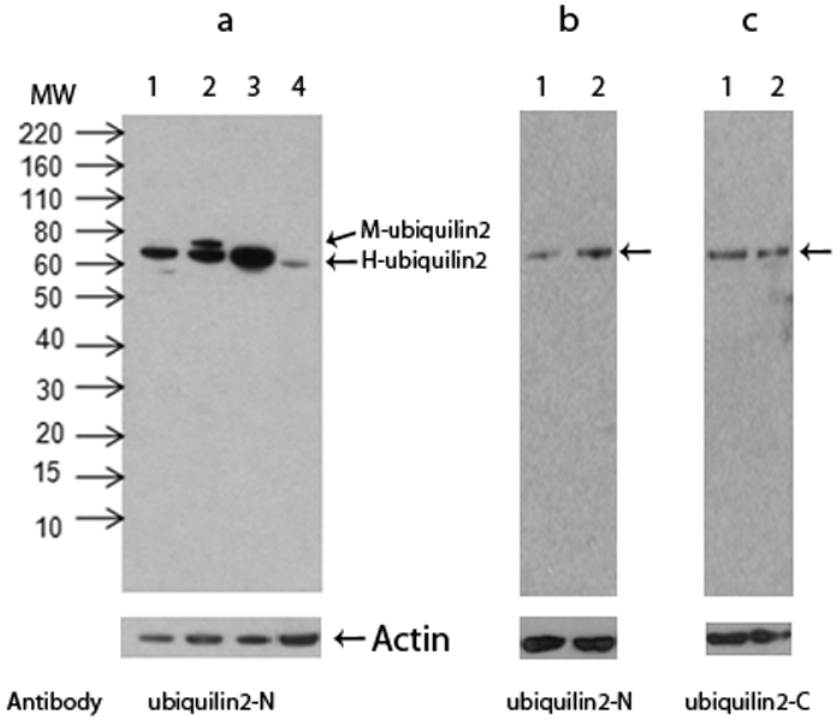


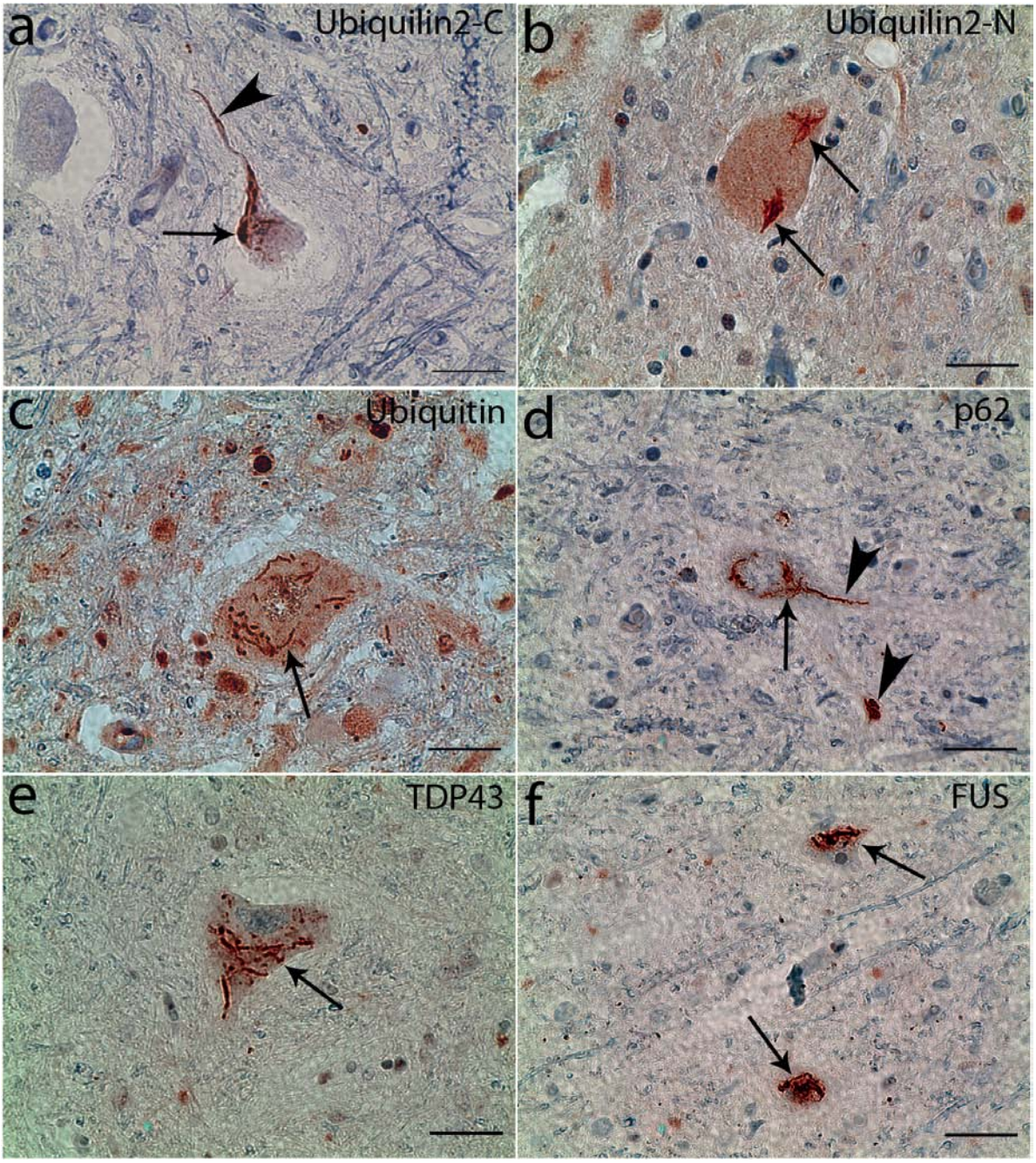
Supplementary Figure 1. Mutations of *UBQLN2* in patients with ALS and ALS/dementia. (a) A mutation, c.1489C>T (p.P497S), was identified in F#9975. The pedigree is shown on the left and sequences are shown on the right. The wild-type sequence is shown in the upper panel. Individuals with mutation in the *UBQLN2* are labeled by (m) and those without mutation are labeled by (n). Shown in the right lower panel is a heterozygous mutation from a female patient (III1). (b) A c.1525 C>T (p.P509S), was identified in F#7700. Shown in the right lower panel is a hemizygous mutation in an asymptomatic case (III2). (c) A c.C1573T (p.P525S) was found in family F#6941. Shown in the right lower panel is a heterozygous mutation from a female patient (III1). (a-c) Probands are indicated with arrows and patients with dementia are indicated with asterisks.



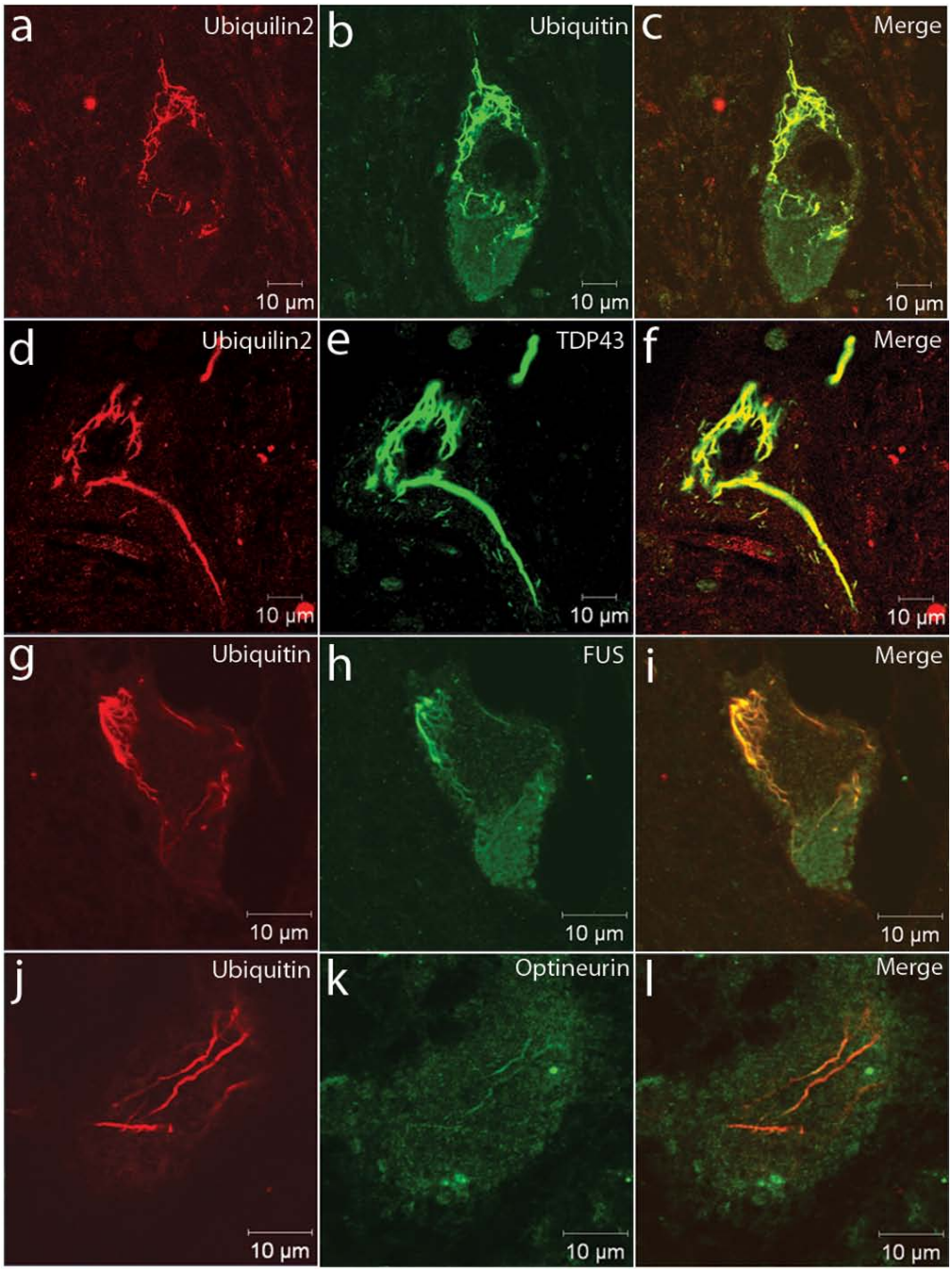
Supplementary Figure 2. Pathology of spinal cord in X-linked ALS. Representative pathology of spinal cord from a patient with *UBQLN2*^{P506T} mutation (F#6316, III4). (a) Myelin staining shows prominent loss of myelin from the corticospinal tract (arrowheads). (b) Hematoxylin and Eosin (H&E) staining shows loss of large neurons in the anterior horn (arrows). (c and d) Glial fibrillary acidic protein (GFAP) staining shows prominent astrocytosis in the anterior horns (arrows).



