Role of X11 and ubiquilin as In Vivo Regulators of the Amyloid Precursor Protein in Drosophila

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Abstract

The Amyloid Precursor Protein (APP) undergoes sequential proteolytic cleavages through the action of β- and γ-secretase, which result in the generation of toxic β-amyloid (Aβ) peptides and a C-terminal fragment consisting of the intracellular domain of APP (AICD). Mutations leading to increased APP steady state levels or alterations in APP cleavage cause familial Alzheimer’s disease (AD). Thus, identification of factors that regulate APP steady state levels and/or APP cleavage by γ-secretase is likely to provide insight into AD pathogenesis. Here, using transgenic flies that act as reporters for endogenous γ-secretase activity and/or APP levels (GAMAREP), and for the APP intracellular domain (AICDREP), we identified mutations in X11L and ubiquilin (ubqn) as genetic modifiers of APP. Human homologs of both X11L (X11/Mint) and Ubqn (UBQLN1) have been implicated in AD pathogenesis. In contrast to previous reports, we show that overexpression of X11L or human X11 does not alter γ-secretase cleavage of APP or Notch, another γ-secretase substrate. Instead, expression of either X11L or human X11 regulates APP at the level of the AICD, and this activity requires the phosphotyrosine binding (PTB) domain of X11. In contrast, Ubqn regulates the levels of APP: loss of ubqn function leads to a decrease in the steady state levels of APP, while increased ubqn expression results in an increase in APP levels. Ubqn physically binds to APP, an interaction that depends on its ubiquitin-associated (UBA) domain, suggesting that direct physical interactions may underlie Ubqn-dependent regulation of APP. Together, our studies identify X11L and Ubqn as in vivo regulators of APP. Since increased expression of X11 attenuates Aβ production and/or secretion in APP transgenic mice, but does not act on γ-secretase directly, X11 may represent an attractive therapeutic target for AD.

Introduction

One of the pathological hallmarks of Alzheimer’s disease (AD) is the accumulation of amyloid plaques consisting of toxic β-amyloid (Aβ) peptides. These peptides arise from the sequential cleavage of the Amyloid Precursor Protein (APP), a type I transmembrane protein, by two proteases known as β- and γ-secretase (Fig. 1A). APP proteolysis by β-secretase generates an APP C-terminal fragment (CTF) known as C99. Subsequent cleavage of C99 by γ-secretase results in the release of Aβ into the lumen and the APP intracellular domain (AICD) into the cytosol, where it can contribute to a transcriptional regulatory complex [1]. In addition to this amyloidogenic pathway, APP can also undergo non-amyloidogenic processing via sequential cleavage by α- and γ-secretase (Fig. 1A). α-secretase cleaves within the Aβ sequence, thereby precluding the formation of Aβ. α-secretase produces an APP CTF known as C83, which also serves as a substrate for γ-secretase activity [1].

γ-secretase activity resides in a multi-protein complex that minimally contains Presenilins, Nicasrin, Aph-1 and Pen-2 [2,3]. Mutations in APP, Presenilin 1 and Presenilin 2 cause familial, early onset AD [4–7]. In addition, the triplication of the APP locus as well as promoter mutations in APP that increase APP expression are associated with AD [8,9]. The function of the AICD may also be crucial for AD pathogenesis, since each time Aβ is generated, AICD is simultaneously released. AICD, in conjunction with two PTB domain-containing proteins (Fe65 and Tip60), can enter the nucleus and regulate the transcription of target genes, including APP itself [10]. In addition, AICD has also been implicated in other processes including cell signaling, apoptosis and calcium homeostasis [11–21]. Therefore, identifying genes that regulate APP steady-state levels, APP cleavage, and
The X11/Mint protein family consists of three mammalian members: X11α and X11β, which are expressed in neurons, and X11γ which is ubiquitously expressed. All three X11 proteins contain a phosphotyrosine binding (PTB) domain, followed by two PSD95/Dlg/ZO-1 (PDZ) domains [22]. Several observations suggest links between X11 and AD. First, X11α and X11β have been found in amyloid plaques in post-mortem AD brains [23,24]. Second, increased X11α and X11β expression in mammalian cells leads to a reduced secretion of extracellular Aβ [25–27], while transgenic mice expressing either X11α or X11β are associated with reduced levels of Aβ [28,29]. Third, X11 proteins physically interact with AICD via their PTB domains [30,31] and inhibit AICD-dependent transcription [32]. Fourth, X11α and X11β overexpression increases APP steady-state levels both in vitro and in vivo, likely due to altered maturation in the secretory pathway or endocytic trafficking of APP [25,27,33,34]. Finally, the X11 proteins bind to presenilin via their PDZ domains [35]. Furthermore, X11α and X11β have been reported to modulate γ-secretase in mammalian cells [36]; however, alterations in the levels of C83 and C99 are not observed in transgenic mice overexpressing X11 [29]. These conflicting observations leave it unclear whether X11 overexpression can regulate γ-secretase activity.

