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Supplementary Information for

Mutant UBQLN2 promotes toxicity by modulating intrinsic self-assembly

Lisa M Sharkey[a,e,1], Nathaniel Safren[a,1], Amit S Pithadia[a,1], Julia E Gerson[a], Mark Dulchavsky[a], Svetlana Fischer[a], Ronak Patel[a], Gabrielle Lantis[a], Naila Ashraf[a], John H Kim[b], Alia Meliki[a], Eiko N Minakawa[c], Sami Barmada[a,e,2], Magdalena I Ivanova[a,b,e,2], Henry L Paulson[a,d,e,2]

a. Department of Neurology, University of Michigan, Ann Arbor, MI 48109-2200, USA
b. Biophysics Program, University of Michigan, Ann Arbor, MI 48109-2200, USA
c. Department of Degenerative Neurological Diseases, National Institute of Neuroscience, Kodaira, Japan
d. Michigan Alzheimer's Disease Center, University of Michigan, Ann Arbor, MI 48109-2200, USA
e. Protein Folding Disease Initiative, University of Michigan, Ann Arbor, MI 48109-2200, USA

1. These authors contributed equally to this work.

2. Co-Corresponding authors

Henry L Paulson
Email: henryp@med.umich.edu
Magdalena I Ivanova
Email: mivanona@med.umich.edu
Sami Barmada
Email: sbarmada@med.umich.edu

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Supplementary Text

Detailed Methods:

Plasmids

The pCMV4-FLAG-UBQLN2 plasmid (p4455 FLAG-hPLIC-2; Addgene plasmid # 8661) was a gift from Peter Howley. The P506T mutation was introduced using site directed mutagenesis (QuikChange II, Agilent Genomics). For bacterial expression and purification, WT and P506T UBQLN2 were cloned into pETite C-His Kan DNA vector (Lucigen). The UBA and UBL domains and ΔUBA and ΔUBL (WT and P506T) were subcloned into the pET28 vector using convenient restriction enzyme sites incorporated into PCR primers used to amplify the target sequences. WT and P506T UBQLN2 had a 6xHis tag at the C-terminus, while UBA, UBL, WT middle domain, ΔUBA, and ΔUBL were tagged at the N-terminus.

For expression in primary neurons the iRFP tag was amplified from the piRFP plasmid (Addgene plasmid #31857 was a gift from Vladislav (1)) and subcloned into the expression vector, pGW1. Full-length UBQLN2, ΔUBA and ΔUBL (WT and P506T) were also subcloned into pGW1-iRFP, C-terminal of the iRFP tag.

To generate the MoPrP-FLAG-UBQLN2 (WT and P506T) transgenic mouse constructs, both wildtype and P506T UBQLN2 cDNA were PCR amplified from the pCMV4 constructs and subcloned into the MoPrP vector (courtesy of David Borchelt, Univ. Florida) using Xho1 sites that were incorporated into the forward and reverse PCR primers.

HEK293 cell transfection

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM, supplemented with 10% FBS, and 100 U/ml penicillin/streptomycin. Cells were transfected with pCMV4-UBQLN2 constructs (WT or P506T) with Fugene 6 Transfection Reagent (Roche) according to the manufacturer's instructions. For visualization of transfected UBQLN2, the cells were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) for 5 mins at RT. They were washed in PB, blocked and permeabilized in PBTGS (0.1M PB, 10% NGS, 0.1% Triton X- 100, 0.05% BSA) at RT for a minimum of 1 hr. The cells were then incubated overnight with 1:1000 anti-FLAG rabbit antibodies in PBTGS (Sigma). Primary incubated sections were then washed and incubated with Alexa Fluor 488 (1:1,000; Invitrogen), stained with DAPI (Sigma) mounted to slides with Prolong Gold Antifade Reagent (Invitrogen), and imaged using an IX71 Olympus inverted microscope. Images were analyzed using ImageJ software and particles were analyzed for number and size. Statistics were performed using GraphPad prism.

Generation of transgenic mice

Both wildtype and P506T UBQLN2 cDNA were PCR amplified using the pCMV4-FLAG-UBQLN2 (WT or P506T) plasmids as templates and cloned into the MoPrP vector (courtesy of David Borchelt, Univ. Florida) using Xho1 sites that were incorporated into the forward and reverse PCR primers. Transgenic mouse founders were generated through the UM Transgenic Animal Core. The MoPrP- UBQLN2 transgene constructs (WT or P506T) were linearized with Not1 and purified. Fertilized (C57BL/6 X SJL)F_2 mouse eggs were collected, microinjected with the linearized vector, and transferred to pseudo-pregnant recipients. When the pups were two-weeks-old, tail biopsies were taken and DNA was extracted and genotyped for the presence of the transgene.

Genotyping

Genomic DNA was prepared from tail biopsies by adding 100 µL of lysis buffer (25 mM NaOH and 0.2 mM EDTA) and incubating at 95° C for 1 hour. After cooling to room temperature, 100 µL of neutralizing buffer (40 mM Tris HCl) was added. After centrifugation at 16.1 x g for 5 min, 2 µL were used in the PCR reaction. PCR of genomic DNA was carried out in a 26 µlLvolume containing 2X GoTaq Green Master Mix (Promega), DMSO, and 10 mM primers. The following primers were used: Ub2 forward 5'-GGA CTA CAA GGA CGA TGA CAA CG-3' and Ub2 reverse 5'-CTG CAT CTG TGG ATT AGC CAT AAT GAG-3'; Internal control primers designed by JAX labs: oIMR7338 5'- CTA GGC CAC AGA ATT GAA AGA TCT-3' and oIMR7339 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'.

PCR conditions were as follows: 94° C for 3 min followed by 35 cycles of 94° C for 30 s, 55° C for 45 s and 72° C for 30 s, followed by 72° C for 5 min and terminating with 4° C. DNA products were separated by standard 1.2% agarose gel electrophoresis and visualized with SYBR Safe (Thermo Fisher). PCR products from mice positive for the transgene show band sizes of 658bp (transgene) and 324bp (internal control). Non- TG mice show a band at 324bp only.