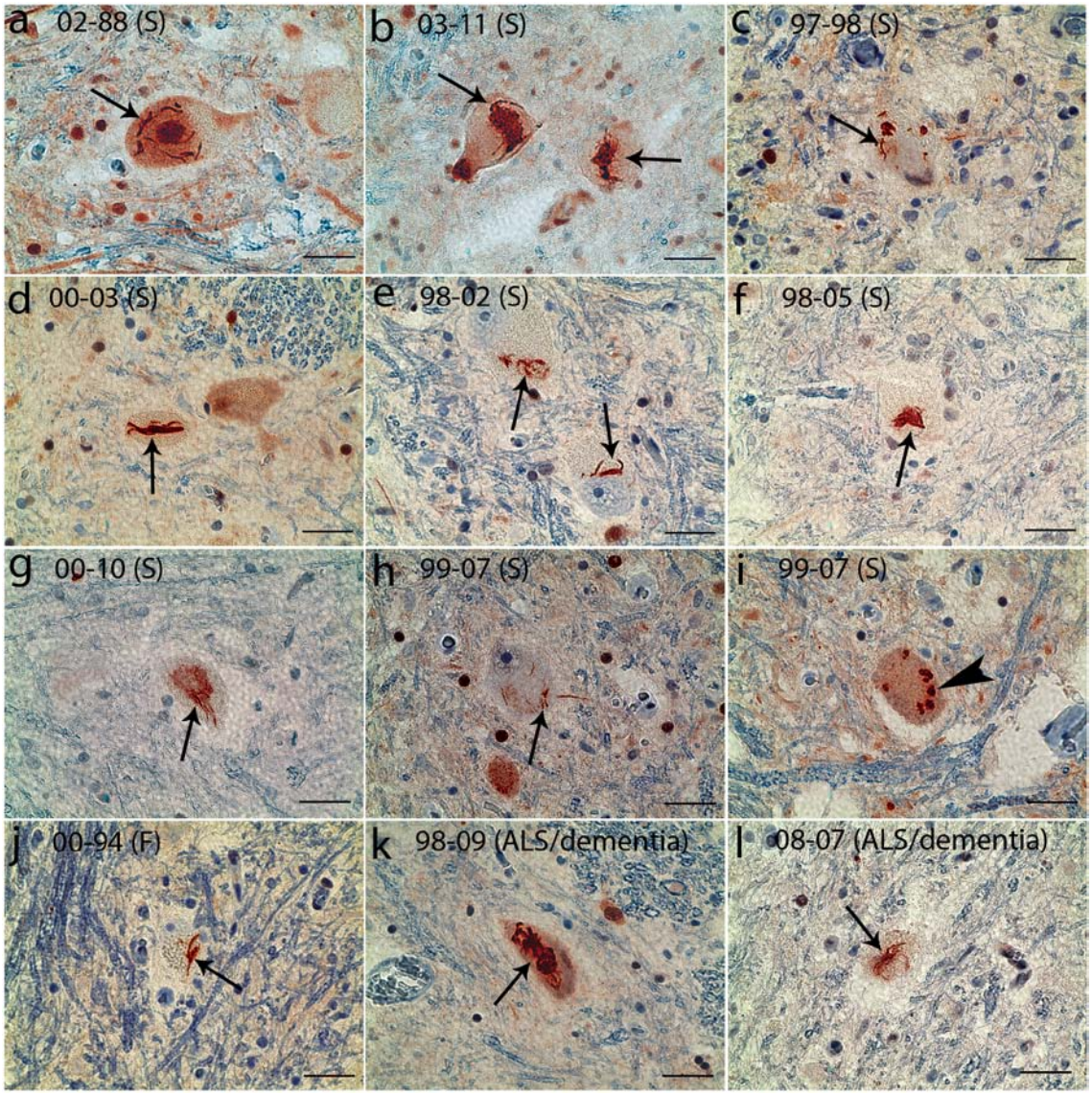
Supplementary Figure 3. Western blot with antibodies to ubiquilin2. (a) A newly developed antibody (ubiquilin2-N) to the N-terminus of ubiquilin2 detects a single band of human or mouse ubiquilin2. Human *UBQLN2*-transfected SH-SY5Y (lane 1), Neuro2a (lane 2) and HEK293 cells (lane 3) and untransfected HEK293 cells (lane 4). Mouse ubiqulin2 (m-ubiquilin2) and human ubiquilin2 (h-ubiquilin2) are indicated. (b and c) The ubiquilin2-N (panel b) and ubiquilin2-C (panel c) antibodies detect a band of expected size (arrows) using human spinal cord homogenates. Lower panel, actin control. Molecular weight is shown on the left in kilodaltons in panel (a).



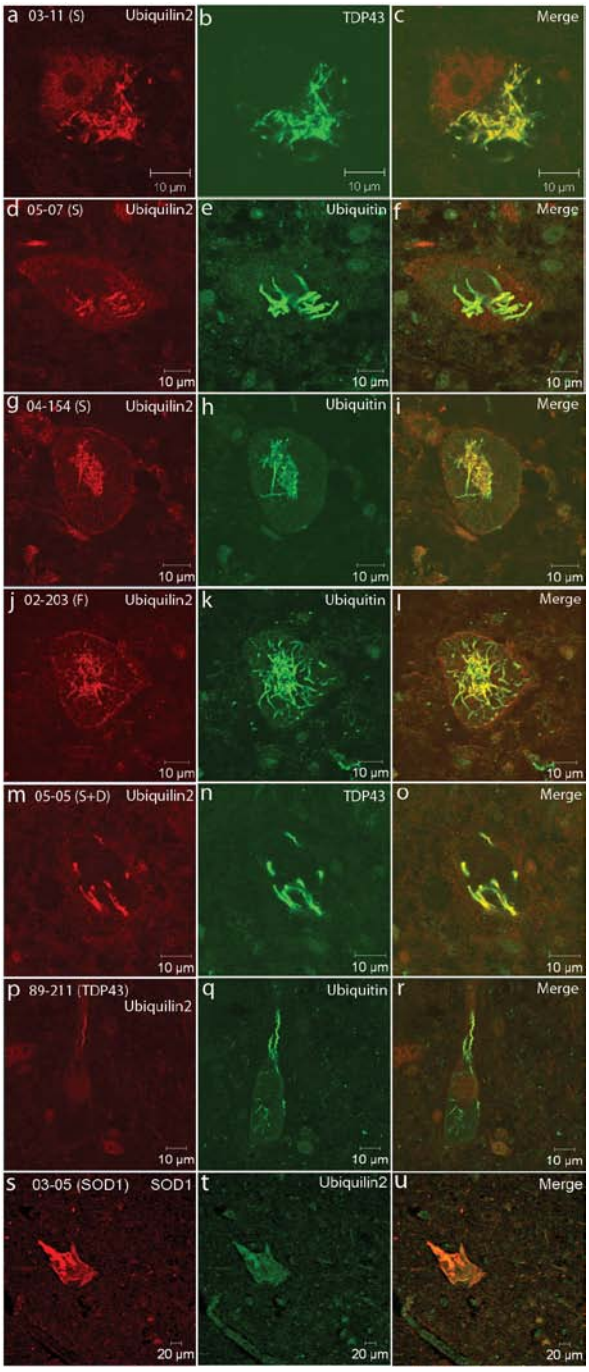
Supplementary Figure 4. Representative immunoreactive skein-like inclusions in spinal motor neurons of a patient with the P506T mutation in *UBQLN2*. The skein-like inclusions are immunoreactive with antibodies against ubiquilin2 (a and b), ubiquitin (c), p62 (d), TDP43 (e) and FUS (f). Ubiquilin2-C indicates a monoclonal antibody generated with a polypeptide at the C-terminus (554-624); ubiquilin2-N indicates a polyclonal antibody raised with a polypeptide at the N-terminus (8-24). Scale bar, 100μm.



