**Isolation and characterization of the *Drosophila* ubiquilin ortholog dUbqln: *in vivo* interaction with early-onset Alzheimer disease genes**

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UBQLN1 variants have been associated with increased risk for late-onset Alzheimer’s disease (AD). We produced transgenic *Drosophila* models that either silence (by RNAi) or overexpress the *Drosophila* ortholog of human UBQLN1, dUbqln. Silencing of dUbqln in the central nervous system led to age-dependent neurodegeneration and shortened lifespan. Silencing of dUbqln in the wing led to wing vein loss that could be partially rescued by mutant rhomboid (rho), a known component of epidermal growth factor receptor signaling pathway. Conversely, overexpression of dUbqln promoted ecotopic wing veins. Overexpression of dUbqln in the eye rescued a small, rough eye phenotype induced by overexpression of *Drosophila* presenilin (dPsn), and also rescuing dPsn-induced malformations in bristles. In contrast, RNAi silencing of dUbqln enhanced the retinal degenerative defect induced by overexpression of dPsn. Finally, co-overexpression of dUbqln and the human amyloid precursor protein (APP) in the eye significantly reduced the levels of full-length APP and its C-terminal fragment. Collectively, these data support *in vivo* functional interaction between UBQLN1 and the AD-associated genes, presenilin and APP, and provide further clues regarding the potential role of UBQLN1 in AD pathogenesis.

**INTRODUCTION**

Alzheimer’s disease (AD), a progressive neurodegenerative disorder, is the most common form of dementia in the elderly. AD is characterized by global cognitive decline accompanied by neuronal and synapse loss, neurofibrillary tangles (NFTs) and senile plaques (1). Recently, it was found that DNA variants in the *ubiquilin 1* (UBQLN1) gene have been associated with increased risk for late-onset AD in a large collection of AD families (2,3). Although some of the AD case-control cohorts have confirmed that genetic variation in UBQLN1 exerts modest effect on risk, age-at-onset and disease duration of AD (4), meta-analyses of the collective case-control studies (www.AlzGene.org) have thus far failed to yield significant summary signals (5) (6–8).

Since the strongest genetic association between UBQLN1 and AD was originally observed in families, where affected and unaffected members share relatively common genetic backgrounds, it is possible that detection of UBQLN1 as a risk factor for AD requires a family-based study design that allows for interaction with other genetic factors. Here, we set out to characterize further the molecular function of UBQLN1 along with an assessment of its interaction with the known AD genes, presenilin and the amyloid precursor protein (APP), using transgenic *Drosophila* models.

UBQLN1 (OMIM#605046; DA41, PLIC-1) (9) belongs to the evolutionarily conserved human ubiquilin family, which consists of at least three other members: UBQLN2 (10), UBQLN3 (11) and UBQLN4 (12). UBQLN1 is ubiquitously expressed and prominently found in neurons where it is...
found both in the nucleus and in the cytoplasm. Pathologically, UBQLN1 has been shown to co-localize with both NFTs and Lewy bodies in brain tissue from AD and Parkinson’s disease (PD) patients, respectively (13). UBQLN1 is also found in purified polyglutamine aggregates in a mouse model of Huntington’s disease (HD) (14). UBQLN1 interacts with early-onset AD-associated gene products presenilin 1 (PS1) and presenilin 2 (PS2); the PS1–UBQLN1 interaction has been detected endogenously in primary neuronal cultures and in brain tissue of healthy controls and AD patients, providing evidence of its physiological relevance (13,15).

Overexpression of UBQLN1 enhances the accumulation of presenilin holoproteins (13,16,17). Moreover, downregulation of UBQLN1 accelerates the maturation and intracellular trafficking of the early-onset familial AD gene product APP (18).

Structurally, ubiquilins contain an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin-associated (UBA) domain, which allow them to associate physically with both the proteasome and ubiquitin ligases. Ubiquilins are designed to link functionally the ubiquitination machinery and the proteasome for protein degradation, including a potential role in the unfolded protein response (UPR) (19). The UBQLN1 UBA domain binds to polyubiquitinated proteins (20). Through the UBL domain, UBQLN1 binds to Rpn3, Rpn10a and Rpn10e proteins in the 19S subunit of the 26S proteasome complex, and interacts with several ubiquitin-interacting motif (UIM) proteins, including epidermolysis-growth factor receptor pathway substrate 15 (Eps15) (21), the co-chaperone human neuron-specific DNAJ-like protein 1a (HSJ1a) and ataxin-3 (AT3), the gene mutated in spinocerebellar ataxia-3 (SCA3 or Machado–Joseph disease; OMIM#109150) (22).

Overexpression of dUbqln was observed in flies overexpressing dUbqln, which drastically reduced dUbqln expression on lifespan, brain morphology and wing development. We then assessed the effects of altered dUbqln expression in lifespan, brain morphology and wing development. In a second set of experiments, we produced double-transgenic fly models to investigate the interaction of dUbqln with the early-onset familial AD genes presenilin (dPsn) and APP (human) in the developing eye of fly.