RNA isolation and qPCR

Total RNA from half of whole brain fractions of mice from Ub2 P506T, Ub2 WT, and Ub WT mouse lines was obtained by an initial extraction using TRIzol Reagent (Invitrogen) followed by purification using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Reverse transcription of 1000 ng of total RNA per sample was performed using the iScriptTM cDNA synthesis kit (Bio-RAD). Endogenous UBQLN2 FLAG transgene, and β-actin (housekeeping) transcript levels were assessed by quantitative real-time polymerase chain reaction using primers. Relative gene expression was determined using the CT method,

normalizing for ®-actin mRNA levels. Primers: Ins Ub2 qPCR F: 5' ATGCTTTACGGCGCATGTAC; Ins Ub2 qPCR R: 5' TGGATCGCGATTTTCTGTGC; Mm_gen_®-actinF: 5' GAACGGACACCATGGGCGGG: Mm_gen_®-actin R: 5' GTGTGTCCCCAAGCCCCACG

Mouse brain tissue harvesting

Animals were deeply anesthetized with a ketamine/xylazine mixture and perfused transcardially with 0.1M phosphate buffer. Brains were dissected and divided sagittally. One half was immediately placed on dry ice and stored at – 80°C for biochemical studies while the other half was fixed in 4% paraformaldehyde at 4°C for 48h, and cryoprotected in 30% sucrose in 0.1M phosphate buffer at 4 °C until saturated. Fixed hemispheres were sectioned at 40 µm sagittally through the entire hemisphere using a sledge microtome (SM200R; Leica Biosystems). Free-floating sections were stored at − 20°C for immunostaining.

Recombinant UBQLN2 Western blots

5 µL of purified WT- and P506T-UBQLN2 protein were loaded (without boiling) on precast NuPAGE 4- 12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked for 2 hours at room temperature with 10% nonfat dry milk in TBS-T buffer. Membranes were then probed overnight at 4 in anti-Ubiquilin-2 antibody (Novus Biologicals; 1:2000). HRP- conjugated goat anti-rabbit IgG (1:5000) and ECL (Pierce) were used to visualize bands.

Detection of transgenic UBQLN2 in UBQLN2 tg brains

Lysates from brain tissue were prepared in RIPA buffer (cat #R0278, Sigma) with protease inhibitors (Roche), PMSF, and PhosphoStop (Roche). Brain tissue was homogenized in a Potter homogenizer, centrifuged (13000 rpm for 30 minutes) and the supernatants were removed. Protein concentration was measured using a BCA assay (cat#23227, ThermoScientific). Samples were prepared in 1x Laemmli sample buffer with DTT, heated at 100°C for 1 min and centrifuged for 1 min at 13000 rpm. Proteins were resolved on 4-12% SDS-polyacrylamide electrophoresis gels and transferred to PVDF membrane, 0.2 um, at 0.35A for 3h at 4°C or for 16h (overnight) at 30V. Membranes were blocked in 5% milk/2.5%BSA/TBS-T for 1 hour at RT on a shaker. Primary antibodies, rabbit ⟨-FLAG (Sigma, cat#F7425) 1:1000, rabbit anti-UBQLN2 1:250 (NBP1- 85639, Novus Biologicals) rabbit anti-α-Tubulin 1:10000 (Cell Signaling, cat# 2144) and mouse anti-GAPDH (1:5,000, MAB374; Millipore) were incubated overnight at 4 °C overnight on shaker. Secondary antibodies were peroxidase-conjugated goat anti-rabbit or goat anti-mouse (1:10000; Jackson Immunoresearch) incubated for 1h at RT on shaker. Bound

primary antibodies were visualized by incubation with a peroxidase- conjugated anti-mouse or anti-rabbit secondary antibody (1:10000; Jackson ImmunoResearch Laboratories) followed by treatment with an enhanced chemiluminescence (ECL)-plus reagent (Western Lighting; PerkinElmer) and exposure to autoradiography films. Band intensities were quantified using ImageJ analysis software (NIH).

Native Blots

Lysates from non-TG and UBQLN2 transgenic mouse brain tissue were prepared in 4x Native Page Sample Buffer (Novus) as per the manufacturer instructions. Protein concentration was quantified with a BCA assay (cat#23227, ThermoScientific). Samples were run on a 4-16% Bis-Tris NativePAGE gel (Novus) using NativePAGE 20x Running Buffer and the Light Blue Cathode Buffer as described by the manufacturer. Gels were transferred to PVDF membranes, 0.2 μm, at 0.35A for 3h at 4°C. Membranes were blocked with 5% milk/2.5% BSA/TBS-T for 1 hour at RT and probed with primary antibody, rabbit ⟨-FLAG (Sigma, cat#F7425) 1:1000 and secondary antibody goat anti-mouse (1:10000; Jackson Immunoresearch), developed and visualized as described above.

Soluble/Insoluble Fractionation blots

Brains were homogenized in PBS with a protease inhibitor cocktail (catalog no. 11873580001; Sigma Aldrich), using a 1:3 dilution of tissue: PBS (w/v). Samples were centrifuged at 9,300 RCF for 10 min at 4°C. Supernatants (PBS-soluble fraction) were aliquoted, snap-frozen, and stored at -80°C until use. For insoluble fractions, pellets were resuspended in PBS with protease inhibitor cocktail (Roche), centrifuged at 9,300 RCF for 10 min at 4°C and supernatants were discarded. Remaining pellet was resuspended in 1% sarkosyl in PBS with protease inhibitor, vortexed for 1 min, and incubated at room temperature for 1 hr. Samples were water sonicated for 5 min and centrifuged for 20 min at 14000 rpm at 4oC. Soluble and insoluble fractions of brain extracts containing 50 µg of total protein were loaded (without boiling) on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked for 2 hours at room temperature with 10% nonfat dry milk in TBS-T buffer. Membranes were then probed overnight at 4 in anti-Ubiquilin-2 antibody (Novus Biologicals; 1:2000), poly-ubiquitinated substrate antibody, FK1 (Enzo; 1:1000), or anti-GAPDH (Millipore; 1:3000) diluted in 5% nonfat dry milk. HRP- conjugated goat anti-rabbit IgG, goat anti-mouse IgG and goat anti-mouse IgM (1:3000) were used for detection as appropriate. ECL (Pierce) was used to visualize bands, which were normalized to corresponding GAPDH levels.

All quantification of immunoblots was performed by densitometric analysis using ImageJ

software (National Institutes of Health). Analyses were completed in triplicate and analyzed by one-way ANOVA with the Bonferroni post hoc test.