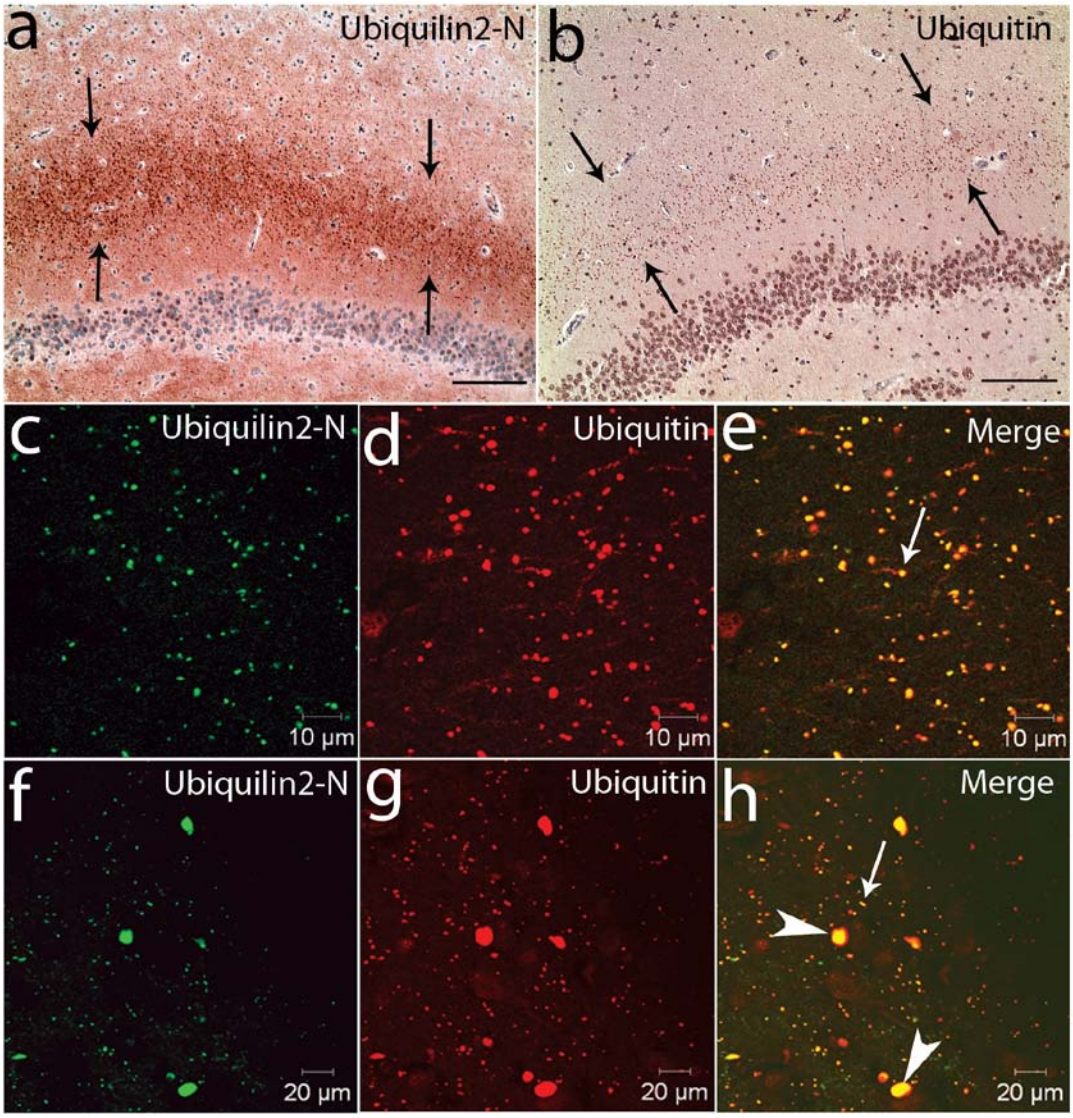
Supplementary Figure 5. Co-localization of ubiquilin2 with other proteins involved in ALS. The skein-like inclusions in spinal cord sections from a patient with P506T mutation were studied using confocal microscopy. The skein-like inclusions were detected with a monoclonal antibody, ubiquilin2-C (a and d), and antibodies to ubiquitin (b, g and j), TDP43 (e) and FUS (h). These inclusions were also weakly immunoreactive for optineurin (k).



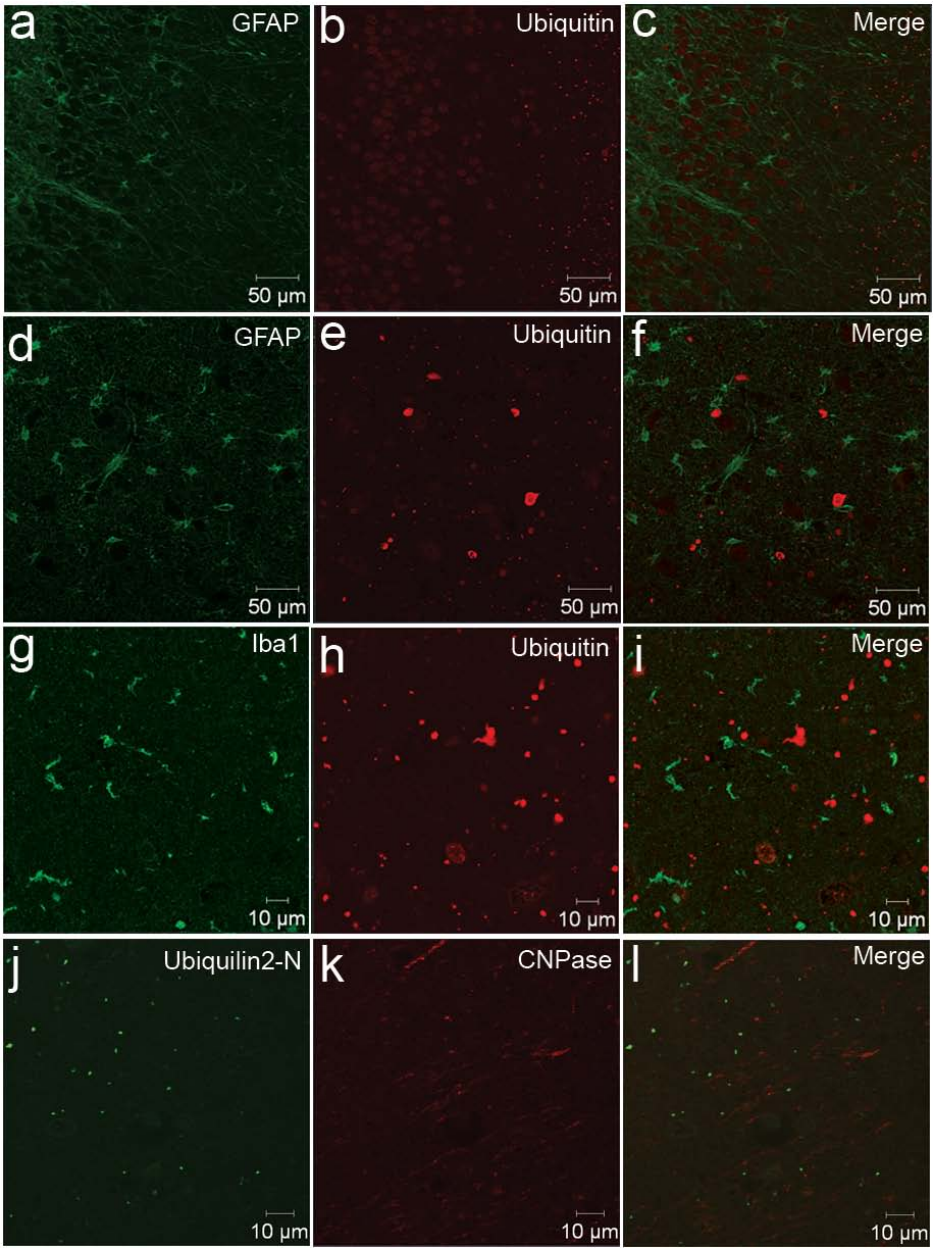
Supplementary Figure 6. Ubiquilin2-immunoreactive inclusions in spinal cord sections from patients with SALS, FALS and ALS/dementia. Human spinal cord sections were analyzed with immunohistochemistry using a monoclonal antibody (ubiquilin2-C) against ubiquilin2. Representative ubiquilin2-immunoreactive inclusions are shown here in anterior horn neurons in patients with SALS (a-i), FALS (j) and ALS/dementia (k and l). Most of the inclusions were skein-like (arrows); some of them appeared to be compact (arrowhead). Both skein-like and compact inclusions could be observed in the same patients (h and i). The representative skein-like and ubiquilin2-positive inclusions are indicated by arrows. Autopsy numbers are indicated at the top of each panel. (S), SALS; (F), FALS. Scale bar, 100μm.