Figure 1. Schematics depicting sites of APP cleavage and GAMAREP. (A) The sequential actions of either α-secretase or β-secretase, which cleaves APP in its extracellular domain, and γ-secretase, which cleaves APP within its transmembrane domain, generate AICD and C83 or C99, respectively. α-secretase cleaves APP within the sequence of Aβ, thus precluding the generation of Aβ. (B) GAMAREP (GMR-C99-Gal4, UAS-grim) contains two components: First, C99-Gal4, a chimeric protein with an N-terminal cleavable signal sequence, transmembrane and intracellular domain (yellow bar) and a C-terminal Gal4 (blue circle) is specifically expressed in the eye and is a substrate for γ-secretase. Second, Grim (purple bar) is expressed under the control of a Gal4-dependent promoter (green bar) as a readout. In the presence of endogenous γ-secretase activity (scissor), the unleashed AICD-Gal4 (yellow bar and blue circle fragment) translocates to the nucleus (dashed curve) and binds to the UAS element and activates (black arrow pointing to the right) Grim expression. This leads to apoptosis in the eye, resulting in flies with small and rough eyes (red ovals depict relative eye size). (C) In the absence of γ-cleavage, Gal4 remains tethered at the membrane and therefore is unable to activate Grim expression. This results in flies with increased eye size (i.e., suppression of GAMAREP phenotype). (D) When APP (C99) levels are downregulated, fewer substrate molecules are available (illustrated as one C99 molecule rather than three in B) to activate Grim-dependent apoptosis. This results in larger (suppressed) eye size. (E) When AICD is retained in the cytoplasm, or its levels or function are reduced (not illustrated here), apoptosis occurs less efficiently and the eyes remain large. (F) When AICD-Gal4 mediated transcription is inhibited, apoptosis is also reduced, resulting in flies with larger (suppressed) eyes. In summary, GAMAREP allows the identification of factors that regulate endogenous γ-secretase activity (as in C), as well as factors that regulate APP steady state levels (as in D), and AICD function, stability and/or cytoplasmic retention (as in E and F). (G) The false positive reporter. Suppression of GAMAREP can also arise from factors that regulate processes other than APP biology, such as mutations modifying GMR- or Gal4-dependent transcriptional activation, or apoptosis, which we collectively named false positives. To eliminate these factors, we employed a “false positive” reporter (GMR-Gal4, UAS-apoptotic gene). True modifiers are those that modify they eye phenotypes of GAMAREP, but not those of the false positive reporter.

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Ubiquilin 1 (UBQLN1) is another gene that has been linked to AD. UBQLN1 encodes a protein with ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains, as well as Sti1 repeats [37], which are often associated with chaperone activity [30]. Several studies suggest links between UBQLN1 and AD. First, in post-mortem AD brains, UBQLN1 is found in neurofibrillary tangles [37], a pathological hallmark of AD along with amyloid plaques [1]. Second, the genomic region containing UBQLN1, 9q22, has been identified as containing one major candidate gene for conferring a predisposition to late-onset AD [39,40]. Some reports [41,42], but not others [43–46], suggest that genetic variants in the UBQLN1 gene, including one known as UBQ-8i that deletes one Sti1 repeat, are associated with increased risk for the more prevalent late-onset forms of AD. Further evidence that UBQ-8i has enhanced toxicity comes from studies in Drosophila demonstrating that expression of human UBQ-8i in flies leads to earlier onset and more severe eye degeneration than does expression of wildtype human UBQLN1 [47]. Third, UBQLN1 binds PS1 and PS2 [37], and the fly homolog of Presenilin binds to both human and fly Ubiquilin (Ubqn), respectively [47]. Drosophila ubqn antagonizes preselin [pos] function in both loss-of-function and gain-of-function studies during development and adult-onset neurodegeneration in vivo [47,48]. Finally, in cultured cells, downregulation of UBQLN1 expression alters APP levels and Aβ secretion by modulating APP trafficking [49]. However, reports of altered UBQLN1 expression on APP processing are conflicting [48–50]. Because increases in APP levels and/or altered APP processing are likely to be important risk factors for AD, it is crucial to clarify the effect of UBQLN1 on APP in an in vivo system. Additionally, since both X11 and UBQLN1 have been implicated as regulators of APP steady state levels and APP processing, it is crucial to assess the extent to which each molecule contributes to the myriad regulations of APP-related processes, and to determine what their net effect is in vivo.

Drosophila melanogaster is a useful model organism for studying neurodegenerative diseases and AD-related processes [51–54]. Drosophila contains homologs of APP [55] and all four components of the γ-secretase complex [56,57]. Expression of human APP in Drosophila results in its cleavage by endogenous α- and γ-secretase activities [58,59]. Furthermore, co-expression of human β-secretase (BACE1) and human APP fully reconstitutes APP β-cleavage [60]. In addition, Drosophila contains homologs of X11/ Mint family proteins [22,61] and UBQLN1 with evolutionarily conserved domain architecture [47,48].