Immunofluorescence

40 ⌠m free floating, fixed brain sections were subjected to a basic antigen retrieval, washed, blocked, and incubated overnight at 4°C in primary antibody supplemented with 0.025% Triton X-100, 0.5% BSA, and 5% serum from the host line for secondary antibodies (donkey or goat). Primary antibodies used in these studies included the following: anti-ubiquitin (Millipore Sigma, MAB1510; 1:500), guinea pig anti-p62 C-terminal (1:200; Progen). rabbit polyclonal anti-TDP43 antibodies (G. Yu and J. Herz, University of Texas, Southwestern,1:5000). Primary incubated sections were then washed and incubated with the corresponding secondary Alexa Fluor 488 or 568 antibodies (1:1000; Invitrogen). All sections were stained with DAPI (Sigma) for 15 min at room temperature, mounted with Prolong Gold Antifade Reagent (Invitrogen), and imaged using an IX71 Olympus inverted microscope or Olympus confocal microscope.

Fluorescence recovery after photobleaching (FRAP)

HEK 293 cells were plated on LAB-TEK II (cat. 155409) borosilicate chambers slides. The day following plating, cells were transfected with 0.5 ug of UBQLN2-GFP using Fugene 6 (Promega). Cells were imaged 24 hours after transfection with a Nikon A-1 confocal microscope using Nikon Elements software with perfect focus engaged. FRAP imaging consisted of three phases: pre-bleach imaging, bleaching, and post-bleach imaging. During pre-bleach imaging, a UBQLN2-GFP punctum was imaged every 2 seconds for 10 seconds. In the bleach phase, an ROI was drawn corresponding to 1/3 to 1⁄2 the area of a granule. This ROI defined the stimulation area for a 488nm laser at 50% power. The postbleach phase consisted of two periods. For the first minute, images were acquired every 5 seconds, while for the subsequent 10 minutes images were acquired every 10 seconds. This was done in order to provide greater temporal resolution during the rising phase of fluorescence recovery.

FRAP analysis was performed in ImageJ. To fix granules in place, stack registration (Rigid Body) was performed. Following thresholding one ROI was generated that corresponded to the pre-bleach signal and another to the postbleach signal. A mask was created from the pre-bleach ROI, inverted, and the postbleach ROI was subtracted from this image. This created a region of bleached signal deemed the FRAP ROI. At each timepoint the integrated density of the FRAP ROI was divided by the integrated density of the pre-bleach ROI. These values were then normalized such that the mean of the five pre-bleach values was set to 1 and the first post-bleach value was set to 0. Values from each granule were then fitted to the equation $I(t)=A(1-e^{-t/\tau})$, where I=normalized fluorescent intensity, A=mobile fraction, t=time, τ=time constant (62).

To quantify complexity, eight observers were asked to score the complexity of UBQLN2 granules on a scale from 1 to 4 based on representative images corresponding to a granule matching each number, where 1= a roughly circular granule of uniform composition, 2=granule with an elongated shape and/or non-uniform composition. 3=non-circular shape with a non-uniform composition but smooth surface, 4= spiculated granule with a complex uneven surface. The observers were blind to the genotype of the granules. The mean of the eight scores was used as a granule's complexity score.

Protein expression

UBQLN2 constructs were transformed in Rosetta (DE3) Escherichia coli bacteria. All LB/agar plates and LB media were supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Transformed cells were grown overnight at 37°C on LB/agar media plates. On the following day, cells were transferred to 100 ml LB starter cultures and allowed to grow for 60-90 min. The starter cultures were then transferred to 2L flasks containing 1L LB media. After the OD reached $A_{600} \approx 0.6$-0.8, cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and collected after 3-5 additional hours of incubation. The bacteria were collected by centrifugation for 6 min at 10,322 x g. Bacterial pellets were stored at -80°C and purified within 1-2 days.

Protein purification

For full-length UBQLN2, on the day of protein purification, pellets (bacteria grown in 1L LB media) were resuspended in 15 ml of pre-chilled lysis buffer containing 25 mM Na phosphate, 0.5 M NaCl, 20 mM Imidazole pH 7.4, EDTA-free cOmplete protease inhibitor cocktail tablet (Roche; 1 tablet per 10ml lysis buffer), 6 µL/mL of saturated phenylmethylsulfonyl fluoride (PMSF), 1mg/ml lysozyme, and 10% glycerol. Bacteria were then lysed using EmulsiFlex B-15 high pressure homogenizer (Avestin). The lysate was centrifuged at 31,000 x g for 25 min. The proteins were precipitated from the supernatant by adding 0.2 g/mL ammonium. The solution was stirred at 4°C for 30 min and centrifuged at 13,000 x g for 25 min. The pellets were redissolved in 17 mL of buffer containing 25mM Na phosphate, 0.5M NaCl, 20mM Imidazole pH 7.4, filtered through 0.22 ⌈m Steriflip vacuum filters (Millipore) and subsequently loaded on a HisTrap HP column (GE Healthcare Life Sciences) using 25 mM Na phosphate, 0.5M NaCl, 20 mM Imidazole pH 7.4 as the binding buffer. The protein was eluted by running a 0-100% gradient of 25mM Na phosphate, 0.5M NaCl, 0.5M Imidazole pH 7.4. For UBA, UBL, and ΔUBL, the lysates from the EmulsiFlex B-15 homogenizer were added to 2.0 mL Ni-NTA agarose (Qiagen) and incubated on the nutator at 4 °C for 1 h. Then the beads with the bound protein were washed twice with 20 ml wash buffer (25 mM sodium phosphate, 500 mM NaCl, 20 mM Imidazole). Beads were then mixed with 5mL of elution buffer (25 mM sodium phosphate, 500

mM NaCl, 500 mM Imidazole) and incubated on nutator for 30 min at 4 °C. The slurry was spun at 700 x g for 3 min and the supernatant with the eluted protein was collected. This was repeated two more times. Proteins were additionally purified on a size exclusion Superdex HiLoad 16/600 200 column (GE Healthcare Life Sciences), which was pre-equilibrated with 5 mM sodium phosphate, 1 mM sodium azide, pH 7.5.

Thioflavin T (ThT) binding assay

ThT assays were carried out with 10 μM protein in 0.1 M NaCl, 25 mM Na phosphate pH 7.5. Prior to the assay proteins were filtered through a 0.22 μm filter. ThT was added to a final concentration of 10 μM. Teflon beads were added into each well of a Falcon 96-well plate (black/clear, flat bottom, Corning, 353219). Then 70 to 100 μl of sample were pipetted into each well. Plates were then incubated at 37°C in a FLUOstar Omega (BMG Labtech Inc) by shaking at 200 rpm using the 'meander corner well shaking' mode. Fluorescence was measured with gain set at 90%, an excitation wavelength of 440 nm and emission wavelength of 490 nm. Two to four technical replicates were measured per sample for a single ThT assay. Data shown in Figures 3 and S2 are averaged over two to four independent experiments done with different protein preparations.