RESULTS

Isolation and characterization of Drosophila ubiquulin (dUbqln)

The human UBQLN1 gene consists of 2861 bp and 11 exons, in which exon 8 is alternatively spliced to create two main transcripts, TV1 (full-length) and TV2 (missing exon 8) (chromosome 9q21.2; Gene ID: 28979). The TV1 gene product, UBQLN1, contains 589 amino acids (9). The three primary human paralogues of UBQLN1, UBQLN2 (chromosome X, GeneID: 28978) (10), UBQLN3 (chromosome 11p15; Gene ID: 50613) (11) and UBQLN4 (chromosome 1q21; Gene ID: 56893) (12) share significant homology and structural similarity with human UBQLN1. In addition, UBQLN4P is a pseudogene of UBQLN4 located on chromosome 3q24 (Gene ID: 285328). Both UBQLN2 and UBQLN3 consist of 1 exon, whereas UBQLN4 consists of 11 exons. A BLAST search of the Drosophila Genome Database using the human UBQLN1 amino acid sequence as the search string revealed a single homolog, CG14224, on chromosome X18E1, as the human paralogues of UBQLN1, UBQLN2 (chromosome X, GeneID: 28978) (10), UBQLN3 (chromosome 11p15; Gene ID: 50613) (11) and UBQLN4 (chromosome 1q21; Gene ID: 56893) (12) share significant homology and structural similarity with human UBQLN1.

Silencing and overexpression of dUbqln in the CNS

We used Elav–GAL4 to drive expression of dUbqln in all neurons throughout the development to investigate the effects of RNAi silencing and overexpression of dUbqln in the fly CNS. Immunostaining with anti-dUbqln revealed dUbqln expression because of dosage compensation. In the third instar larval CNS of control flies with Elav–GAL4 driver alone (Elav–GAL4/+), compared with control flies, dUbqln expression was moderately decreased in flies in which dUbqln was silenced (UAS–dUbqln–RNAi; Elav–GAL4); increased accumulation of dUbqln was observed in flies overexpressing dUbqln (UAS–dUbqln; Elav–GAL4) (Fig. 2A). Double immunostaining with an anti-Elav antibody as control showed similar Elav expression levels in the CNS of all the tested flies (data not shown).

Under standard culture condition (25°C), compared with control flies (Elav–GAL4/+), male flies silencing dUbqln driven by Elav–GAL4 (UAS–dUbqln–RNAi; Elav–GAL4) suffered premature death. The flies were born normally, but lifespan began to decline shortly after eclosion and significantly declined by the time the flies were 30 days old (P = 0.01, Fig. 2B). At 50 days, >70% of the control flies were still alive, whereas >80% of the UAS–dUbqln–RNAi; Elav–GAL4 flies were already dead (Fig. 2B). Decreased lifespan was consistently observed in two independent UAS–dUbqln–RNAi lines (Fig. 2B). In contrast, there was no significant difference in lifespan between female UAS–dUbqln–RNAi; Elav–GAL4 and control flies (data not shown). This gender discrepancy is most likely because of higher expression in males where the X-chromosome-linked Elav–GAL4 increases dUbqln–RNAi expression because of dosage compensation. The phenotypic differences in lifespan among male and female flies may also possibly reflect unknown male-specific
factors in transgenic animals, as documented in prior studies. For example, co-expression of Appl and the bovine tau gene has been reported to cause a more pronounced decrease in male viability than in female transgenic flies using the ApplG1a–GAL4 driver (25). Moreover, overexpressing APP and the β-site APP cleaving enzyme (BACE) driven by GMR–GAL4 has been reported to lead to larger and more extensive Aβ plaques in male flies compared with female flies (26). Histologic studies with transmission electronic microscope (TEM) revealed severe widespread neurodegeneration in
the CNS of 30-day-old male flies in which $dUbqln$ was silenced (UAS–$dUbqln$–RNAi; Elav–GAL4), compared with control flies (Fig. 2C showed TEM images from the central body complex regions of brain). We also observed multilamellar structures throughout the brain, and the neuropil contained multiple membrane-bound vacuoles (Fig. 2C). Under standard culture conditions, there were no statistically significant differences in the lifespan of control flies and those overexpressing $dUbqln$ in the CNS (UAS–$dUbqln$; Elav–GAL4) (Fig. 2B).

