Supplementary Information

Drosophila longevity assays—Flies were maintained with the standard cornmeal-yeast medium (Nutri-Fly BF, Genesee scientific) supplemented with propionic acid and all crosses were performed at 23°C except where indicated. Longevity analysis and climbing assay were modified from methods described previously (63). For longevity analysis, flies were aged at 30°C and survival was recorded every day. For each individual experiment, lifespan of elav/+ flies was recorded to account for environmental variations on the flies. Food was changed every 2-3 days. Log-rank (Mantel-Cox) test was performed for survival analysis.

Supplementary data

Figure S1. (A) Alignment of N-terminal regions of UBQLN1 and UBQLN2. Orange corresponds to UBQLN2 specific PXXP motifs. Blue corresponds to UBL domain. **(B)** Solubility of UBQLN2 is higher than UBQLN1 in 1% Triton-X 100 buffer. Solubility was measured by densitometric analysis and normalized to total (sum of soluble and pellet) input (n=5, *p-value*=0.017).

Figure S2. Schematic of CRISPR mediated knockout of *UBQLN2* in MEFs.

Figure S3. ALS mutations in UBQLN2 are additive and expose amino-terminal epitopes. *UBQLN2*$^{-/-}$ MEFs were transfected with the indicated Myc-tagged UBQLN2 expression constructs. The cells were lysed with NP-40 buffer, and separated into soluble and pellet fractions and analyzed by immunoblotting with the indicated antibodies. The NP-40 soluble fractions were immunoprecipitated with α-Myc antibody and immunoblotted with α-Myc, α-UBQLN2 and α-Ub antibodies.

Figure S4. (A) UBQLN2 ALS mutants show enhanced Ub association *in vitro*. GST-tagged UBQLN2^{WT}, UBQLN2^{P497H}, UBQLN2^{P525S}, UBQLN2^{P3X} and UBQLN2^{P4X} expressed in an *E. coli* were incubated with monoUb-conjugated agarose beads and input and bound fractions analyzed by immunoblotting with α-GST or stained with Coomassie blue. **(B)** UBA mutation of UBQLN2 diminished Ub binding. GST-tagged UBQLN2^{WT}, UBQLN2^{P497H}, UBQLN2$^{P497H/UBA*}$, UBQLN2^{P4X} and UBQLN2$^{P4X/UBA*}$ expressed in an *E. coli* were incubated with monoUb-conjugated agarose beads and input and bound fractions analyzed by immunoblotting with α-GST.

Figure S5. Expression of wild-type and ALS mutant UBQLN2 proteins leads to an increase in cellular Ub. Head extracts from GMR>UBQLN2^{WT}, and GMR>UBQLN2^{P497H}, GMR>UBQLN2^{P525S}, and GMR>UBQLN2^{P4X} flies were separated into soluble and insoluble fractions in RIPA buffer and immunoblotted with α-UBQLN2, α-Ub, and α-β-tubulin antibodies at different times post-eclosion.

Figure S6. Expression of UBQLN2 ALS mutants leads to bristle loss. **(A)** Cuticle imprints and external eye images from 10 day-old flies of GMR>Gal4, GMR>UBQLN2^{WT}, GMR>UBQLN2^{P497H} and GMR>UBQLN2^{P525S} exhibiting a loss of bristle phenotype. **(B)** Quantification of interommatidial bristle loss in GMR>Gal4, GMR>UBQLN2^{WT}, GMR>UBQLN2^{P497H}, and GMR>UBQLN2^{P525S} flies.

Figure S7. Genetic interactions between UBQLN2 and other ALS genes. External eye images from homozygous GMR>UBQLN2^{WT} and GMR>UBQLN2^{P525S} flies crossed to flies expressing

wild-type or ALS-mutant alleles of TDP-43 or FUS or a fly line expressing 30 copies of a GGGGCC HRE representative of expansions from the C9ORF72 locus.

Figure S8. Colocalization between UBQLN2^{P4X} and Ub. Whole brains from Elav>UBQLN2^{P4X} flies were stained with α-UBQLN2 and α-Ub antibodies and imaged by confocal microscopy.

Figure S9. UBQLN2^{ALS} mutants exhibit age-related colocalization with p62. **(A-C)** Localization patterns of wild-type and ALS mutant UBQLN2 proteins with p62 in different age flies. Whole brains of 0, 7, 14, 21 day old-flies from UBQLN2^{WT} (A), UBQLN2^{P497H} (B), and UBQLN2^{P4X} (C) flies were stained with α-UBQLN2 and α-p62 antibodies and imaged by confocal microscopy.

Figure S10. Representative NMJ images from Elav>Gal4, Elav>UBQLN2^{WT}, Elav>UBQLN2^{P497H}, and Elav>UBQLN2^{P4X} larvae. Larval NMJs were stained with HRP and DLG antibodies as described in Materials and Methods and imaged by confocal microscopy.

Figure S11. Lifespan of indicated fly lines expressing UBQLN2 transgenes from 51D and 85Fb integration sites under control of Elav. Elav>Gal4 and Elav>UBQLN2 flies of indicated genotypes were maintained at 30°C and survival assessed by Kaplan-Meier analysis. N= 50-100 male flies.

Figure S12. UBQLN2^{P4X} mutants exhibit reduced climbing ability. Climbing ability of Elav>Gal4 and Elav>UBQLN2^{P4X} flies was measured at 10-days post-eclosion as described in Materials and Methods.