Transmission electron microscopy (TEM)

Negatively stained specimens for TEM were prepared by applying 5 μL of protein sample to hydrophilic 400 mesh carbon-coated Formvar support films mounted on copper grids (Ted Pella, Inc., 01702-F). The samples were allowed to adhere for 4 min, rinsed twice with distilled water, and stained for 60-90 sec with 5 μL of 1% uranyl acetate (Ted Pella, Inc.). All samples were imaged at an accelerating voltage of 80 kV in a JEOL JSM 1400 Plus (JEOL)**.** Grids from two to four independent experiments were examined for native and aggregated samples.

Primary neuron culture, transfection, imaging and analysis

Primary mixed cortical neurons were dissected from embryonic day 20 rat pups and plated at $6x10^5$ cells/mL. On the fourth day, *in vitro* neurons were transfected using lipofectamine 2000 (Invitrogen). Each well was co-transfected with 100ng of either pGW1-iRFP or a pGW1-UBQLN2-iRFP plasmid and 100ng of pGW1- mApple using 0.5uL of lipofectamine. Lipofectamine-DNA complexes were added to the cells and incubated for 20 minutes before being replaced with Neurobasal media (Gibco) supplemented with B27 (Gibco). Neurons were tracked longitudinally using an automated fluorescence microscopy platform. The Nikon Eclipse Ti inverted microscope equipped with a PerfectFocus system, a high numerical aperture 20X objective lens, and an Andor iXon3 897 EMCCD camera was used to acquire images. A

Lambda XL Xenon lamp (Sutter) with a liquid light guide was used for sample illumination. Shutter, filter, and stage movements were all controlled by μManager software running custom Beanshell scripts. Neuronal tracking was accomplished using custom ImageJ/Fiji macros and Python scripts which identified neuronal cell bodies based on morphology and fluorescence intensity. Upon a loss of fluorescence, rounding of the cell body, or retraction of neuronal processes, a cell was identified as having died. Statistical analyses were performed using the survival package and custom scripts in *R*. To calculate the coefficient of variation (CV) on a single- cell basis, the following equation was used:

$CV = SD_{Cy5} / \mu_{Cy5}$,

where SD is standard deviation of fluorescence intensity in the Cy5 channel (representing iRFP fluorescence) for each cell, and μ_{Cy5} is the mean fluorescence intensity (integrated density / area) in the Cy5 channel for each cell. All images were background subtracted using the rolling ball method in ImageJ/Fiji prior to CV calculation.

Primary neuron Immunofluorescence

For measuring the degree of UBQLN2 overexpression in primary neuron experiments DIV4 rat primary cortical neurons were co-transfected with 100ng pGW1-mApple and 100ng pGW1-iRFP-WT-UBQLN2. The following day neurons were fixed with 4% paraformaldehyde and then stained with rabbit anti-UBQLN2 (1:50; NBP1-85639, Novus Biologicals), followed by an Alexa Fluor 488 secondary antibody (1:500; Invitrogen). Hoechst (1:2000; Invitrogen H3569) was added to stain nuclei.

WT-UBQLN2 & L619A-UBQLN2 + Ubiquitin pulldown

Full length His-tagged UBQLN2 wild-type and L619A were expressed as described. Bacterial cell pellets (bacteria grown in 1L LB media) were resuspended in 10 ml of pre-chilled lysis buffer containing 1x TBS, EDTA-free cOmplete protease inhibitor cocktail tablet (Roche; 1 tablet per 10ml lyses buffer), 6 μL/mL of saturated phenylmethylsulfonyl fluoride (PMSF), 1mg/ml lysozyme, and 10% glycerol. Bacteria were then lysed using a Sonic Dismembrator 60 (Fisher Scientific). The lysate was centrifuged at 12,000 rpm for 25 min. The lysate was then filtered through 0.22 ⌈m Steriflip vacuum filters (Millipore) and subsequently loaded on pre- equilibrated Ni-NTA agarose (QIAGEN). The protein and resin was washed with 1x TBS with 5 mM imidazole (20 mL) and eluted with 1x TBS with 300 mM imidazole. Protein quality was assessed by SDS-PAGE followed by Coomassie blue staining. Proteins were dialyzed into 1x TBS and stored at -80 ^{o}C until further use.

0.1 mg/mL WT or L619A UBQLN2 and 0.01 mg/mL Poly-Ubiquitin/Ub3-Ub7 WT Chains (K48 linked) (R&D Systems) were incubated on a Labquake tube rotator (Fisher Scientific) at room temperature for 1 hr. The solution was added to Ni-NTA agarose and the beads were washed with 1x TBS (10 mL total). The complex was eluted with 3 mL 1x TBS with 300 mM imidazole. The flow-thru and elution samples were assayed on a Bio-Rad TGX 4-20% gradient gel and analyzed by Western blot. Membranes were probed with rabbit polyclonal Ubiquilin-2 antibody (Novus Biologicals) and rabbit polyclonal Ubiquitin antibody (#3933, Cell Signal Technology).

Figures:

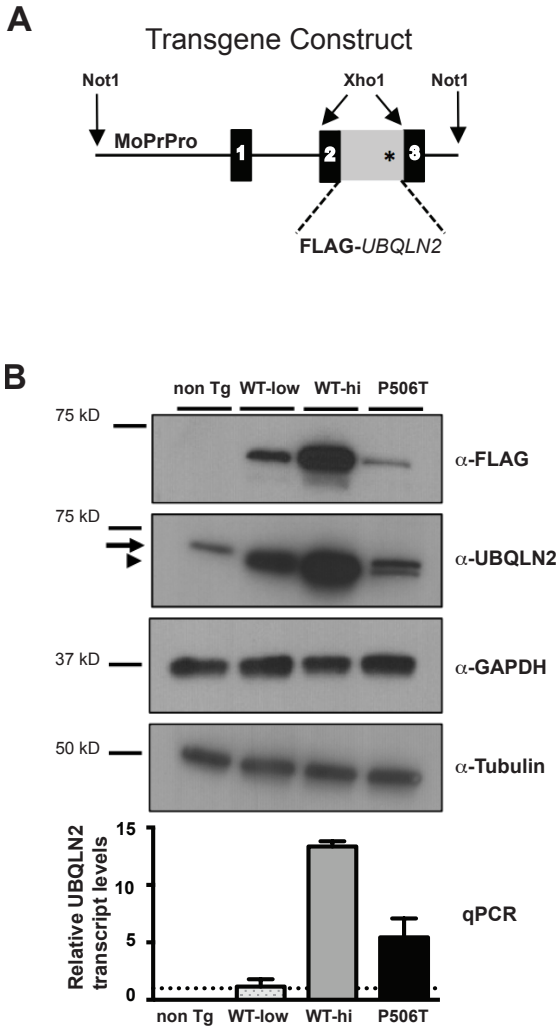

Figure S1: Three transgenic mouse lines were generated to overexpress either WT UBQLN2 (high and low levels) or the mutant P506T UBQLN2.

A) Mouse prion promoter construct used to generate transgenic mouse lines expressing FLAG-tagged UBQLN2.

B) Western blot of whole brain lysates from non-transgenic and transgenic UBQLN2 mouse lines, run on SDS-PAGE and probed with anti-FLAG and anti-UBQLN2 antibodies. Anti-UBQLN2 antibodies detect two closely spaced bands: endogenous murine UBQLN2 (arrow) and FLAG-tagged human UBQLN2 (arrowhead). qPCR quantification of brain UBQLN2 transcript levels for each mouse line is depicted below each respective Western blot lane.

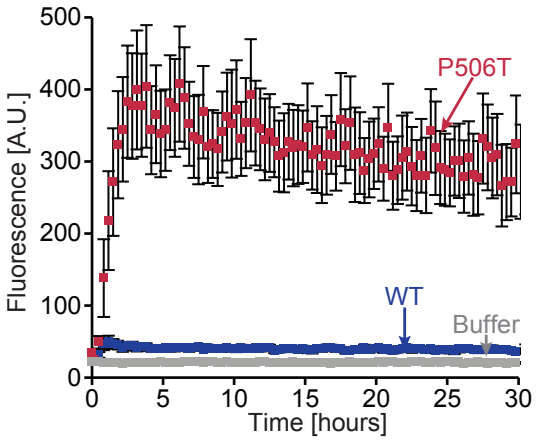

Figure S2: Thioflavin-T assay monitoring kinetics of aggregation and amyloid formation for UBQLN2 WT and P506T

In four of eight in vitro incubations, P506T-UBQLN2 developed ThT fluorescence immediately after beginning the assay, as shown here. The other four incubations show a lag phase, as in Fig. 3.

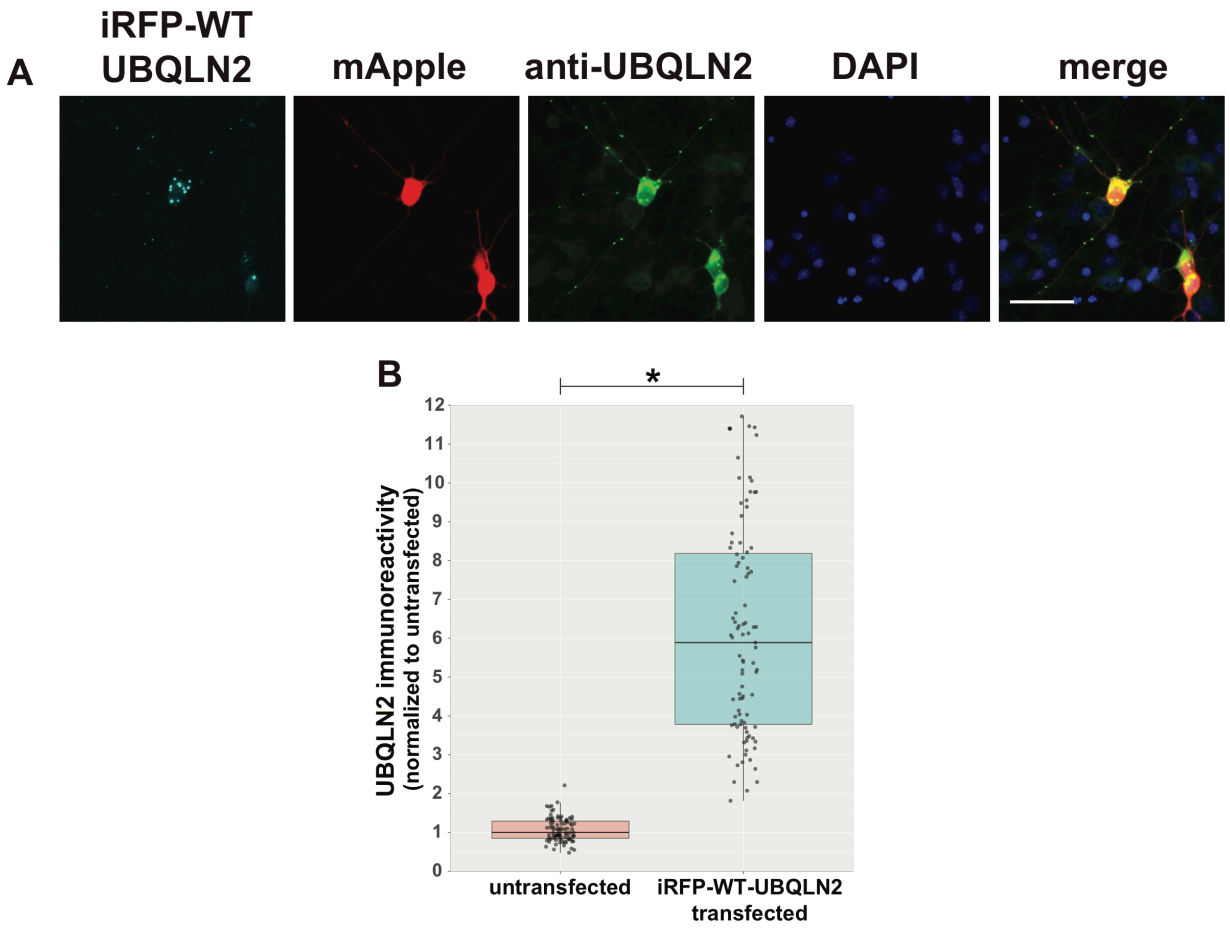

Figure S3. Levels of UBQLN2 over-expression in primary neurons.