**dUbqln regulates Drosophila wing vein patterning**

In *Drosophila*, the adult wing and thoracic body wall arise from the larval wing disc. The wing veins are stereotyped and easy to score, making them ideal for studying pattern formation. Accordingly, a large number of mutants of genes affecting the wings pattern have been previously isolated, e.g. *Notch* (27). To assess the potential role of $dUbqln$ in wing development, we ectopically silenced (by RNAi) or overexpressed $dUbqln$ in different patterns in the wing disc using three wing-specific GAL4 drivers, Sd-GAL4, En-GAL4 and Ptc-GAL4. The scalloped (*sd*) gene is expressed in the dorsal/ventral boundary of the developing wing imaginal disc that structures the growth of the entire wing (28). The engrailed (*en*) gene is a ‘selector’ gene that is expressed in all posterior developmental compartments to confer a ‘posterior identity’ on cells for the wing development (29). Thus, engrailed–GAL4 (En–GAL4) drive targeted transgene expression only in the posterior compartment of the wing.
Finally, patched (ptc) is a segment polarity gene that is expressed in both compartments centering at the anterior/posterior border (30).

To assess the effects of RNAi silencing or overexpression of dUbqln in transgenic flies, we carried out western blot analysis using protein lysates isolated from the third instar larval wing discs (Fig. 4). Silencing of dUbqln by Sd–GAL4 (UAS–dUbqln–RNAi; Sd–GAL4) in wing discs led a 63% decrease in dUbqln expression compared with control flies (Fig. 3). Wild-type (WT) wings have five longitudinal (L1–5) and two cross veins: anterior cross vein (acv) and posterior cross vein (pcv), creating distinct intervein sectors (Fig. 4A). In addition, there is a marginal (M) vein encompassing the length of the wing margin (Fig. 4A). RNAi silencing of dUbqln with Sd–GAL4 (UAS–dUbqln–RNAi; Sd–GAL4) resulted in three types of phenotypes: (i) loss of the partial L4, L5, entire acv and pcv; (ii) a thickened L3 vein, particularly in the distal portion of the margin area of the wing; (iii) notches in the wing margin (Fig. 4B). The phenotypes of UAS–dUbqln–RNAi; Sd–GAL4 flies showed variable penetrance in male and female flies. Compared with female flies, male UAS–dUbqln–RNAi; Sd–GAL4 flies were semi-lethal, as only 30% (89/300) of adult progenies were male and 70% (211/300) were female (as opposed to the expected ~50% of each gender). All the male flies and ~5% (10/211) of the female flies exhibited all three severe phenotypes, as described above. The majority of the female flies (95%, 201/211) exhibited only a weak phenotype as evidenced by loss of pcv or both pcv and acv veins. This suggests that similar to the dosage effect in the CNS, dUbqln silencing likely caused dose-dependent wing vein loss as the X-chromosome linked Sd–GAL4 drove a higher level of UAS–dUbqln–RNAi expression in male versus female flies. Consistent with the en and ptc gene expression patterns, silencing of dUbqln by En–GAL4 (UAS–dUbqln–RNAi; En–GAL4) led to loss of the entire pcv, partial acv and L5 veins and weakened distal portion of L4 and L3–4 M veins in the posterior portion of the wing (Fig. 4C). Silencing of dUbqln with Ptc–GAL4 resulted in loss of the entire acv, narrowing of the intervein sector between L3 and L4 veins in all flies, compared with WT controls (Fig. 4D).

In contrast to the RNAi silencing results in wing, overexpression of dUbqln with the Sd–GAL4 driver resulted in the development of ectopic veins near L2 vein and bridges between L1 and L2 veins in all the flies. Ecotropic veins were also observed near the pcv and at the distal part of L5 vein (Fig. 4E). Overexpression of dUbqln driven by En–GAL4 caused ectopic veins primarily near pcv and at the distal part of L5 vein, but in only 2% (4/200) of the UAS–dUbqln; En–GAL4 flies (Fig. 4F). Overexpression of dUbqln by Ptc–GAL4 led to no significant phenotypes (data not shown). The observed wing vein loss phenotypes are similar to those caused by mutant rhomboid (rho), which encodes a known component of epidermal growth factor receptor (EGFR) signaling pathway. We used an established UAS–rho transgenic (31) to overexpress full-length WT rho by En–GAL4. This resulted in loss of partial pcv and acv and weakened distal portion of L4 and L3–4 M veins (Fig. 5A). The homozygous null mutant rho^{ve-1} (27) showed loss of partial L3, L4 and L5 and weakened L2–4 M veins (Fig. 5B), whereas heterozygous rho^{ve-1/+} exhibited only a weakened distal portion of L5 vein (Fig. 5C). Next, we created double-transgenic flies with heterozygous rho^{ve-1/+} and RNAi silencing of dUbqln by En–GAL4. This led to a normal acv and partially restored pcv and L5 vein, while the distal portion of L4 and L3–4 M veins corresponding to the
posterior portion of the wing remained weakened (Fig. 5D), compared with RNAi silencing of dUbqln by En–GAL4 alone (Fig. 4C). These data suggest that heterozygosity for mutant rho can partially rescue wing vein loss induced by RNAi silencing of dUbqln.