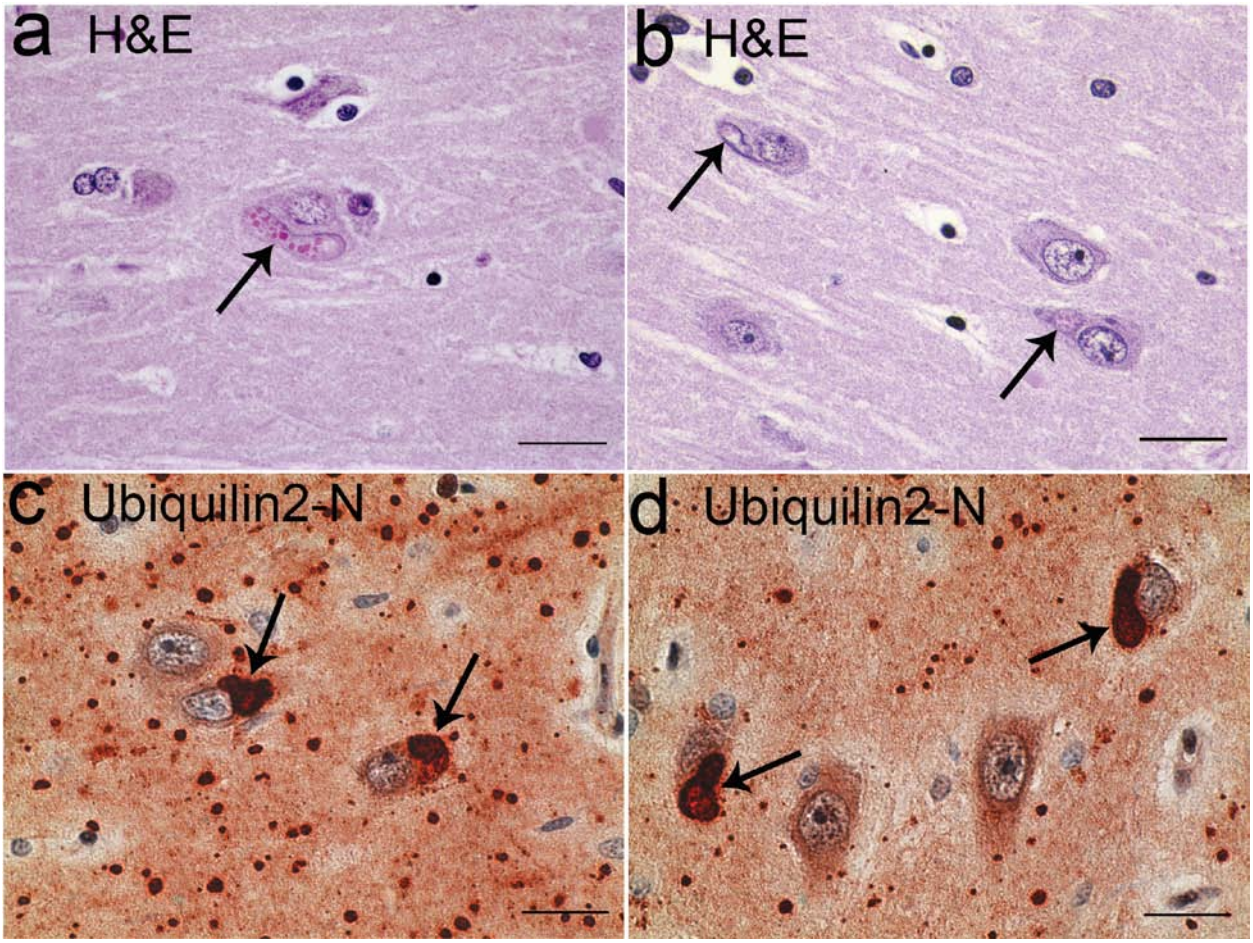
Supplementary Figure 7. Co-localization of ubiquilin2-immunoreactive inclusions with other proteins involved in ALS in the spinal cord sections from cases with SALS, FALS and ALS/dementia. Shown here are representative ubiquilin2-immunoreactive inclusions (detected with ubiquilin2-C) that are co-localized with ubiquitin and TDP43 in patients with SALS (a-i), FALS (j-l), sporadic ALS/dementia (m-o), TDP43-linked ALS (TDP43-G298S, p-r). In a SOD1-linked ALS case (SOD1-G85R), the SOD1-immunoreactive inclusions are also positive for ubiquilin2. Autopsy numbers are indicated at the top of each panel. (S), SALS; (F), FALS; (S+D), SALS/dementia.



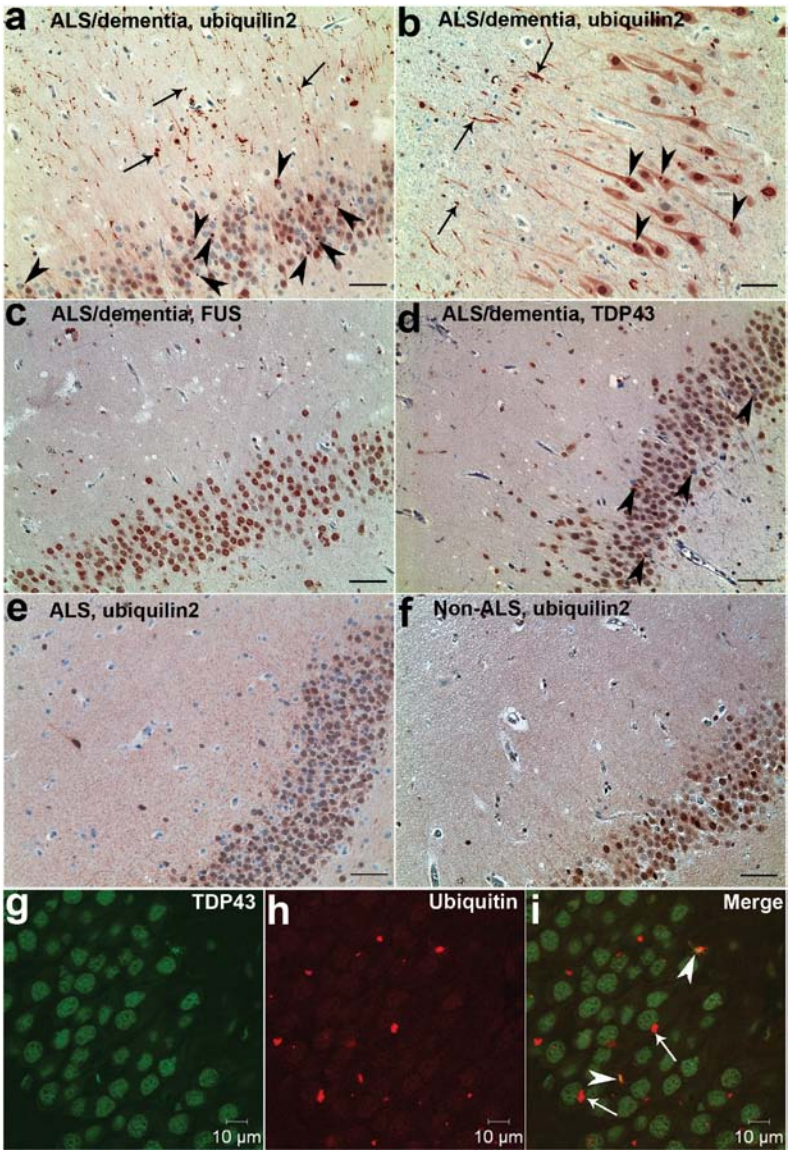
Supplementary Figure 8. Co-localization of ubiquilin2 and ubiquitin in inclusions in the hippocampus. Brain sections from the hippocampal region of a patient with a *UBQLN2*^{P506T} mutation were analyzed using immmunohistochemistry (a and b) and confocal microscopy (c-h). Antibodies against ubiquilin2 N-terminus (ubiquilin2-N) (a), or ubiquitin (b) were used. Similar to the inclusions detected with ubiquilin2-C monoclonal antibody, the immunoreactive inclusions are observed using ubiquilin2-N polyclonal antibody (a) and ubiquitin in the molecular layer of the fascia dentata. Arrows in panels (a and b) indicate the middle region of the molecular layer with inclusions that are positive with antibodies to ubiquilin2 (a) and ubiquitin (b). Scale bar, 200μm. For confocal microscopy (c-h), antibodies against ubiquilin2 (ubiquilin2-C, green) and ubiquitin (red) were used. Co-localization of ubiquilin2 and ubiquitin in the inclusions are shown in the middle region of the molecular layer of the fascia dentata (c-e) and the pyramidal layer of the CA1 region (f-h) in the hippocampus. Representative neuritic and cytoplasmic inclusions are indicated by arrows and arrowheads, respectively.