A) Primary neurons co-transfected with iRFP-WT-UBQLN2 (cyan) and mApple (red) immunostained using anti-UBQLN2 antibodies (green). Scale bar=100 µM. B) Transfected cells (n=100) displayed a 6.1-fold increase in UBQLN2 immunoreactivity relative to untransfected cells (n=95). Plot shows median (horizontal lines), interquartile range (box), min/max (vertical lines), and values for individual neurons (dots). *p<.0001, student's T-test.

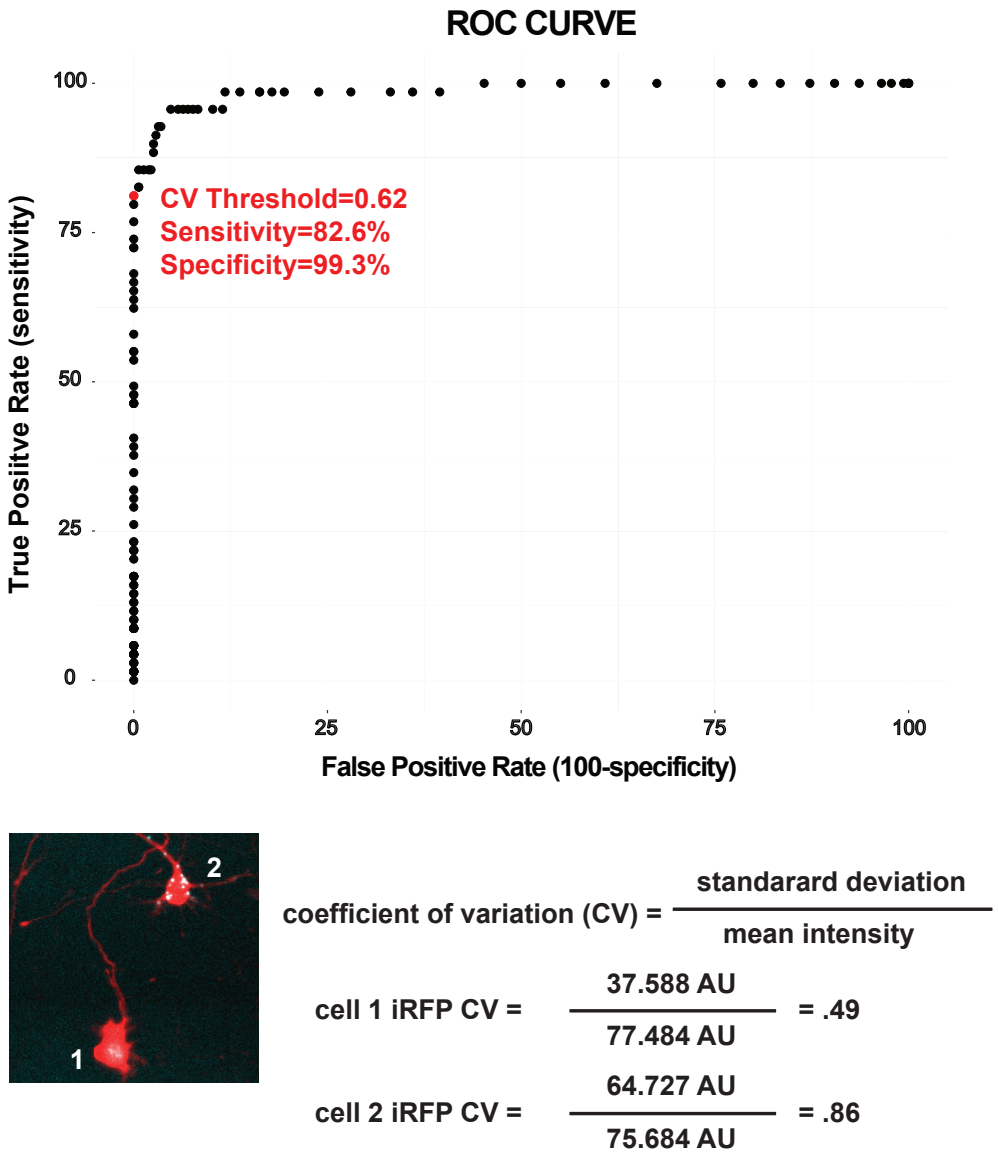

Figure S4. Receiver operating characteristic curve (ROC curve) of iRFP coefficient of variation (CV) for prediction of UBQLN2-iRFP puncta.

552 neurons were manually scored for the presence or absence of UBQLN2 puncta. The percentage of true positives: [(total # of cells with puncta - # of cells with puncta above CV value)/(total # of cells with puncta)] and false positives [(total # of cells without puncta-# of cells without puncta above CV value)/(total # of cells without puncta)] were recorded for a range of CV values. A cut-off value of 0.62 was chosen as the ideal threshold value to minimize false positives (99.36% specificity) while maintaining high sensitivity (82.6%).

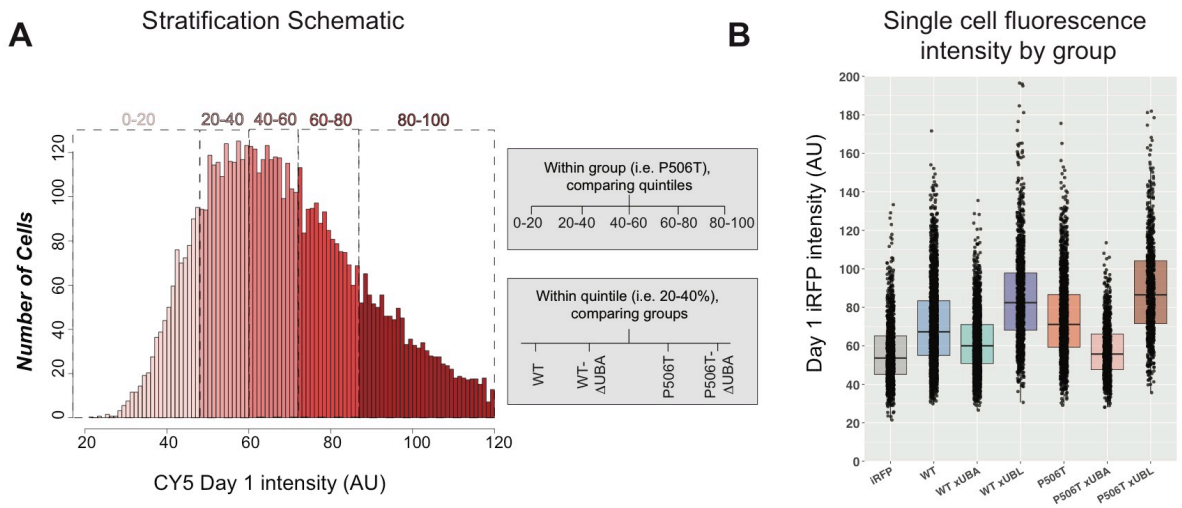

Figure S5: Dose dependent relationship between UBQLN2 expression and aggregation.