We engineered transgenic flies that act as living reporters for γ-secretase activity in the eye [59] (Fig 1B-F). These flies (GMR-C99-Gal4, UAS-grm), known as gamma-secretase activity and ΔAPP level reporters (hereafter called GAMAREP), express a chimeric protein which contains a signal sequence, followed by a fusion of the C99 fragment of APP to the yeast transcription factor Gal4. This C99-Gal4 protein is expressed specifically in the developing eye in the presence of a Gal4 responsive element that drives an apoptosis-inducing gene known as grm [62,63]. In the presence of endogenous γ-secretase activity (Fig 1B), AICD-Gal4 is liberated from the membrane, migrates to the nucleus and activates transcription of grm. The resulting apoptosis generates flies with small and rough eyes [59]. When γ-secretase activity is impaired (Fig. 1C), Gal4 remains tethered to the membrane, resulting in less apoptosis. Therefore, the size and roughness of the eye inversely correlates, in a semi-quantitative manner, with the level of endogenous γ-secretase activity (high levels of activity results in flies with small eyes while low levels are associated with nearly normal eyes). These flies act as sensitive reporters for modest changes (two-fold) in the levels of known γ-secretase components [59]. Though originally intended to identify regulators of γ-secretase activity [59], the eye phenotypes of GAMAREP flies are also expected to be sensitive to genetic perturbations that alter the levels of APP (Fig 1D), AICD function, and/or the transcriptional activity of AICD-Gal4 (Fig 1E, F). To discriminate factors that regulate transgene expression levels, Gal4-dependent transcriptional efficiency or apoptosis, we also generated a false positive reporter (GMR-Gal4, UAS-apoptotic gene) (Fig 1G). True modifiers will be identified as factors that modify only GAMAREP eye phenotypes, and not those of the false positive reporter. Here we report the identification of X11L and ubqn as regulators of APP in Drosophila.

Results

Increased expression of X11L suppresses GAMAREP eye phenotypes

We carried out a genetic screen (to be described in detail elsewhere) in which GAMAREP flies were crossed with flies carrying single insertions of the EP P element on the X chromosome. EP elements carry a Gal4 responsive promoter pointing outwards from the end of the transposon. Thus, when inserted near the 5’ end of a gene, EP elements can drive nearby gene overexpression in a Gal4 dependent manner. From this screen, we identified one suppressor line, which contains an EP element insertion in the 5’ region of the X11L gene (CG5675) (Fig 2A). To confirm that the GAMAREP suppression is due to overexpression of X11L, we showed that eye-specific overexpression of X11L (GMR-X11L) (Fig 2E) indeed suppressed the GAMAREP small eye phenotype (Fig 2E compared to D), indicating that X11L acts specifically on C99, not on GAL4 or apoptotic genes. To demonstrate that the mechanism we uncovered for X11L is shared by human X11 (a), we expressed human X11 (a) specifically in the fly eye (Fig 2H). Strikingly, expression of human X11 (a) also suppressed the small eye phenotype of GAMAREP (Fig 2F), but not that of the false positive reporter (Fig 2J). This suggests that Drosophila X11L and human X11 (a) show functional conservation and that mechanisms of action discovered in flies are likely to be relevant to humans.

X11L overexpression regulates APP at the level of AICD

To determine how increased X11L expression regulates APP in the GAMAREP system, we established assays for APP levels and cleavage in Drosophila. Full length human APP (APP695) or C99 was myc-tagged at the C-terminus and expressed in the eye. Tissue lysates were examined by Western blotting with an anti-Myc antibody to follow the fate of APP (Fig 3A) or C99 (Fig 3B). APP-Myc or C99-Myc was cleaved by endogenous α- and γ-secretase in both systems, yielding an AICD-Myc fragment (γ-secretase cleaved) and C83 (an α-secretase cleaved fragment) (Fig 3A and 3B). Furthermore, although no endogenous BACE activity has been definitively demonstrated in Drosophila, expression of human BACE in the presence of APP resulted in the generation of C99, in addition to C83 and AICD (Fig 2C). RNAi-mediated silencing of any one of the four components of the γ-secretase complex, grm, nct, aplh-1 or pen-2, resulted in attenuated AICD generation and an increase in the levels of CTFs (γ-secretase substrates) (Fig 3A and 3B). In addition, overexpression of a dominant negative form of...
Our observation that overexpression of fly and human X11 does not alter γ-secretase mediated cleavage of APP stands in contrast to a previous report [36]. To explore this issue further, we asked...
whether X11L overexpression could regulate γ-cleavage of Notch, another substrate of γ-secretase. Notch is a transmembrane receptor that mediates cell-cell communication events in multiple developmental contexts [65,66]. Following cleavage of its extracellular domain (the S2 cleavage), Notch requires γ-secretase-mediated cleavage (denoted S3) to release the Notch intracellular domain (NICD), and activate downstream signaling events [67]. To analyze Notch processing, a transmembrane version of Notch representing the S2 cleaved fragment was Myc-tagged at its C-terminus and specifically expressed in the fly eye. Silencing of any one of the four γ-secretase components led to decreased γ-cleavage of Notch, as demonstrated by decreased levels of NICD, and an increase in the levels of uncleaved Notch (C83 and C99). In contrast, expression of either X11L or human X11a results in any alteration in the CTF levels. Overexpression of X11L does not significantly inhibit γ-secretase activity in vivo.

ubqn acts to stabilize the steady-state levels of APP

To study ubqn function in Drosophila, we generated two RNAi constructs targeted to two independent regions of ubqn (the coding region and the 5′-untranslated region, respectively), and used these transgenic flies to carry out tissue-specific silencing [47]. As described previously, ubiquitous expression of RNAi-ubqn resulted in a loss of detectable Ubqn protein [47]. Both ubqn RNAi

interactions between X11L and psn in vivo. RNAi-mediated silencing of psn specifically in the eye (GMR-RNAi-psn) resulted in flies with small, rough eyes (Fig. 5C). This is likely due to impaired cleavage of Notch since partial loss of Notch function also results in small, rough eyes [68]. As expected, overexpression of psn partially suppressed the rough eye phenotype associated with GMR-RNAi-psn, while expression of PsnD279A enhanced this phenotype (data not shown). In contrast, expression of either Drosophila X11L or human X11 did not result in enhancement of the GMR-RNAi-psn eye phenotype (Fig. 5D and 5E), suggesting that overexpression of X11L does not significantly inhibit γ-secretase activity in vivo.