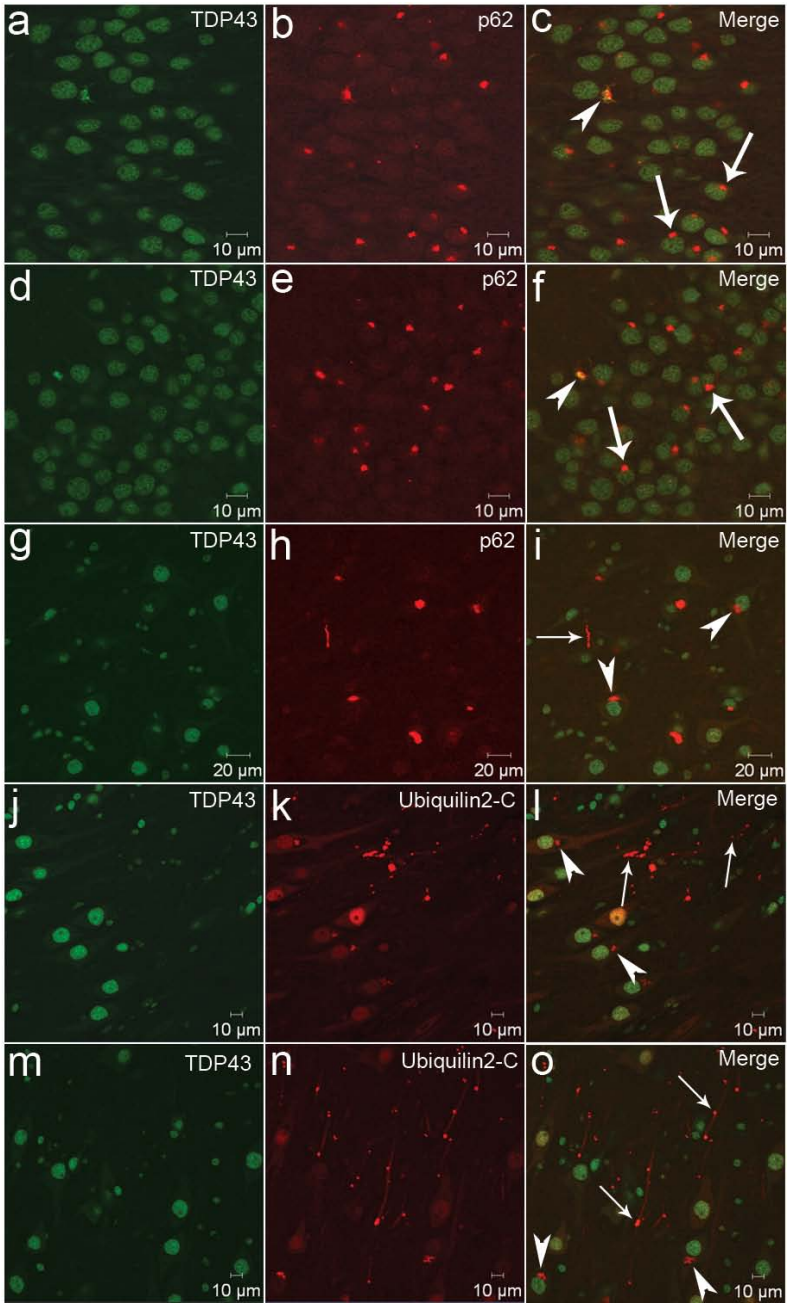
Supplementary Figure 9. No co-localization of ubiquitin/ubiquilin2 inclusions with major glial markers. Hippocampal sections of a patient with P506T mutation were stained with antibodies to glial markers, including GFAP for astrocytes, Iba1 for microglia and CNPase for oligodendrocytes. Inclusions were detected with antibodies to ubiquitin or ubiquilin2 (ubiquilin2-N). Images shown are from molecular layer (a-c, and j-l) and from CA3 region of hippocampus (d-f and g-i).



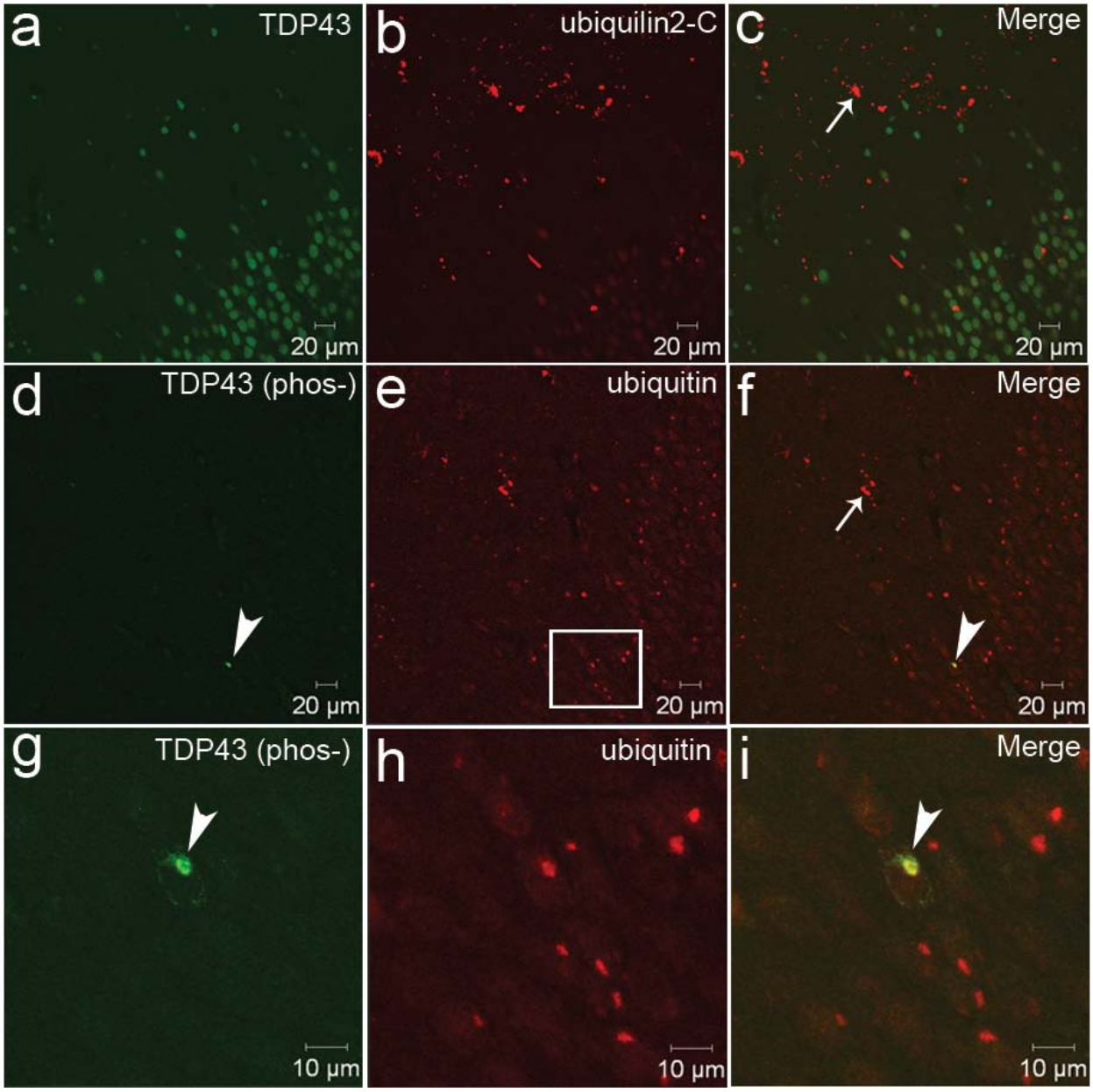
Supplementary Figure 10. Membrane-bound perikaryal structure in hippocampal pyramidal neurons. (a and b) Membrane-bound perikaryal structure containing large eosinophilic granules of varying sizes are indicated (arrows) in the hippocampal pyramidal neurons in the CA1 region of hippocampal pyramidal layer (Hematoxylin & Eosin staining). (c and d) Immunoreactivity of the membrane-bound perikaryal structures with ubiquilin2-N antibody is shown by immunohistochemical staining. The membrane-bound perikaryal structures are indicated by arrows. Scale bar, 100μm.