A) Schematic depicting two stratification approaches used in subsequent analyses. For both, cells are rank ordered along a continuous variable such as iRFP fluorescence intensity, and the population is then divided into quintiles. Within a genotype, different quintiles can be compared to each other to assess dose dependency, as in Figure 4D. Alternatively, different genotypes can be compared within a quintile in order to ensure accurate comparison of cells showing similar UBQLN2 expression, as in Figure 4E.

B) Boxplot illustrating the distribution of day 1 UBQLN2 expression (iRFP intensity) between groups. Each black circle represents a single cell. Black lines within each box represent the median iRFP intensity.

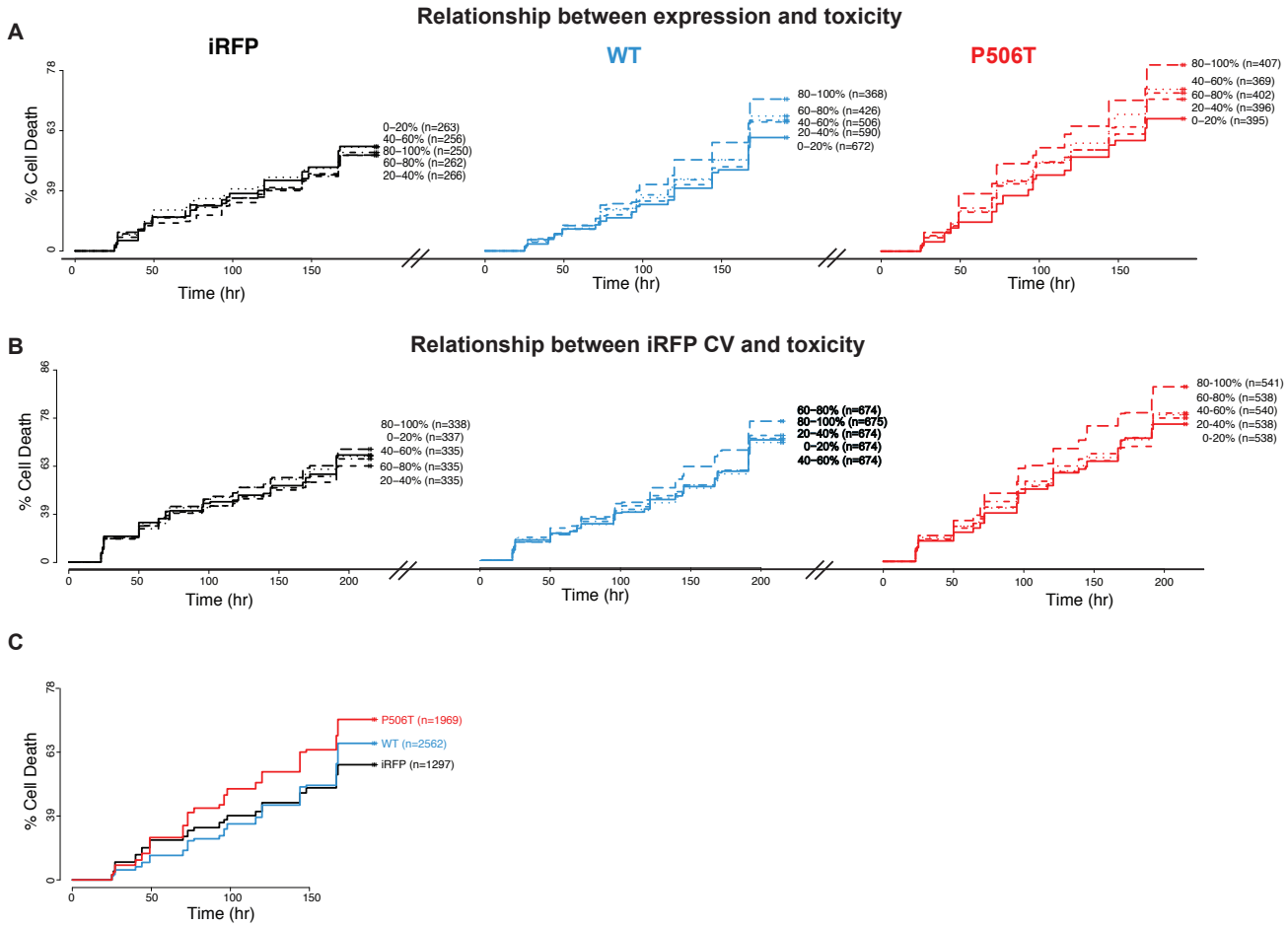

Figure S6: Dose dependent relationships between UBQLN2 expression and aggregation and toxicity.

A) Relationship between expression and toxicity. Neurons expressing iRFP, iRFP-WT-UBQLN2 or iRFP-P506T-UBQLN2 were rank-ordered based on day 1 iRFP intensity, and neurons were then stratified into quintiles. Survival of neurons in each quintile was compared using cox-proportional hazards analysis with the lowest quintile serving as a reference group.

B) A similar analysis was performed as in A but rank-ordering neurons based on day 1 iRFP fluorescence CV. The hazard ratios and statistics for A and B are presented in Table S4.

C) Cumulative risk of death upon stratifying by both expression and CV illustrates toxicity associated with both WT-UBQLN2 (hazard ratio=1.14, p= 4.7E-3) and P506T-UBQLN2 (hazard ratio=1.48, p= 4.22E-15) relative to iRFP that is independent of expression level or aggregation propensity.