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transgenes silenced ubqn expression to similar levels and gave identical phenotypes in all experiments [47]. Using the same RNAi hairpins, we carried out eye-specific silencing of ubqn (GMR-RNAi-ubqn); effects confirmed by Western blotting of head lysates using anti-Ubqn antibodies (Supplementary Fig. S1). Though silencing of ubqn function did not lead to any overt developmental phenotypes (Fig. 6C), it strongly suppressed the GAMAREP phenotype (Fig. 6B compared with 6A). Importantly, silencing of ubqn did not alter the false positive reporter eye phenotypes (Fig. 6D compared with 2D), suggesting that the suppression of GAMAREP was specifically due to an effect on C99.

We next investigated how ubqn loss-of-function suppressed GAMAREP. APP-Myc was co-expressed with human BACE in the eye. Silencing of ubqn resulted in a reduction of steady state levels of full length APP as well as its proteolytic fragments (C99, C83 and AICD) in the eye (Fig. 6G). Importantly, the reduction of full length APP and its cleaved forms occurred largely propor-
mentary Fig. S1). However, ubqn overexpression (GMR-Gal4, UAS-ubqn) led to an increase in APP levels (Fig. 6I). As with silencing of ubqn, ubqn overexpression increased the steady state levels of the full length and γ-secretase cleaved fragments of APP largely in proportion, suggesting that perturbation of Ubqn levels does not affect γ-cleavage of APP (Fig. 6I). Together, we concluded that the suppression of the GAMAREP phenotype caused by loss of ubqn function is most likely the result of a decrease in APP steady-state levels. While loss of ubqn strongly suppressed the GAMAREP phenotype, we did not observe a significant enhancement of GAMAREP upon ubqn overexpression. This is most likely due to the fact that our GAMAREP reporter is much less sensitive in detecting enhancers than suppressors. This is based on multiple screens carried out to identify modifiers of GAMAREP, from which we isolated a dozen GAMAREP suppressors but no enhancers (unpublished observations).

How Ubqn regulates APP levels is unclear, but could involve direct interactions between these proteins. To explore this hypothesis, we asked if Ubqn can bind to APP directly. We mixed purified GST and GST-Ubqn fusion proteins with lysates from Schneider 2 (S2) cells transfected with Myc-tagged C99. Indeed, Myc-C99 specifically bound GST-Ubqn, but not GST alone (Fig. 6J). Interestingly, in addition to native-sized C99, GST-Ubqn preferentially bound to high molecular weight products, which are likely polyubiquitinated forms of C99 (Fig. 6J). Deletion of the UBA domain, but not the UBL domain, significantly abrogated the interaction between Ubqn and C99 (Fig. 6J), indicating that the UBA domain is required for this interaction.

**Discussion**

Here, we report the identification of two APP regulators, X11L and Ubqn, using a living reporter, GAMAREP. GAMAREP allows us to effectively identify regulators of γ-secretase activity, APP steady-state levels and/or AICD function. Both the steady-state levels and the processing of APP have been demonstrated to be important in the pathogenesis of AD. However, the function of AICD, a much less studied APP fragment, may also be crucial for AD pathogenesis, since AICD is generated along with Aβ and may have functions including transcriptional regulation, signaling, apoptosis and calcium homeostasis. Importantly, when a protein regulates multiple aspects of APP, either directly or indirectly, the combination of GAMAREP, AICDREP, and our in vivo cleavage assays allows us to uncover the relative contributions of these regulatory inputs. This is of particular relevance in this study since both X11 and UBQLN1 have been proposed to have multiple functions affecting myriad pathways in various systems. Therefore, it is important to determine in vivo which pathways play predominant roles in events related to AD pathogenesis. Our data suggest that the predominant role of X11L is to regulate APP at the level of the AICD by a process requiring its PTB domain, whereas the major role of Ubqn is to regulate APP levels, likely through a direct physical interaction with APP that is dependent on the UBA domain of Ubqn.

As a modifier of AICDREP, X11L does not regulate AICD levels. Following γ-cleavage, AICD is thought to migrate into the nucleus. It is likely that X11L overexpression causes cytoplasmic retention of AICD based on the following observations. In mammalian cultured cells, X11L overexpression can reduce nuclear localization of the AICD with Tip60 [69]. Moreover, X11L can shuttle between the nucleus and cytoplasm to regulate AICD transcriptional targets [70]. Our functional studies using AICDREP suggest that the PTB domain of X11L is essential for X11L’s ability to regulate AICD. Therefore, our hypothesis is that X11L

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**Figure 5. X11L overexpression does not regulate the effect of γ-secretase activity on Notch.** (A–B) Western blotting of adult head lysates from various transgenic flies expressing the S2-cleaved Notch fragment (a γ-secretase substrate). (A) Eye-specific silencing of any of the four component of the γ-secretase complex results in a reduction of NICD levels and an increase in the levels of uncleaved Notch. (B) Overexpression of either X11L or human X11α fails to alter S2-cleaved Notch levels or NICD levels, in contrast to expression of PsnDN. (C–E) Overexpression of either Drosophila X11L (D) or human X11α (E) does not modify the eye phenotype due to eye-specific knockdown of psn (C). Insets show a close-up view of the ommatidia.