Interaction of dUbqln with dPsn

Previous studies (13) have shown UBQLN1 to co-localize and interact with both human PS1 and PS2, which are represented by a single ortholog in Drosophila, dPsn (32). Next, we tested for interaction between dPsn and dUbqln, by examining the effects of RNAi silencing or overexpression of dUbqln on a rough eye phenotype that is induced by overexpression of dPsn. For this purpose, we used the GMR–GAL4 to drive gene expression in the eye (33). Compared with control fly eye with GMR–GAL4 drive alone (GMR–GAL4/+, Fig. 6A), neither overexpression (UAS–dUbqln; GMR–GAL4), nor silencing (UAS–dUbqln–RNAi; GMR–GAL4) of dUbqln alone in the eye led to any detectable abnormalities.
In agreement with previous studies (32), flies overexpressing two copies of full-length WT dPsn driven by GMR–GAL4 (2XUAS–dPsn; GMR–GAL4) exhibited a smaller, rough eye phenotype. At eclosion, these flies possessed significantly fewer as well as malformed interommatidial bristles compared with controls (Fig. 6D). Most parts of the eye lacked the mechanosensory bristles normally surrounding each facet of the compound eye. Additionally, some bristles on the edge of the eye were abnormal in shape characterized by bubble- and balloon-like lesions (as well as some double spindles), roughly 3–5 μm in diameter (Fig. 6D). To our knowledge, these malformed ‘bubble-bristles’ have not been described previously.

Next, we investigated the effects of co-overexpression of dUbqln and dPsn on the rough eye and ‘bubble-bristle’ phenotype. Relative to overexpression of dPsn alone, co-expression with dUbqln led to an increase in size of the fly eye. In addition, the irregular ommatidial packing and fusion were reduced, and the number of ommatidia returned nearly to normal. Most strikingly, the ‘bubble-bristle’ phenotype was completely rescued. However, in many facets of the eye, we observed tufting: duplicated or triple bristles in single interommatidial regions (Fig. 6E). In contrast to the co-expression results, RNAi silencing of dUbqln dramatically enhanced the dPsn-induced rough eye phenotype, leading to an even smaller and rough eye than that observed with dPsn overexpression alone (Fig. 6F). Moreover, dUbqln silencing in addition to overexpression of dPsn caused a loss of eye pigmentation, suggesting the loss of pigment cells. Scanning electron microscopy (SEM) revealed that RNAi silencing of dUbqln on a background of dPsn overexpression caused a much more severe degenerative phenotype as evidenced by numerous visible holes (2–5 μm in diameter) on a small, rough eye. In addition, the ommatidia were very irregular and mostly fused with very few bristles (Fig. 6F).

The interommatidial bristles are mechanosensory organs in the fly peripheral nervous system. The decision of Drosophila ectodermal cells to adopt the sensory organ precursor (SOP) fate is controlled by two families of basic helix–loop–helix (bHLH) proteins. The proneural activators, e.g. achaete (ac) and scute (mammalian achaete–scute homolog-1, HASH-1 in human) promote neural development, whereas the Hairy/Enhancers of split (E(spl)) repressors (Hairy/Enhancers of split homolog-1, HES-1 in human) suppress it (34,35). Thus, we then investigated the effects of overexpression of dUbqln and/or dPsn on ac expression levels by immunostaining pupae eyes with an anti-ac antibody (Fig. 7). Compared with control flies (GMR–GAL4/þ) (Fig. 7A), increased expression of dPsn moderately decreased ac expression (Fig. 7B), consistent with the adult eye phenotype of malformed and reduced interommatidial bristles. In contrast, co-overexpression of dUbqln and dPsn resulted in significantly increased ac expression in the interommatidial region (Fig. 7C). Meanwhile, RNAi silencing of dUbqln further decreased ac expression in the eye of flies overexpressing dPsn (Fig. 7D). These data suggest that dUbqln and dPsn can regulate proneural activator ac expression.

dUbqln regulates APP processing

Recently, RNAi silencing of UBQLN1 in various cell lines was shown to accelerate the maturation and intracellular trafficking
Figure 7. Immunohistochemical staining of pupae eyes with anti-achaete (secondary anti-mouse-Alexa488, green, shown as gray scale picture) by confocal microscopy. (A) Controls (GMR–GAL4/+). (B) Flies overexpressing dUbqln (UAS–dUbqln; GMR–GAL4) showed normal ommatidia structure; ac was expressed in the interommatidial regions. (C) Flies overexpressing dPsn (2xUAS–dPsn; GMR–GAL4) possessed small, rough eyes with reduced numbers of interommatidal bristles that were often severely malformed. The few bristles present were abnormal and immature forming various bubbles, balloons and spindle; a couple consisted of double spindles, ~3–5 μm in diameter. (D) Co-overexpressing dUbqln with dPsn (UAS–dUbqln/2xUAS–dPsn; GMR–GAL4) partially rescued fly eye size, reduced the degree of irregular ommatidial packing and fusion and restored bristle to normal shaft shape but tufting. (E) Silencing dUbqln (UAS–dUbqln–RNAi/2xUAS–dPsn; GMR–GAL4) enhanced the dPsn-induced rough eye phenotype to cause a smaller and lighter color rough eye with more fused ommatidia, apoptosis-like abnormalities, few or no bristles and many visible holes (2–5 μm in diameter).

