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A reporter for amyloid precursor protein γ-secretase activity in *Drosophila*

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A key event in the pathogenesis of Alzheimer's disease (AD) is the deposition of senile plaques consisting largely of a peptide known as β-amyloid (Aβ) that is derived from the amyloid precursor protein (APP). A proteolytic activity called γ-secretase cleaves APP in the transmembrane domain and is required for Aβ generation. Aberrant γ-secretase cleavage of APP underlies the majority of early onset, familial AD. γ-Secretase resides in a large multi-protein complex, of which Presenilin, Nicastrin, APH-1 and PEN-2 are four essential components. Thus, identifying components and pathways by which the γ-secretase activity is regulated is crucial to understanding the mechanisms underlying AD pathogenesis, and may provide new diagnostic tools and therapeutic targets. Here we describe the generation of *Drosophila* that act as living reporters of γ-secretase activity in the fly eye. In these reporter flies the size of the eye correlates with the level of endogenous γ-secretase activity, and is very sensitive to the levels of three genes required for APP γ-secretase activity, *presenilin*, *nicastrin* and *aph-1*. Thus, these flies provide a sensitized system with which to identify other components of the γ-secretase complex and regulators of its activity. We have used these flies to carry out a screen for mutations that suppress γ-secretase activity and have identified a small chromosomal region that contains a gene or genes whose products may promote γ-secretase activity.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease of the brain that manifests as a decline of memory and other cognitive functions. A critical pathogenic event leading to AD is the progressive accumulation of amyloid plaques consisting primarily of the amyloid β peptide (Aβ) (reviewed in 1,2). Aβ is derived from the amyloid precursor protein (APP), a type 1 transmembrane protein, through the action of two proteolytic activities known as β-secretase and γ-secretase (Fig. 1A). The sequential action of β-secretase, which cleaves APP in the extracellular domain, and γ-secretase, which cleaves APP within the transmembrane domain, generates Aβ peptides and releases the intracellular domain of APP (AICD) from membrane association (Fig. 1A, reviewed in 1–4). γ-Secretase generates two major Aβ peptides of 40 or 42–43 residues in length, differing in the length of their C-termini. The longer forms of Aβ, Aβ42–43, are prone to aggregate and are thought to seed the formation of amyloid plaques (reviewed in 3–5). Understanding how γ-secretase-mediated APP cleavage is achieved is crucial to understand AD pathogenesis. γ-Secretase also cleaves other signaling proteins within their transmembrane domains (reviewed in 6). One of the most notable ones is Notch, a key transmembrane receptor that plays important roles in cell–cell communication both during development and in adulthood (reviewed in 7). Cleavage of Notch by γ-secretase is essential for Notch function and this function of γ-secretase is evolutionarily conserved (6,7).

γ-Secretase activity resides in a large multi-protein complex (8), the exact molecular weight and composition of which is still being elucidated (reviewed in 9,10). Recent observations indicate that the activity of four proteins, Presenilin, Nicastrin, APH-1 and PEN-2, are necessary for γ-secretase activity (reviewed in 10). The human genome encodes two Presenilins, Presenilin 1 (PS1) and Presenilin 2 (PS2). Presenilins bind APP and probably provide the active catalytic core of γ-secretase or act as intimate cofactors (reviewed in 2,6,11–14). Mutations in *PS1* and *PS2* are associated with the majority of familial AD cases (4). Nicastrin is a transmembrane glycoprotein isolated via its ability to bind PS1 (15) and to promote *Notch* signaling