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overexpression leads to the cytoplasmic retention of AICD via a direct physical interaction between the PTB domain of X11L and AICD.

We have also showed that, in contrast to prior studies [36], X11L overexpression does not inhibit γ-secretase activity in vivo. In Drosophila, none of the APP cleavage products, C83, C99 or AICD, is affected by X11L overexpression. Moreover, X11L overexpression does not inhibit γ-secretase activity in vivo towards another key substrate, Notch, at the level of cleavage, or at the level of genetic interaction with psn. In a prior study, King et al concluded that X11 inhibits γ-secretase activity, based largely on the findings that extracellular Aβ levels were decreased upon X11 overexpression [36]. They interpreted this to be due to decreased Aβ production by γ-secretase inhibition. However, decreased extracellular Aβ levels could also result from decreased Aβ secretion or increased Aβ degradation. In fact, another in vivo study suggested that although X11L overexpression led to reduced Aβ levels, levels of C83 and C99 (direct γ-secretase substrates) did not increase [29]. Since overexpression of X11L can increase the steady-state levels of APP, likely by modulating APP trafficking to certain compartments in the secretory pathway [25,27,33,34], an alternative interpretation of work by King et al is that X11L overexpression decreases Aβ via its modulation of APP trafficking, rather than by regulating γ-secretase cleavage. Therefore, these prior studies are, in fact, consistent with our data and interpretation.

Our data also suggest that the predominant function of ubqn is to regulate APP levels, not γ-secretase activity. We have established that Ubqn can bind to and stabilize APP. In contrast to the selective reduction of AICD and increase in CTFs seen following the knockdown of any one of the four γ-secretase components, both ubqn loss-of-function and ubqn overexpression modify the levels of APP and its cleavage fragments largely in proportion. Our results are consistent with findings made by Zhang et al and different from Hiltunen et al; possible reasons for these differences are discussed in Zhang et al [50].

In this study, we did not observe any alterations in γ-secretase activity on APP in response to the silencing of ubqn or ubqn overexpression, similar to observations made by others [48–50]. However, we have previously shown that Ubqn binds to Psn and antagonizes psn function both during development and during adult-onset eye neurodegeneration in Drosophila [47]. There are several ways these observations can be reconciled. γ-secretase activity is known to vary depending upon the substrate and the tissue in which it acts [71]. It is possible that ubqn antagonizes psn activity such that its effect on γ-secretase is more evident on
substrates other than APP in the eye. As a result, the modification of the γ-cleavage of APP by ubqn, if any, might be below the detection limits of our assay. Alternatively, it is well established that in addition to being the catalytic core of the γ-secretase complex, Psn can also function in a γ-secretase independent fashion [72,73]. Therefore, it is possible that ubqn primarily inhibits γ-secretase independent functions of Psn, leaving γ-secretase dependent activity largely intact. In either case, the primary effect of ubqn on APP appears to be the regulation of APP steady state levels, and not inhibition of the γ-cleavage of APP.

Here, using GAMAREP and AICDREP, in conjunction with in vivo cleavage analysis, we have identified factors that regulate APP. Further screens using these tools are likely to identify other proteins which may have implications for AD pathogenesis. Since increased expression of X11 attenuates AP production or secretion in APP transgenic mice [28,29], but does not act on γ-secretase directly (this work), X11 may present an attractive therapeutic target for AD. Similarly, since reduced Ubqn levels result in a modest decrease in APP levels, whereas expression of both human proteins which may have implications for AD pathogenesis. Since in vivo cleavage analysis, we have identified factors that regulate APP transgenic mice [28,29], but does not act on γ-secretase independent activity largely intact. In either case, the primary effect of ubqn on APP appears to be the regulation of APP steady state levels, and not inhibition of the γ-cleavage of APP.

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Materials and Methods

Molecular Biology

A microRNA-based technology [74] was used for RNAi silencing. To silence pen, act, and pen-2, the respective coding regions were independently targeted. PCR products of these microRNA precursors were cloned into pGMR. To generate GMR-X11L, GMR-X11L, the X11 coding sequence was PCR amplified from the EST clone, LD29081, and subcloned into each vector. To generate GMR-human-X11, a clone of X11 (kindly provided by Declan McLoughlin) was subcloned into the EcoR1 and NotI sites of pGMR. To generate GMR-X11AP, the insert of UAS-X11APT [75] was subcloned into pGMR vector. To generate GMR-AICD-Gal4, the nucleotide region encoding the predicted human AICD fragment was PCR amplified and fused in-frame, upstream of an S. cerevisiae Gal4 sequence. To make GMR-ubqn, GMR-RNAi-ubqnCDs and GMR-RNAi-ubqnUTR, the inserts from UAS-ubqn, UAS-RNAi-ubqnCDs and UAS-RNAi-ubqnUTR [47] were subcloned into pGMR vector, respectively. To generate pGMR-APP-9My and GMR-AICD-9My, coding sequence of APP95 or AICD was PCR amplified and cloned in-frame upstream of a 9xMyc sequences that has been previously subcloned into pGMR vector. GMR-ubqn was also generated by PCR and subcloned to generate pM-T-C99-ubqn vector and GMR-C99-ubqnMy, respectively. To make pGEX-ubqnAUBL, a PCR fragment encoding UbqnAUBL was subcloned into the EcoR1 and NotI sites of the modified pGEX4T-1 vector. pGEX-ubqn and pGEX-UbqnAUBL, were described previously [47]. All cloned PCR products were confirmed by DNA sequencing.