Figure 6. Modifying effects of RNAi silencing and overexpression of dUbqln on a dPsn overexpression-induced rough eye phenotype by GMR–GAL4. Fly eye images were obtained by stereomicroscopy and SEM (magnification, ×140, ×1000, ×1700 and ×4000; Scale bar at ×4000 images: 5 μm). (A) Controls (GMR–GAL4/+). (B) Flies overexpressing dUbqln (UAS–dUbqln; GMR–GAL4) showed normal eye images. (C) Flies silencing dUbqln (UAS–dUbqln–RNAi; GMR–GAL4) showed normal eye images. (D) Flies overexpressing dPsn (2xUAS–dPsn; GMR–GAL4) possessed small, rough eyes with reduced numbers of interommatidal bristles that were often severely malformed. The few bristles present were abnormal and immature forming various bubbles, balloons and spindle; a couple consisted of double spindles, ~3–5 μm in diameter. (E) Co-overexpressing dUbqln with dPsn (UAS–dUbqln/2xUAS–dPsn; GMR–GAL4) partially rescued fly eye size, reduced the degree of irregular ommatidial packing and fusion and restored bristle to normal shaft shape but tufting. (F) Silencing dUbqln (UAS–dUbqln–RNAi/2xUAS–dPsn; GMR–GAL4) enhanced the dPsn-induced rough eye phenotype to cause a smaller and lighter color rough eye with more fused ommatidia, apoptosis-like abnormalities, few or no bristles and many visible holes (2–5 μm in diameter).
of full-length APP to the cell surface, which, in turn, enhanced the secretion of sAPP (α and β forms) as well as Aβ (18). We asked whether overexpression of dUbqln can affect APP processing in fly eye. The ortholog of human APP (hAPP) in fly, dAppl, demonstrates 23% identity and 17% similarity to hAPP with strongest conservation in the –NH₂ and –COOH termini of the gene products (Fig. 8A). Transgenic flies expressing dAppl and hAPP were previously shown to be able to provide similar levels of rescue of the behavioral deficits in flies with a null mutation of dAppl, demonstrating a functional conservation between dAppl and hAPP (36). Compared with hAPP, dAppl lacks some important features in human APP synthesis...
flies. We also observed that silencing of degeneration and a significantly shortened lifespan in male showed that co-overexpression of dUbqln and full-length WT hAPP (APP695) using an established transgenic line UAS–APP
dUbqln in control adult fly head as a 58 kDa product. Using actin to control for protein load, dUbqln expression in the eyes of flies overexpressing dUbqln (UAS–dUbqln; GMR–GAL4) was increased by 277% over endogenous levels (Fig. 8B). Using an anti-human APP antibody, A8717 (Fig. 8A), we detected the production of full-length APP (hAPP-FL) and a single δ-cleaved APP C-terminal fragment (hAPP–δ-CTF; 13 kDa) in the eyes of flies overexpressing hAPP (Fig. 8C). hAPP–δ-CTF can be generated in the processing of hAPP in transgenic flies (which lack BACE), and is consistent with previous studies of these same transgenic hAPP flies (26). A similar form of δ-cleaved APPδ95 was previously reported in cultured rat neurons (39); however, the protease responsible for δ-secretase cleavage remains unknown. Overexpression of hAPP resulted in roughly a 140% increase in hAPP-FL, and an abundant accumulation of the hAPP–δ-CTF, which was otherwise undetectable in control flies (Fig. 8C), suggesting that majority of hAPP holoprotein was processed by δ-cleavage in Drosophila eye. Co-overexpression of dUbqln and hAPP in the eye led to a reduction in the level of hAPP–FL to 74%, and a more significant reduction in the ratio of hAPP–δ-CTF/hAPP–FL to 34% compared with flies overexpressing hAPP alone (Fig. 8C). These data suggest that co-overexpression with dUbqln significantly affects APP levels and the generation of hAPP–δ-CTF.

DISCUSSION

We have isolated the Drosophila ortholog of human UBQLN1, dUbqln, and made several transgenic fly models that either silence (by RNAi) or overexpress dUbqln, either alone or in combination with rho, dPsn or hAPP. Overexpression of dUbqln alone in CNS had no obvious effects. However, silencing of dUbqln in CNS led to age- and dose-dependent neurodegeneration and a significantly shortened lifespan in male flies. We also observed that silencing of dUbqln in the wing led to wing vein loss, which can be partially rescued by mutant rho, whereas overexpression of dUbqln promoted ectopic wing veins. Neither overexpression, nor silencing of dUbqln led to any detectable effects on the eye. However, overexpression of dUbqln partially rescued a small, rough eye phenotype induced by overexpression of dPsn. In contrast, silencing of dUbqln enhanced the retinal neurodegenerative phenotype induced by overexpression of dPsn. Finally, we showed that co-overexpression of dUbqln and hAPP in eye significantly reduced the levels of hAPP–FL and production of hAPP–δ-CTF, compared with flies overexpressing hAPP alone.