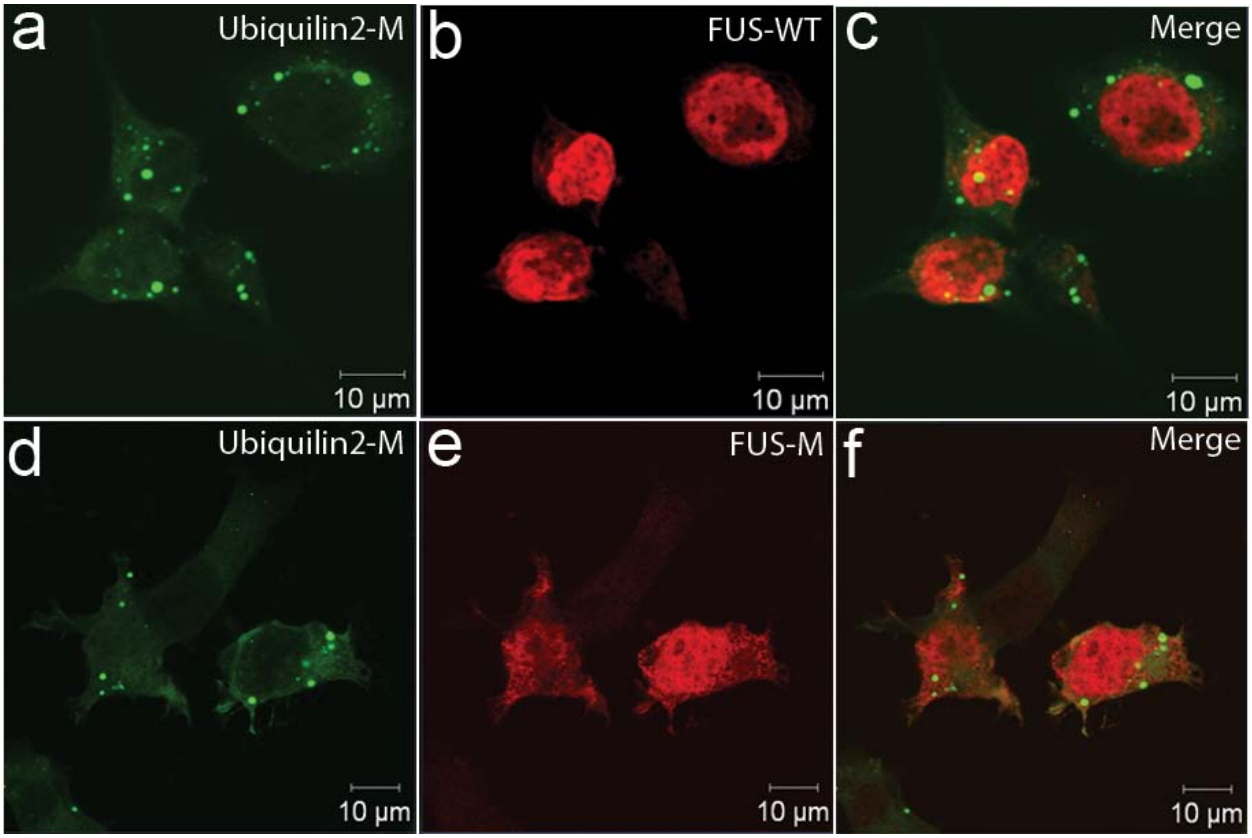
Supplementary Figure 11. Ubiquilin2 pathology in the hippocampus of ALS/dementia cases without *UBQLN2* mutations. Brain sections from the hippocampal regions of a patient without *UBQLN2* mutations were analyzed with immunohistochemistry and confocal microscopy using antibodies against ubiquilin2 (ubiquilin2-C), FUS, TDP43 and ubiquitin (a-d). The ubiquilin2-positive inclusions are present in the molecular layer and in the dentate granule cells of the fascia dentata (a), and CA regions (CA3 is shown) (b). The representative neuritic and cytoplasmic inclusions are indicated by arrows and arrowheads, respectively. No FUS pathology is present in fascia dentata (c). No TDP43 inclusions are present in the molecular layer, but TDP43 inclusions are present in some dentate granule cells (arrowheads), which show no nuclear staining of TDP43 (d). Ubiquilin2 inclusions are not present in ALS cases without dementia (e), nor in non-ALS control cases (f). Some ubiquitin-positive inclusions are also TDP43-positive (g-i) in dentate granule cells in ALS/dementia cases, which show no nuclear TDP43 distribution (arrowheads). However, the cells with ubiquitin inclusions, but without TDP43 inclusions show normal nuclear distribution of TDP43 (arrows). Scale bar, 100µm in panels (a-f).



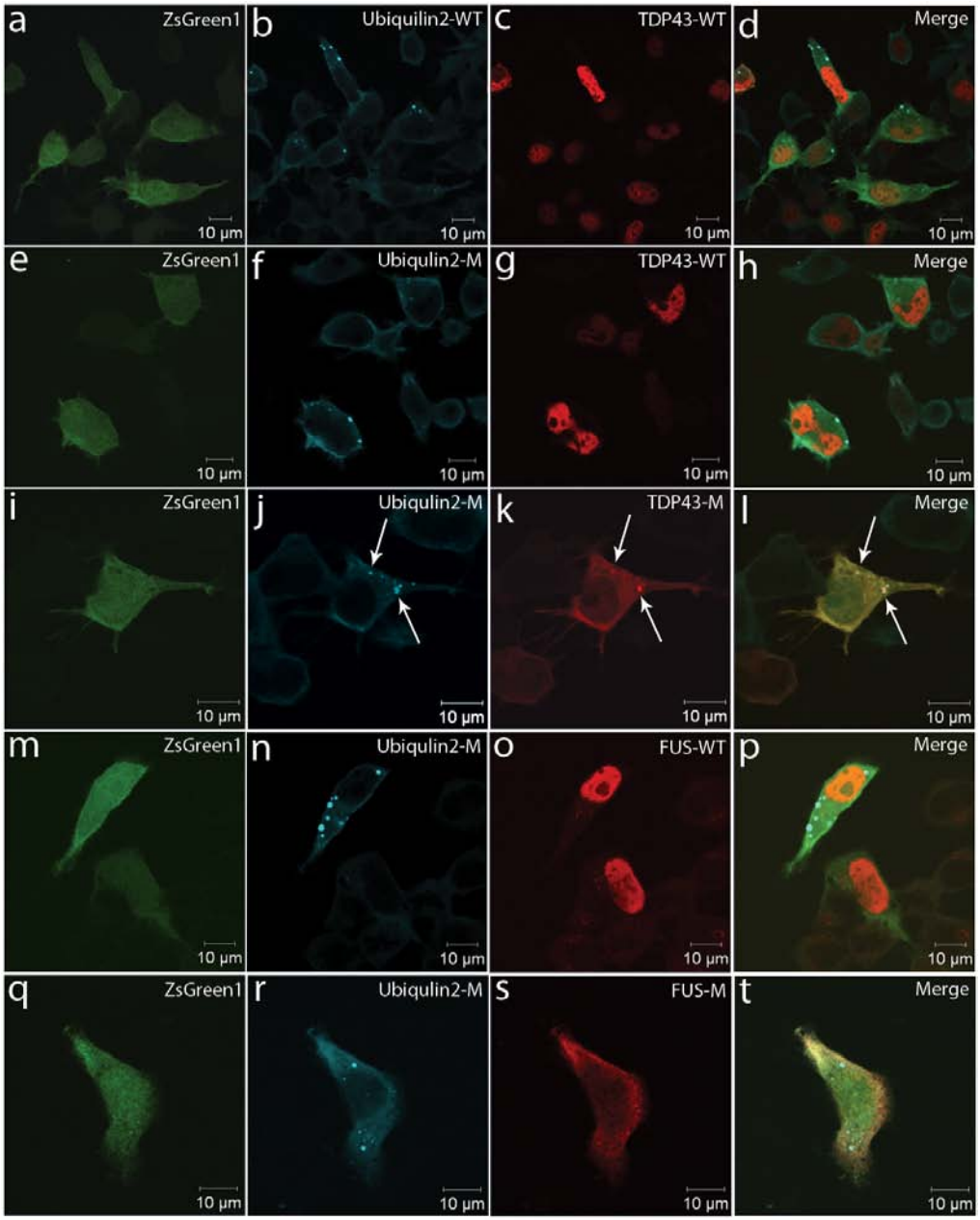
Supplementary Figure 12. TDP43-independent ubiquilin2-containing inclusions in the hippocampus of an ALS/dementia case. Hippocampal sections of an ALS/dementia case without a *UBQLN2* mutation were stained with antibodies to TDP43 (green) and p62 or ubiquilin2-C (red). Most of the dentate granule cells with p62-positive inclusions show normal nuclear distribution of TDP43 (arrows) (a-f). A few cells with TDP43 inclusions show no nuclear distribution of TDP43 (arrowheads) (a-f). Neuritic (arrows) and cytoplasmic (arrowheads) inclusions are prominent in CA regions (CA3 is shown) (g-o). These inclusions are positive for p62 (g-i) and ubiquilin2 (j-o), but negative for TDP43 (g-o).