Drosophila Genetics and Strains

For the X chromosome EP screen, individual lines of females carrying a single inserted EP element were individually crossed to GAMAREP males. Progeny carrying one copy of an EP insertion and one copy of GAMAREP were examined, and their eye size compared with control flies derived from a cross of wildtype females to GAMAREP males. For experiments involving transgenic flies, multiple independent fly lines were generated (Rainbow Transgenic Flies) and tested for each transgene. UAS-BACE flies [60] were obtained from Rita Reifegerste via Doris Kretzschmar, and UAS-bid flies [64] were obtained from Bruce Hay. GMR-Psn [257] was described previously [59].

Scanning Electron Microscopy

Freshly sacrificed flies were mounted on their side with one eye upward on white tape using clear nail polish. All flies were placed on a rotating platform to permit for orientation under vacuum and were imaged at 180× magnification and 100 psi using a Hitachi 2460N scanning electron microscope. Analysis of eye phenotypes was performed as described previously [59].

Antibody Generation

A fusion of GST-TEV to Drosophila X11L residues 452–775, corresponding to a region within the N-terminal domain, with an intervening TEV protease recognition site, was purified from E. coli lysates. Glutathione agarose-retained proteins were cleaved by TEV protease to remove GST, and used to immunize rabbits (Imgenex).

Lysate Preparation and Western Blotting

Heads from age and sex-matched adults were disrupted in lysis buffer, complete protease inhibitor cocktail (Roche) using a sonicator-3000 from MISONIX. Samples were sonicated. Samples were boiled, centrifuged, and total protein from 4 heads per genotype was analyzed by Western blotting. Antibodies used were anti-Myc (Upstate), anti-Ubqn [47], anti-X11L and anti-Tubulin (Sigma).

S2 Cell Culture and Transfection

S2 cells were grown in Schneider’s Drosophila medium (Invitrogen) supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin at room temperature. Transfections were carried out using MaxFect transfection reagent (Molecula). Typically, 1.5×10⁶ cells plated in a 12-well dish were transfected with 0.7 – 1 μg total plasmid DNA plus 5 μL MaxFect reagent. Metallothionein promoter expression was induced with 0.5 mM copper sulfate 24 hours after transfection. S2 cell lysates were prepared by harvesting transfected cells (~5×10⁶) in 1 mL lysis buffer, followed by incubation on ice for 10 minutes and centrifugation (16,000×g, 10 min) to pellet insoluble debris.

GST Pulldown Assay

10 μg GST fusion proteins purified from E. coli lysates were retained on 15 μL glutathione beads, and mixed with 400 μg S2 cell lysate in 1mL total volume. Retained proteins were eluted by boiling in Laemmli sample buffer, and detected by Western blotting with anti-Myc antibody (Covance) [47].

Supporting Information

Figure S1. The expression levels of X11L and ubqn transgenes. (A) We have generated polyclonal antibodies against X11L. X11L overexpression is accomplished using either an eye-specific promoter (GMR-X11L) or the eye-specific driver using the UAS-GAL4 system (GMR-Gal4, UAS-X11L). Western blots of lysates from fly heads overexpressing X11L or lysates from Schneider 2 cells overexpressing X11L (MT-X11L) reveal a band of predicted size using anti-X11L antibodies. (B) Western blots of lysates from fly heads expressing RNAi-ubqn or ubqn using anti-Ubqn antibodies. Silencing of ubqn significantly reduces Ubqn levels, while ubqn overexpression increases Ubqn levels. A non-specific band (*) serves as protein loading control. Found at: doi:10.1371/journal.pone.0002495.s001 (1.06 MB TIF)
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Author Contributions

Conceived and designed the experiments: MG GG RF AG. Performed the experiments: MG GG RF AG. Analyzed the data: MG GG RF AG. Contributed reagents/materials/analysis tools: JW HY. Wrote the paper: MG.