UBQLN1 is a UBL protein that binds ubiquitinated proteins and proteasome, thereby acting as a chaperone for protein degradation (19). Overexpression of human UBQLN1 has been reported to interfere with ubiquitin-dependent proteasomal degradation of p53 and IkBα (20). The UBL domain of UBQLN1 binds to UIMs of AT3, HSI1a and Esp15 to form complexes involved in the clearance of misfolded aggregation-prone proteins and endocytosis. Knocking down either UBQLN1 or Eps15 was shown to affect aggresome formation (22). Inefficient proteasome degradation of abnormal protein aggregates has been proposed to be a contributory factor to neuropathogenesis in human neurodegenerative disorders (40). Interestingly, UBQLN1 has been detected in abnormal protein aggregates in brains of patients with AD, PD and brains of a mouse model of HD (13,14). Furthermore, UBQLN1 has been shown to interact with the mammalian target of rapamycin (mTOR) protein kinase, which is known to control cell cycle progression, cell growth and protein degradation (41). mTOR is sequestered in polyglutamine aggregates of HD in cell models, transgenic mice and human brains; sequestration of mTOR impairs its kinase activity and induces autophagy (42). Autophagic cell death has been well implicated in human neurodegenerative diseases, including AD (43), PD (44) and polyglutamine diseases (42). UBQLN1 may provide a functional link between the ubiquitin–proteasome pathway and autophagy via mTOR interaction. Loss of function of one or more of these UBQLN1-related activities may explain severe neurodegeneration and shortened lifespan in flies with reduced dUbqln expression via RNAi silencing. The pathological features of widespread vacuolization and neurodegeneration in brains of flies silencing dUbqln in CNS resemble those previously observed in the brains of flies overexpressing WT and mutant human Tau in the CNS (43), and mutants of other Drosophila genes, including swiss cheese mutant (45), spongecake mutant similar to these seen in spongiform encephalopathies, e.g. Creutzfeldt–Jakob disease and eggroll mutant resembling ones found in lipid storage diseases, e.g. Tay–Sachs disease (46).

By characterizing phenotypes induced by RNAi silencing or overexpression of dUbqln in the wing, we have shown for the first time that dUbqln can regulate wing vein patterning. Notch and EGFR signaling pathways play important roles in the patterning and differentiation of the wing veins in the developmental process in Drosophila. Notch receptor cleavage within the plasma membrane by γ-secretase results in the release and translocation of the Notch intracellular domain (NICD) into the nucleus to execute Notch signaling. This leads to expression of one of the Enhancer of split genes, E(spl)mβ, which, in turn, prevents vein formation (27,47,48). A wing with reduced Notch activity exhibited classic defect of combination both notches in the wing margin and thickened L3 or L5 wings (49), similar to that observed in flies in which dUbqln was silenced in the wing (Fig. 4B). This suggests that dUbqln may regulate Notch signaling, which would be consistent with the fact that Notch is a substrate for γ-secretase, in which presenilin is a known UBQLN1 interactor.
Signaling via EGFR and its downstream effectors is also required throughout the process of wing vein formation. rho and a group of other genes, args, rolled, Dsor1(Mek), l(1)ph, Star and veinlet, encode known or suspected components of the EGFR signaling pathway (50), mutations in each of which can cause loss of the L4 and other wing veins (27,51,52). In our study, RNAi silencing of dUbqln by En–GAL4 in the wing produced phenotypes similar to that caused by either overexpression of rho (En-GAL4) or mutant rho. In addition, heterozygosity for mutant rho partially rescued wing vein loss induced by silencing of dUbqln. These data suggest that dUbqln may be a novel component of the EGFR signaling pathway regulating vein formation. Moreover, these findings would imply that rho acts upstream to repress dUbqln activity.

EGFR is a cell-surface receptor tyrosine kinase, and activation of the EGFR results in the initiation of a diverse array of cellular pathways in development, tissue repair, normal cellular homeostasis and survival. Aberrant signaling patterns of EGFR have been linked to the progression of a diversity of diseases, including cancer, atherosclerosis, asthma, fibrosis and three major neurodegenerative disorders, AD, HD and PD. In neuronal tissue, EGFR can promote neuronal stem cell proliferation, control astrocyte survival and inhibit apoptosis (53,54). EGFR activity is significantly lower in AD brains than that in non-AD control brains (55). APP Swedish mutant neurons demonstrated impaired inactivation, degradation and ubiquitination of EGFR during EGF stimulation (56). Cells deficient in PS1 and PS2 suppressed EGFR downstream intracellular Akt and MEK/ERK signaling pathways (57). Moreover, expanded polyglutamine peptides in huntingtin have been shown to antagonize EGFR signaling in Drosophila glia (58). The neurotrophic activity of EGF is also impaired in patients with PD (59). With regard to neurodegeneration, EGFR-deficient mice develop progressive neurodegeneration accompanied by massive apoptotic cell death, affecting both neurons and glia in the forebrain, resulting from increased apoptosis in cortical astrocytes mediated by Akt and caspase-dependent mechanism (53,60).