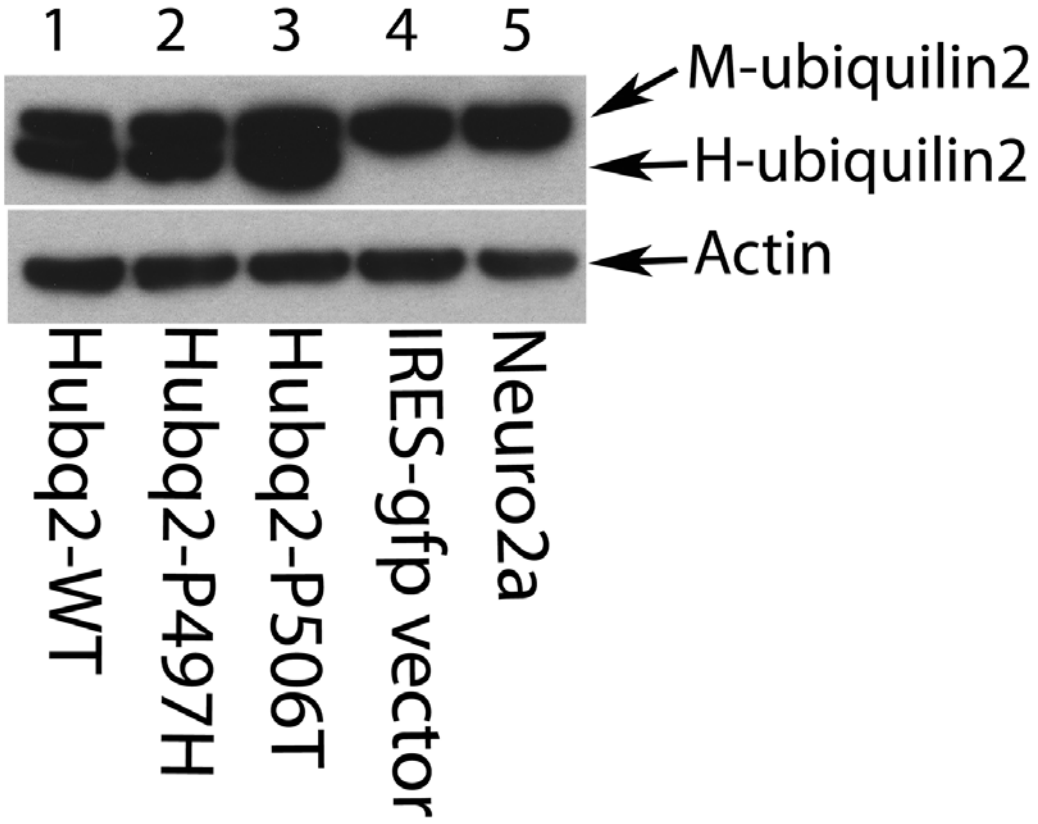
Supplementary Figure 13. TDP43-independent ubiquilin2-containing inclusions in the hippocampus of ALS/dementia cases. The hippocampal sections of an ALS/dementia case without ubiquilin2 mutations were stained with antibodies to TDP43 (green) and ubiquilin2-C or ubiquitin (red). An antibody to phosphorylated TDP43 (pS409/410), which is specific to the TDP43 in the inclusions, is indicated by TDP43 (phos-) in panel (d). Panels (g-i) are higher power images of the boxed areas from panels (d-f). The ubiquilin2/ubiquitin positive neuritic inclusions (arrows) in the dentate molecular layer and majority of the cytoplasmic inclusions are negative for TDP43. An inclusion detected with TDP43 (phos-) is indicated with an arrowhead (d-i).



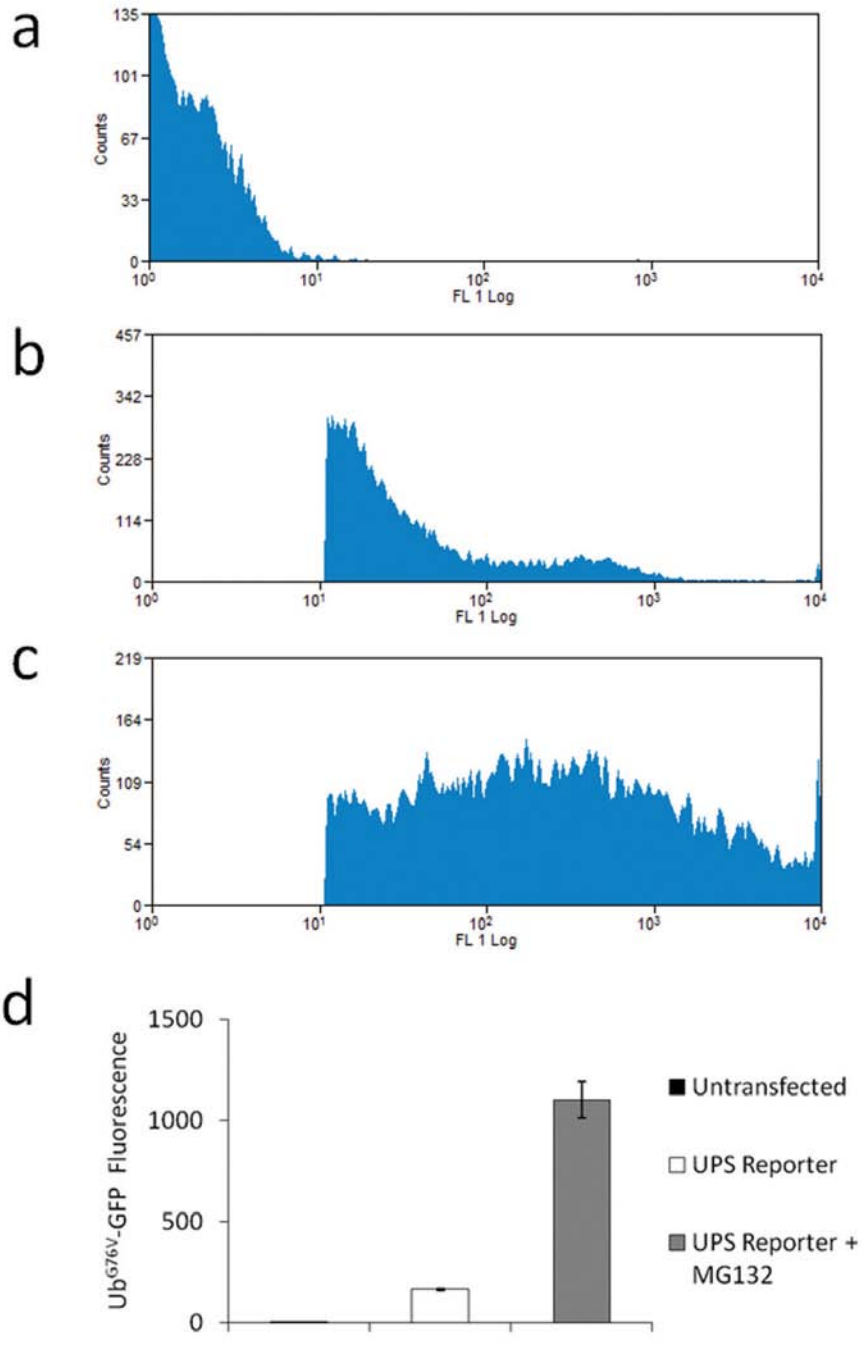
Supplementary Figure 14. No apparent co-localization of ubiquilin2 and FUS. Neuro2a cells were transfected with mutant ubiquilin2 (P497H), and wild-type FUS or ALS-linked mutant FUS (R495X). Ubiquilin2 is GFP-tagged and FUS is myc-tagged. WtFUS is almost exclusively distributed in the nuclei. ALS-linked mutant FUS (R495X) shows nuclear and cytoplasmic distribution. The ubiquilin2-positive inclusions appear to be negative for FUS.



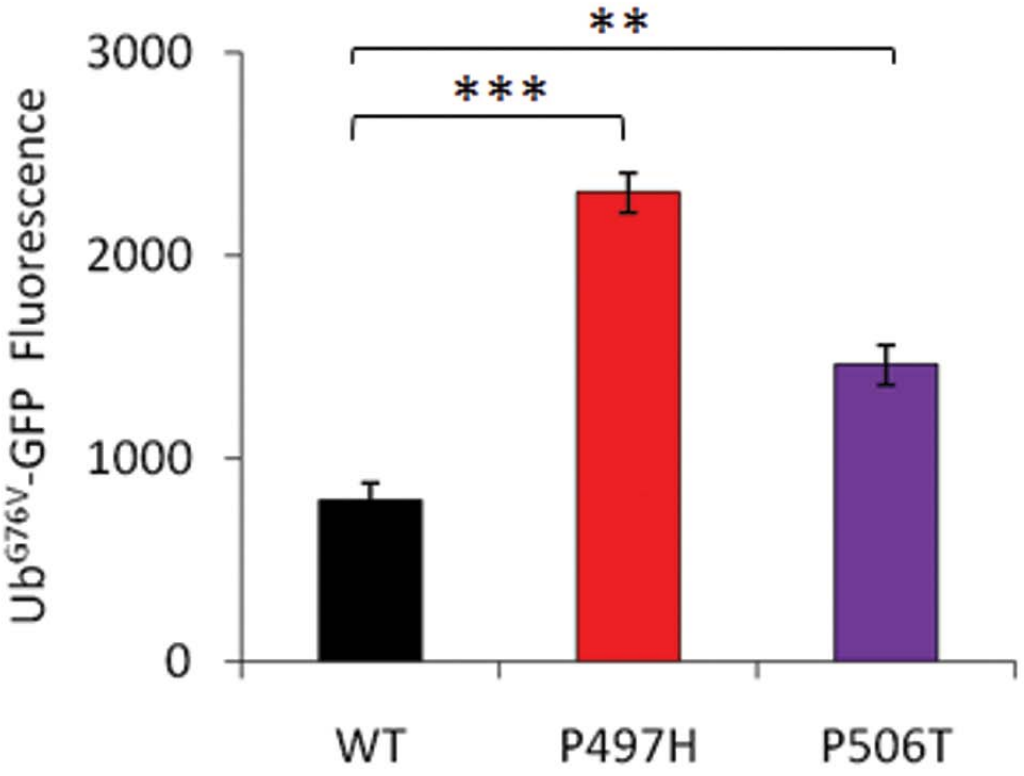
Supplementary Figure 15. Co-localization of ubiquilin2 and C-TDP43. Neuro2a cells were transfected with different expression constructs. The ubiquilin2 constructs were cloned into pIRES2-ZsGreen1 vector, so that ubiquilin2 is expressed in a tag-free fashion, and cells expressing exogenous human ubiquilin2 are labeled green. Co-transfection of ubiquilin2-pIRES2-ZsGreen1and FUS-myc or TDP43-cherry constructs shows that the ALS- and dementia-linked TDP43 fragment (aa218-414, C-TDP43) is co-localized with ubiquilin2-P497H in the cytosolic inclusions (i-l) (arrows).