References

1. Selkoe DJ (2001) Alzheimer’s disease: genes, proteins, and therapy. Physiol Rev 81: 741–766.
2. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, et al. (2003) Reconstitution of gamma-secretase activity. Nat Cell Biol 5: 486–488.
3. Iwatsubo T (2004) The gamma-secretase complex: machinery for intramembrane proteolysis. Curr Opin Neurobiol 14: 379–383.
4. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer’s disease. Nature 349: 704–706.
5. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, et al. (1995) Candidate gene for the chromosome 1 familial Alzheimer’s disease locus. Science 269: 973–977.
6. Rogelj B, Mitchell JC, Miller CC, McLoughlin DM (2006) The X11/Mint family of adaptor proteins. Brain Res Rev 52: 305–315.
7. Sherrington R, Rogelj B, Auger EA, Levesque G, Ibeda M, et al. (1993) Familial Alzheimer’s disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer’s disease type 3 gene. Nature 376: 775–778.
8. Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Alves da Costa C, et al. (2004) Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease. Am J Hum Genet 75: 936–946.
9. Cao X, Sudhof TC (2001) A transcriptionally [correction of transcriptively] active complex of APP with F0v65 and histone acetyltransferase Tip60. Science 293: 115–120.
10. Lee JH, Lau KF, Rogelj B, Turner RS, Levy E, et al. (2004) The UBQLN1 polymorphism, UBQ-8i, at 9q22 is not associated with Alzheimer’s disease amyloid precursor protein interactions: mutational analysis of the YENPTY motif of amyloid precursor protein. Mol Cell Biol 24: 6229–6231.
11. Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Alves da Costa C, et al. (2005) Presenilin-dependent transcriptional control of the A beta-degrading enzyme nephrilysin by intracellular domains of beta APP and APPL. Neuron 46: 541–554.
12. Ryan KA, Pimplikar SW (2005) Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. J Cell Biol 171: 327–337.
13. Zhang YW, Wang R, Liu Q, Zhang H, Liao FF, et al. (2007) Presenilin/gamma- secretase-dependent processing of beta-amyloid precursor protein regulates EGF receptor expression. Proc Natl Acad Sci U S A 104: 16163–16168.
14. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, et al. (1991) The UBQLN1 polymorphism, UBQ-8i, at 9q22 is not associated with Alzheimer’s disease amyloid precursor protein interactions: mutational analysis of the YENPTY motif of amyloid precursor protein. Mol Cell Biol 24: 6229–6231.
15. Lee JH, Lau KF, Rogelj B, Turner RS, Levy E, et al. (2004) The UBQLN1 polymorphism, UBQ-8i, at 9q22 is not associated with Alzheimer’s disease amyloid precursor protein interactions: mutational analysis of the YENPTY motif of amyloid precursor protein. Mol Cell Biol 24: 6229–6231.
16. Brouwers N, Sleegers K, Engelborghs S, Bogaerts V, van Duijn CM, et al. (2006) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 315: 557–565.
17. Guo J, Ganguly A, Feldman RM, Guo M (2008) ubiquilin antagonizes presenilin and amyloid precursor protein intracellular domain (AICD). Mol Biol Cell 18: 201–210.
18. Blacker D, Bertram L, Saunders AJ, Moscarillo TJ, Albert MS, et al. (2003) Results of a high-resolution genome screen of 437 Alzheimer’s disease families. Hum Mol Genet 12: 239–246.
19. Borg JP, Ooi J, Levy E, Margolis B (1996) The phosphotyrosine interaction family of adaptor proteins. Brain Res Rev 23: 49099–49104.
20. Brouwers N, Sleegers K, Engelborghs S, Bogaerts V, van Duijn CM, et al. (2006) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 315: 557–565.
21. Guo J, Ganguly A, Feldman RM, Guo M (2008) ubiquilin antagonizes presenilin and amyloid precursor protein intracellular domain (AICD). Mol Biol Cell 18: 201–210.
22. King GD, Pearson RW, Steinhilb ML, Zoghbi HY, et al. (2000) Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation. J Cell Biol 151: 847–862.
23. Blacker D, Bertram L, Saunders AJ, Moscarillo TJ, Albert MS, et al. (2003) Results of a high-resolution genome screen of 437 Alzheimer’s disease families. Hum Mol Genet 12: 23–32.
24. Myers A, Wazirz DeVinzie F, Holmpan M, Hanshore M, Crook R, et al. (2002) Full genome screen for Alzheimer disease: stage II analysis. Am J Med Genet 114: 235–244.
25. Bertram L, Hilburn M, Parkinson M, Ingelsson M, Lange C, et al. (2005) Family-based association between Alzheimer’s disease and variants in UBQLN1. N Engl J Med 352: 884–894.
26. King GD, Bernat EM, Bruffey KE, DeKooij ST (2006) Genetic association of ubiquilin with Alzheimer’s disease and related quantitative measures. Mol Psychiatry 11: 273–279.
27. Kaur MA, Martin ER, Bronson PG, Browning-Large C, Doraizsamy PM, et al. (2006) Lack of association between UBQLN1 and Alzheimer disease. Am J Med Genet Psychiatriy Genet 141: 208–213.
28. Saura M, Novosnyy P, Hinnen M, Kruse JS, Cherny S, et al. (2006) Ubiquitin 1 polymorphisms are not associated with late-onset Alzheimer’s disease. Ann Neurol 59: 21–26.
29. Brouwers N, Sherges K, Engelnborgo S, Bogaerts V, van Duijn CM, et al. (2006) The UBQLN1 polymorphism, UBQ-8i, at 9q22 is not associated with Alzheimer’s disease with onset before 70 years. Neurosci Lett 392: 72–74.
30. Olafsdottir F, Chau J, Shi J, Thaker U, et al. (2006) Association study of the ubiquilin gene with Alzheimer’s disease. Neurol Dis 22: 691–693.
31. Ganguly A, Feldman RM, Guo M (2008) ubiquilin antagonizes presenilin and promotes neurodegeneration in Drosophila. Hum Mol Genet 17: 293–302.
32. Li A, Xie Z, Dong Y, McKay RM, McKee ML, et al. (2007) Isolation and characterization of the Drosophila ubiquilin ortholog dUBqln. In vivo
interaction with early-onset Alzheimer disease genes. Hum Mol Genet 16: 2626–2639.