Eps15 and its related protein Eps15R are key components of the clathrin-mediated endocytic pathway involved in EGFR endocytosis and trafficking, essential for terminating EGFR signaling (61). Parkin, the gene product of the parkin gene for a common familial form of PD, binds to Eps15 via UBL–UIM interaction, which is required for parkin-mediated Eps15 ubiquitination. By ubiquitinating Eps15, parkin interferes with the ability of the Eps15 UIMs to bind ubiquitinated EGFR, delaying EGFR internalization and degradation and promoting PI3K–Akt signaling; thereby, in parkin deficient cells, EGFR endocytosis and degradation are accelerated (62). Similar to parkin, UBQLN1 interacts with both Eps15 and Eps15R and recruits Eps15 into ubiquitin-rich cytoplasmic aggregates (21). The UBL–UIM interaction between UBQLN1 and Eps15 plays an important role in regulating the UPR and aggresome formation (21,22). The loss of UBL domain of UBQLN1 has a dominant negative effect on aggregate transport and localizes Eps15 to aggregate (22). Therefore, it is interesting to speculate that loss of UBQLN1 might have similar effects to those resulting from loss of parkin, i.e. in the absence of UBQLN1, Eps15 binds ubiquitinated EGFR leading receptor downregulation; decreased EGFR signaling, in turn, induces apoptosis. Conversely, overexpression of UBQLN1 in HeLa cells reduced protein aggregates and polyglutamine-induced cytotoxicity (63); we found that overexpression of dUbqln can partially rescue overexpression of dPsn-induced retinal neurodegenerative phenotype, and EGFR overexpression leads to an increased cell proliferation and tumor growth in various malignant tumors (64).

Overexpression of WT and mutant dPsn has previously been shown to induce apoptosis in the Drosophila eye (32). dUbqln silencing in the context of overexpression of dPsn caused significant neuron loss in the eye. Similar retinal neurodegenerative phenotypes have been observed in fly eyes overexpressing other genes, including Tau associated with Tau neurotoxicity (65), and other two ubiquilin interacting genes: AT1 associated with SCA1 (66) and AT3 associated with SCAC3 (67). Moreover, silencing dUbqln in addition to overexpression of dPsn caused a loss of eye pigmentation, suggesting the loss of pigment cells. This phenotype is similar to that caused by a mutation in the garnet gene (68).

The dPsn- and dPsn/dUbqln-overexpressing flies exhibited aberrations in the interommatial bristles derived from SOP cells in the proneural cluster. The SOP formation is triggered by inhibiting the expression of ac and scute in its neighboring cells through lateral inhibition, which requires the interaction of the gene products for Delta (DI) (ligand) and Notch (N) (receptor) (69), as well as the HES proteins, which are direct transcriptional targets of Notch (70). Loss of dPsn has previously been demonstrated to lead to impaired lateral inhibition in proneural clusters, resulting in enlarged SOP territories and increased proneural ac activities (71). In mammalian cells, UBQLN1 interacts with HASH-1, the mammalian homolog of ac. Co-overexpression of UBQLN1 with HASH-1 in neuronal cells led to significant accumulation of HASH-1 and co-localization with HASH-1 in the nucleus (72). In our study, overexpression of dPsn caused bristle malformations with reduced ac expression, suggesting that increased expression of dPsn may elevate γ-secretase activity and result in increased NICD release, thereby potentiating Notch signaling. This defect could be reversed by co-overexpressing dUbqln with dPsn, which may have served to increase levels of the proneural activator, ac. The bristle tufting phenotype in fly eyes co-overexpressing dUbqln and dPsn was similar to that observed in the fly eye mutants for the neurogenic gene neutralized (nue), which is required for the determination of cell fates within the proneural cluster. Cells mutant for nuel autonomously adopt a neural fate when adjacent to WT cells (73). In addition, mutant clones of two other neurogenic genes DI and N involved in lateral inhibition also exhibit specific defects in bristle development. Mutant DI clones show tufting, whereas both tufting and balding have been observed in N clones (74). Collectively, these data further support the notion that both dPsn and dUbqln are able to modulate proteins in the bHLH transcriptional complex most likely via their effects on Notch signaling and ac levels.

UBQLN1 plays a central role in regulating the proteasomal degradation of various proteins (19). Therefore, even minor changes in the expression and/or function of this protein could potentially affect the steady-state levels of multiple
protein targets. RNAi silencing of UBQLN1 was previously found to increase the intracellular trafficking of APP to the cell surface leading to enhanced secretion of sAPP, Aβ40 and Aβ42 (18). Here, we co-overexpressed dUbqln and hAPP in the fly eye photoreceptor neuron, and demonstrated that co-overexpression of dUbqln can moderately reduce levels of hAPP–FL and dramatically reduce levels of hAPP–β-CTF. These data add in vivo support to the previous finding that dUbqln modulates APP processing. Either increasing or decreasing steady-state UBQLN1 levels in the brain may therefore affect APP trafficking and processing, thereby influencing the generation of Aβ.