Supplementary Figure 16. Similar transfection efficiency of wild-type and mutant ubiquilin2 expression constructs. Cell lysates from Neuro2a cells transfected with ubiquilin2 constructs were immunoblotted with a ubiquilin2 antibody (ubiquilin2-N). Similar expression levels of human ubiquilin2 are shown. Mouse endogenous ubiquilin2 is indicated by M-ubiquilin2, and exogenous human ubiquilin2 is indicated by H-ubiquilin2. Actin is used as an internal control. Expression vector without human ubiquilin2 insert (IRES-GFP) and non-transfected cells were also used as controls.



Supplementary Figure 17. Flow-cytometric analysis of cells transfected with the UPS reporter substrate (Ub^{G76V}-GFP). The top three panels show results from one representative experiment using untransfected cells (a), Ub^{G76V}-GFP transfected cells (b), and Ub^{G76V}-GFP transfected cells treated for 24 hours with 5 μM of the proteasomal inhibitor, MG-132 (c). Incubation with MG-132 causes a marked increase in Ub^{G76V}-GFP fluorescence (FL1) intensity (d). Data are averaged from three independent experiments. Mean GFP fluorescence intensities are given. Error bars, means ± s.e.m.



Supplementary Figure 18. Mutations in ubiquilin2 lead to ubiquitin-mediated impairment of proteasomal degradation. Ub^{G76V}-GFP fluorescence intensity was quantified by FACS 48 hours post-transfection in SH-SY5Y cells that were transiently transfected with either wild-type (WT) or mutant ubiquilin2. Data are averaged from at least three independent experiments. **p<0.01 and ***p<0.001 indicating significant differences when compared to WT-UBQLN2 (two-tailed Student's *t* test). Error bars, means ± s.e.m.

Supplementary Table 1. Two-point Lod scores for F#186

LOCUS θ=	0.000	0.050	0.100	0.150	0.200	0.300	0.400
DXS1055	-3.053	1.598	1.848	1.833	1.697	1.218	0.549
DXS573	**3.664**	**3.348**	**3.020**	**2.677**	**2.319**	**1.549**	**0.694**
DXS8023	**3.367**	**3.074**	**2.770**	**2.452**	**2.119**	**1.403**	**0.609**
DXS988	**4.797**	**4.393**	**3.972**	**3.532**	**3.071**	**2.076**	**0.968**
DXS991	**4.971**	**4.551**	**4.113**	**3.655**	**3.176**	**2.143**	**0.992**
DXS8029	**4.927**	**4.510**	**4.074**	**3.619**	**3.142**	**2.113**	**0.966**
DXS9736	**4.982**	**4.562**	**4.124**	**3.667**	**3.187**	**2.154**	**1.003**
DXS981	**4.638**	**4.248**	**3.841**	**3.415**	**2.969**	**2.003**	**0.926**
DXS1275	-1.317	2.949	2.867	2.642	2.347	1.616	0.744

Note: Two-point linkage analysis was conducted using the MLINK component of the LINKAGE software package (Lathrop GM, Lalouel JM. 1984). The trait was modeled as X-linked dominant with age dependent penetrance and with disease gene frequency of 0.001.

Supplementary Table 2. Clinical information

Family A: # 186				
Individual	Gender	Age at onset (years)	Duration (months)	Phenotype
I:2	F	45	≈ 48	ALS
II:2	F	62	60	ALS
II:4	F	58	84	ALS
II:6	F	62	≈ 60	ALS
II:7	M	19	24	ALS
III:1	M	25	59	ALS
III:3	M	33	≈ 36	ALS
III:4	F			OBLIGATE: died age 72, asymptomatic
III:5	F	52	≈ 84	ALS
III:6	F	53	47	ALS
III:9	M	33	24	ALS
III:10	M	35	≈ 48	ALS
IV:1	F	37	40	ALS/dementia
IV:2	F			OBLIGATE: living age 71, asymptomatic
IV:3	M	49	25	ALS
IV:4	F	47	33	ALS
IV:5	F	42	24	ALS
IV:7	M	29	26	ALS
IV:8	M	38	≈ 24	ALS
V:1	F	41	36	ALS
V:3	M	47	Living at 43	ALS
Family B: # 9975				
I:1	M	29	24	ALS
II:2	F	44	55	ALS/dementia
II:3	F	25	48	ALS
II:4	F	40	46	ALS
II:6	F	43	12	ALS/dementia
III:1	F	41	25	ALS/dementia
III:4	F	37	Living at 51	ALS/dementia
III:5	M	29	19	ALS
Family C: #6316				
I:2	F	42	≈60	ALS
II:1	F			OBLIGATE: living age 50, asymptomatic
II:3	F	40	84	ALS/dementia
III:1	M	16	Living at 180	Dementia/UMN signs

III:4	M	22	≈ 60	ALS/dementia
Family D: # 7700				
II:4	F	53	36	ALS
II:5	F	59	27	ALS
Family E: # 6941				
II:2	F			OBLIGATE: died age 94, asymptomatic
II:3	F			OBLIGATE: died age 78, asymptomatic
III:1	F	71	58	ALS
III:2	M	70	11	ALS
Male: mean age of onset 33.9 years, mean duration 43.1 months				
Female: mean age of onset 47.3 years, mean duration 48.5 months				

Note: Additional information of ALS/dementia cases in F# 6316

III-1. This patient had a normal childhood. At age of 16 years, he started to show mood swings, outbursts and memory problems. He was evaluated in a Memory Disorders Clinic at age of 18 years, where he was diagnosed with behavioral variant of FTD. His cognitive function was equivalent to that of a 9-11 year old. By early 20s, the patient showed mild stiffness in upper extremities, and "tremor" and stiffness in lower extremities. In the middle of 20s, the patient started using a walker within the house and wheelchair for longer distances. By late 20s, he exclusively used wheelchair. The patient was examined by one of us (K.M.B) when he was 29 years old. Most significant cognitive problem was insight (no insight into medical condition, difficulty understanding complexity of his condition) and memory. He had trouble with time and following a story line. He could follow simple commands and carry on simple conversation. He could not read, write or draw anything. The patient could stand, took a few steps with significant assistance. He had spasticity in all extremities, but significantly more in legs. The patient showed pseudobulbar signs. He had no difficulty in using arms/hands. Dexterity and coordination were good in upper extremities. He had decreased range of motion & reduced strength in all muscles of lower extremities (4/5 bil, except for no dorsiflexion of ankles or toes), plantar flex 1-2 bil. No apparent wasting or fasciculation were noted anywhere. Smile was intact, but he could not whistle or blow out candle. He had spastic dysarthria, and urinary and occasional bowel incontinence. Reflexes were increased to 4+ in all extremities. Hoffman's sign was present in the upper extremities and he had sustained clonus at the ankles. No sensory abnormality was observed. Saccades could not be tested because the patient could not follow instructions.

III-4. This patient had a normal childhood. He started to show similar cognitive dysfunction as patient III-1, difficulty walking, dysarthria and dysphagia by age of 22 years. The symptoms were progressive and the patient died at age of 27 years. Pathological analysis of autopsy sample from this patient revealed prominent loss of myelin from the corticospinal tract, loss of large neurons and astrocytosis in the anterior horns (as shown in supplementary figure 2). The spinal

cord and hippocampus autopsy samples from this patient were also used for immunostaining with various antibodies in this study.

II-3. This patient is the mother of the case III-4. The patient had a normal childhood and started to show behavioral dysfunction in her early 40s, and rapidly progressed to global dementia. Motor neuron signs were similar to those in III-1. She required tube feeding in her last year of life. She died at age of 47 years. Analysis of spinal cord and hippocampal autopsy samples revealed similar pathology observed in patient III-4.