49. Hiltunen M, Lu A, Thomas AV, Romano DM, Kim M, et al. (2006) Ubiquln 1 modulates amyloid precursor protein trafficking and abeta secretion. J Biol Chem 281: 32240–32253.

50. Zhang C, Khandelwal DJ, Chakraborty R, Cullar TL, Sarangi S, et al. (2007) An AICD-based functional screen to identify APP metabolism regulators. Mol Neurodegener 2: 15.

51. March JL, Thompson LM (2006) Drosophila in the study of neurodegenerative disease. Neuron 52: 169–178.

52. Sang TK, Jackson GR (2005) Drosophila models of neurodegenerative disease. NeuroRx 2: 430–446.

53. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, et al. (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 441: 1162–1166.

54. Dodson MW, Guo M (2007) Pink1, Parkin, DJ-1 and mitochondrial dysfunction in Parkinson’s disease. Curr Opin Neurobiol 17: 331–337.

55. Luo LQ, Martin-Morris LE, White K (1990) Identification, secretion, and neural expression of APPL, a Drosophila protein similar to human amyloid protein precursor. J Neurosci 10: 3849–3861.

56. Hu Y, Fortini ME (2003) Different cofactor activities in gamma-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex. J Cell Biol 161: 685–690.

57. Takasugi N, Tomita S, Hayashi I, Tsuruoka M, Niimura M, et al. (2003) The role of presenilin cofactors in the gamma-secretase complex. Nature 422: 438–444.

58. Fossgreen A, Bruckner B, Czech C, Masters CL, Beyreuther K, et al. (1998) Transgenic Drosophila expressing human amyloid precursor protein show gamma-secretase activity and a blistered-wing phenotype. Proc Natl Acad Sci U S A 95: 15707–15712.

59. Guo M, Hong EJ, fernandes J, Zipursky SL, Hay BA (2003) A reporter for amyloid precursor protein gamma-secretase activity in Drosophila. Hum Mol Genet 12: 2689–2698.

60. Greeve I, Kretzschmar D, Tschape JA, Beyreuther K, et al. (2004) Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic Drosophila. J Neurosci 24: 3099–3106.

61. Hase M, Yagi Y, Taru H, Tomita S, Sumioka A, et al. (2002) Expression and characterization of the Drosophila X11-like/Mint protein during neural development. J Neurochem 81: 1223–1232.

62. Hay BA, Hub JR, Guo M (2004) The genetics of cell death: approaches, insights and opportunities in Drosophila. Nat Rev Genet 5: 911–922.

63. Hay BA, Guo M (2006) Gasap-Dependent Cell Death in Drosophila. Annu Rev Cell Dev Biol 22: 623–650.

64. Yoo SJ, Hub JR, Muro I, Yu H, Wang L, et al. (2002) Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat Cell Biol 4: 416–424.

65. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signalling: cell fate control and signal integration in development. Science 284: 779–776.

66. Guo M, Jan LY, Jan YN (1996) Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17: 27–41.

67. Bray Sj (2006) Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7: 678–689.

68. Cagan RL, Ready DF (1989) Notch is required for successive cell decisions in the developing Drosophila retina. Genes Dev 3: 1099–1112.

69. von Roth RC, Kohli BM, Boset J, Meier M, Suzuki T, et al. (2004) The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor. J Cell Sci 117: 4435–4448.

70. Sumioka A, Saito Y, Sakuma M, Araki Y, Yamamoto T, et al. (2008) The X11L/X11beta/MINT2 and X11L2/X11gamma/MINT3 scaffold proteins shuttle between the nucleus and cytoplasm. Exp Cell Res 314: 1155–1162.

71. Loewer A, Soha P, Beyreuther K, Paro R, Merdes G (2004) Cell-type-specific processing of the amyloid precursor protein by Presenilin during Drosophila development. EMBO Rep 5: 405–411.

72. Parks AL, Curtis D (2007) Presenilin diversifies its portfolio. Trends Genet 23: 140–150.

73. Vetrivel KS, Zhang YW, Xu H, Thimakaran G (2006) Pathological and physiological functions of presenilins. Mol Neurodegener 1: 4.

74. Chen CH, Huang H, Ward CM, Su JT, Schaeffer LV, et al. (2007) A Synthetic Maternal-Effect Selfish Genetic Element Drives Population Replacement in Drosophila. Science 316: 397–600.

75. Ashley J, Packard M, Ataman B, Budnik V (2005) Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. J Neurosci 25: 3943–3953.

76. Vichna S, Hertenstein A, Betchinger J, Knoblach JA, Gert de Courth H, et al. (2006) The adapter protein X11/Lalphi/Dmmit interacts with the PDZ-binding domain of the cell recognition protein Rst in Drosophila. Dev Biol 299: 296–307.