In summary, we have recapitulated some key features of AD and other human neurodegenerative disorders including premature death and late-onset, age-dependent neurodegeneration, using transgenic Drosophila models. We have also shown that dUbqln regulates wing vein patterning and interacts with rho, indicating a potential role of dUbqln in EGFR signaling. Moreover, we generated in vivo evidence for interaction of dUbqln and Dpsn in the eye and provided further evidence for the ability of UBQLN1 to modulate APP processing. Collectively, these data provide in vivo support for the functional interaction between UBQLN1 and AD-associated genes, presenilin and APP. The transgenic dUbqln fly models described here should be very useful in future genetic screens aimed at identifying genes regulating dUbqln and dUbqln-related functional pathways in AD and other neurodegenerative diseases.

MATERIALS AND METHODS

Identification of dUbqln, expression constructs and transgenic flies

The dUbqln gene was identified by BLAST search of the Drosophila Genome Database using the human UBQLN1 amino acid sequence as the search string, which revealed a single homolog, CG14224, on chromosome X18E1. Total fly RNA was isolated from the W 1118 whole adult flies using Trizol reagent and cDNA was synthesized using SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis (Invitrogen).

For establishing a gain of function dUbqln model, the UAS–dUbqln construct was made by cloning full-length WT dUbqln cDNA into the pUAST vector as EagI–XbaI fragment. The full-length dUbqln was obtained by RT–PCR amplification from total fly RNA by use of the following primers: 5’-GCTCATCGGTCAGAAATTAAGC and 3’-GTGCATAGCAGCTCCATC ATGGAQACGC using the conditions as described above. Two inverted dUbqln exon 2s were cloned into pWiz vector AvrII 5’ splicing and Nhe 3’ splicing sites. The sequencing analyses confirmed the UAS–dUbqln–RNAi construct contained two inserted dUbqln exon 2s with reversed directions for forming loopless hairpin RNA following splicing to silence expression of dUbqln in transgenic flies.

To produce dUbqln transgenic flies, the UAS–dUbqln and UAS–dUbqln–RNAi constructs were injected into W 1118 embryo as described previously (Genetic Services Inc.) (76). At least two independent transgenic lines were tested for each experiment and consistent results in repeated experiments were recorded. Fly culture and crosses were carried out according to standard procedures. The following fly strains were used: GMR–GAL4 (33), Elav–GAL4 (78), Sd–GAL4 (28), En–GAL4 (29), Ptc–GAL4 (30), UAS–rho (31), rho w–1 (27), 2XUAS–dPsn; GMR–GAL4 (32) (full-length WT dPsn + 14, kindly provided by Dr. Mark E. Fortini) and UAS–APP shos (38) (kindly provided by Dr. Toshiharu Suzuki).

Phenotype scoring, immunostaining and western blot analyses

For survival analyses, 20–30 flies were placed in a food vial and at least 250 flies were prepared for each genotype. Flies were changed to new vials and counted every 2–3 days, up to 85 days. The survival rate among experiment groups was compared using Student’s t-test. A total of 10–15 fly brains from 30-day-old flies for each genotype were fixed, embedded and sectioned for ultrastructure analyses by TEM (Philips CM10 electron microscope), as described previously (79). For immunostaining, the third instar CNS and wing disc were fixed in 4% paraformaldehyde in PBS, blocked in 4% goat serum and probed with primary and secondary anti-bodies accordingly. Wings from adult flies were dissected in isopropanol and mounted in Canada Balsam mounting medium. Eyes from 1–3-day-old adult fly were examined for phenotype with a Nikon stereo microscope and a JEOL 5600LV SEM as described previously (32). The following anti-bodies were used: rabbit-anti-dUbqln (Abgent, against C-terminal amino acid sequence S489-Q503, 1:200 dilutions for immunostaining and 1:3000 for western blotting), mouse anti-Elav (Developmental Studies Hybridoma Bank, 1:200), mouse anti-actin (Developmental Studies Hybridoma Bank, 1:1000) and anti-human APP antibody (Sigma, A8717, 1:2000). The anti-mouse or anti-rabbit Alexa 488 or 546 (Molecular Probes, 1:200) were used as secondary antibodies for fluorescence staining.

Western blot analyses were performed and repeated at least twice using protein isolated from 1–3-day-old fly heads and the third instar larval wing disc, and the intensity of signals was analyzed using image program Quantity One, version 4.6.2. In brief, the levels of analyzed proteins were normalized using the levels of dActin for loading differences controls and...
for the quantification in total protein amounts and presented as the percentage of those in the transgenic flies in comparison with that in controls.

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Conflict of Interest statement. None declared.

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