Tables:

Table S1. Aggregation Propensity with increasing expression

	Expression Percentile	Hazard Ratio	*P* value
WT	0-20	1	1
	20-40	2.54	**3.40E-05**
	40-60	3.83	**5.50E-05**
	60-80	4.87	**1.94E-13**
	80-100	7.33	**2.00E-16**
WT ∆UBL	0-20	1	1
	20-40	1.79	**3.70E-04**
	40-60	2.83	**2.24E-11**
	60-80	3.13	**1.49E-13**
	80-100	4.79	**2.00E-16**
P506T	0-20	1	1
	20-40	1.63	**5.45E-03**
	40-60	2.62	**6.28E-09**
	60-80	3.3	**9.40E-14**
	80-100	6.43	**2.00E-16**
P506T ∆UBL	0-20	1	1
	20-40	1.41	**7.00E-02**
	40-60	1.67	**5.90E-03**
	60-80	1.67	**5.10E-03**
	80-100	2.1	**2.69E-05**

Table S2. Relative risk of puncta formation

Group	Hazard	*P* value
WT	1	NA
WT ΔUBA	6.70E-03	**1.74E-12**
WT ΔUBL	3.14	**2.00E-16**
P506T	2.31	**2.00E-16**
P506T ΔUBA	0.02	**6.99E-09**
P506T ΔUBL	2.74	**2.00E-16**
iRFP	6.80E-03	**6.20E-07**

Table S3. Relative toxicity by genotype

Group	Hazard	*P* value
iRFP	1	NA
WT	1.19	**6.84E-05**
WT ΔUBA	1.5	**2.00E-16**
WT ΔUBL	1.91	**2.00E-16**
P506	1.63	**2.00E-16**
P506T ΔUBA	1.33	**5.81E-08**
P506T ΔUBL	1.48	**1.18E-12**

Table S4. Dose dependent effects of intensity and aggregation on toxicity

		intensity		CV	
	Percentile	Hazard ratio	*P* value	Hazard ratio	*P* value
iRFP	0-20	1	NA	1	NA
	20-40	0.91	0.4	0.92	0.36
	40-60	1.01	0.89	1.02	0.84
	60-80	0.94	0.57	0.94	0.7
	80-100	0.93	0.56	1.06	0.52
WT	0-20	1	NA	1	NA
	20-40	1.1	0.17	1.06	0.4
	40-60	1.16	**0.04**	0.98	0.76
	60-80	1.14	0.08	1.02	0.81
	80-100	1.35	**2.30E-04**	1.16	**2.00E-02**
P506T	0-20	1	NA	1	NA
	20-40	1.15	0.09	1.03	0.59
	40-60	1.22	**0.02**	1.06	0.38
	60-80	1.19	**0.04**	1.09	0.19
	80-100	1.42	**3.56E-05**	1.29	**1.70E-04**

Table S5. Relative toxicity by genotype assessing expression and oligomerization as covariates

Group	Variable	Hazard	*P* value
iRFP	expression	-	0.97
	oligomerization	-	0.46
WT	expression	1.05	**0.00604**
	oligomerization	1.04	**0.035**
WT ΔUBA	expression	-	0.09
	oligomerization	-	0.76
WT ΔUBL	expression	-	0.06
	oligomerization	-	0.59
P506T	expression	1.04	**0.02**
	oligomerization	1.06	**0.0026**
P505T ΔUBA	expression	-	0.5
	oligomerization	-	0.63
P506T ΔUBL	expression	0.91	**0.000805**
	oligomerization	-	0.13

Table S6. Relative toxicity stratified by both expression and CV

Multivariate cox-proportional hazards analysis was performed to determine the degree to which expression and oligomerization affect toxicity. For each genotype, cells were rank ordered by day 1 expression and CV, then stratified into quintiles in order to compare both variables along the same scale. The hazard ratio represents the slope of the dose-dependent toxicity observed for each variable, excluding the contribution of the other variable. Hazard ratios are reported for those groups (in bold type) achieving statistical significance ($p<.05$).

Group	Hazard	*P* value
iRFP	1	NA
WT	1.14	**4.70E-03**
WT ΔUBA	1.37	**7.10E-12**
WT ΔUBL	1.74	**2.00E-16**
P506T	1.48	**4.22E-15**
P5606T ΔUBA	1.31	**2.14E-07**
P506T ΔUBL	1.34	**1.93E-06**

Table S7. Contribution of oligomerization to toxicity

Group	vs. diffuse	*P* value	vs. WT diffuse	*P* value
WT diffuse	NA	NA	-	NA
WT puncta	1.09	0.22	-	0.23
WT ΔUBL diffuse	NA	NA	1.55	**2.00E-16**
WT ΔUBL puncta	1.07	0.3	1.6	**3.00E-15**
P506T diffuse	NA	NA	1.28	**2.30E-09**
P506T puncta	1.19	**0.01**	1.55	**7.33E-15**
P506T ΔUBL diffuse	NA	NA	1.43	**6.56E-10**
P506T ΔUBL puncta	0.72	**2.59E-04**	-	0.5

Table S8. Classification of particles observed by TEM.

ThT, Fibrillar and Puncta columns indicate relative abundance, from absent (-) to very high (****). Far right column describes the morphology of observed species.

UBQLN2 Constructs	ThT fluorescence	Fibrillar species	Puncta in neurons	Species observed by TEM
WT UBQLN2	*	* (rare)	*	round fusiform
P506T UBQLN2	**	**	**	fibrillar and round fusiform
WT-ΔUBL	**	***	***	short fibrillar
P506T-ΔUBL	***	***	***	short and long fibrillar, amorphous
WT-ΔUBA	***	-	-	circular and small amorphous
P506T-ΔUBA	**	-	-	amorphous and short clumped-fibrillar
UBL domain	-	-	N/D	circular with variable size
UBA domain	****	****	N/D	long amyloid-like fibrillar

Supporting Movie Captions:

Move S1. WT FRAP Example Video. Video demonstrating florescence recovery after photobleaching (FRAP) of a UBQLN2-WT-iRFP granule in HEK293 cells. Scale bar 2μm.

Movie S2. P506T FRAP Example Video -video demonstrating FRAP of a UBQLN2-P506T-iRFP granule in HEK293 cells. Scale bar 2μm.

Movie S3. WT Fusion Example Video -Video depicting the fusion of UBQLN2-WT-iRFP granules in HEK293 cells. Scale bar 2μm.

Movie S4. P506T Fusion Example Video -Video depicting the fusion of UBQLN2-P506T-iRFP granules. Scale bar 2μm.

References:

1. Filonov GS*, et al.* (2011) Bright and stable near-infrared fluorescent protein for in vivo imaging. *Nature biotechnology* 29(8):757-761.