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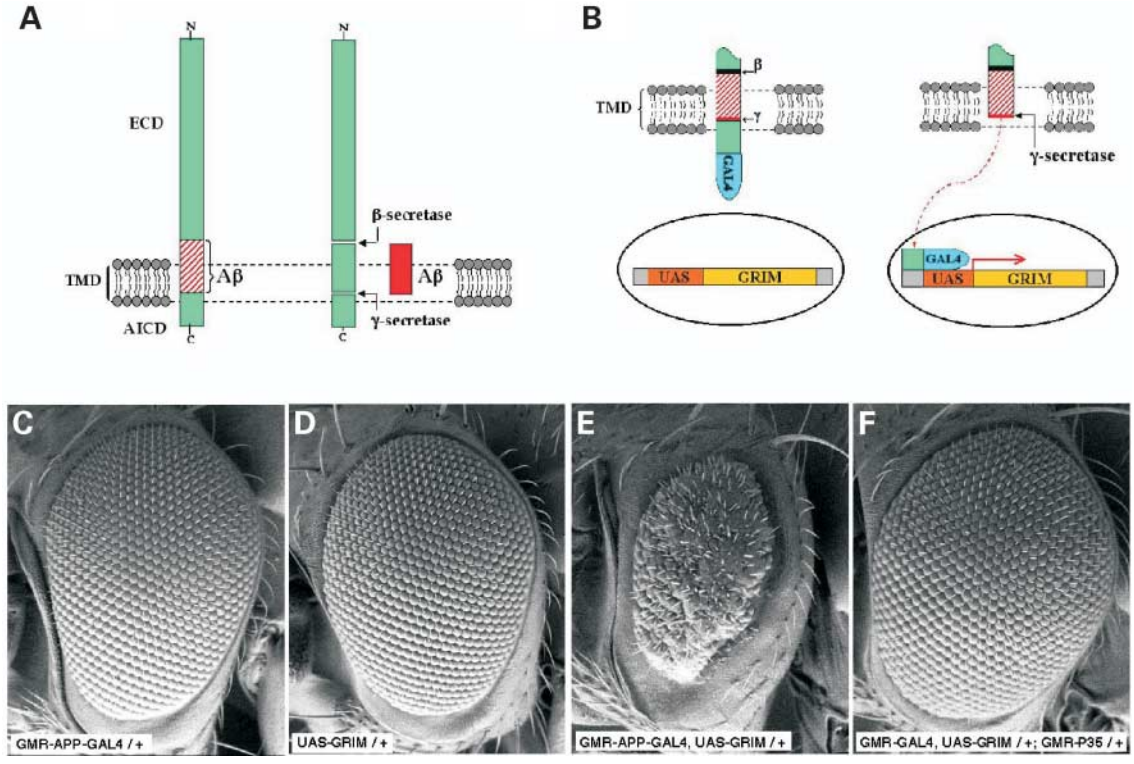

Figure 1. A fly eye-based reporter for γ-secretase activity. (**A**) A schematic illustration of APP cleavage sites. The sequential action of β-secretase, which cleaves APP in the extracellular domain (ECD), and γ-secretase, which cleaves APP within the transmembrane domain (TMD), generates Aβ. (**B**) Schematic illustrating a γ-secretase reporter. The reporter contains two components: a chimeric protein, APP-GAL4, as a substrate and the UAS-GRIM construct as an output. APP-GAL4 is specifically expressed in the eye. In the absence of γ-secretase mediated APP cleavage (left panel), no cell death will be observed *in vivo*. In the presence of γ-secretase (right panel), the unleashed APP fragment and GAL4 are translocated to the nucleus and activate GRIM-dependent cell death. (**C**–**F**) Scanning electron micrographs of adult fly eyes of various genotypes. The genotypes are as follows: (C) GMR–APP–GAL4/+; (D) UAS–GRIM/+; (E) GMR–APP–GAL4, UAS–GRIM/+; (F) GMR–p35/+; GMR–APP–GAL4, UAS–GRIM/+. Expression of GMR–APP–GAL4/+ (C) or UAS–GRIM/+ (D), in isolation resulted in flies with wildtype-appearing eyes. In contrast, Expression of both GMR–APP–GAL4 and UAS–GRIM (reporter) results in adult flies with small eyes (E), indicative of retinal cell death. Retinal cell death present in the reporter flies is eliminated and eye size restored to normal by coexpression of the caspase inhibitor baculovirus p35 (F).

in *C. elegans* (16). APH-1, a polytopic membrane protein originally identified as a regulator of *Notch* signaling in *C. elegans* (17,18), binds to Presenilins and Nicastrin (19–21). PEN-2, a small protein that spans the membrane twice, was also first identified as a regulator of *Notch* signaling in *C. elegans* (18). These four proteins, when coexpressed in *S. cerevisiae*, which lacks endogenous γ-secretase activity, are sufficient to create an active γ-secretase complex (22; reviewed in 10). This finding suggests that these four proteins are the minimal core components of the γ-secretase, although it does not exclude the possibility that factors in yeast may also contribute to formation of the active γ-secretase complex. These four proteins regulate the stability and maturation of each other in tissue culture (19–26). How this regulation occurs is unknown. It is also unknown if other signaling pathways regulate γ-secretase activity by modifying the assembly or activity of these proteins.

Drosophila γ-secretase and its four known components are functionally conserved. *Drosophila* has an endogenous γ-secretase activity for APP and Notch (27), and its mechanism of action through formation of a high molecular weight complex with Presenilin is also conserved (28). *Drosophila* has a single homolog each of *presenilin* (*psn*) (29–31), *nicastrin* (*nct*) (32–34), *aph-1* and *pen-2* (18). Loss of *psn* or *nct* function in *Drosophila* results in *Notch*-like phenotypes (32–37). Removing *aph-1* or *pen-2* in *Drosophila* Schneider 2 (S2) culture cells via RNA interference (RNAi) suppresses γ-cleavage of both APP and Notch (18). Together, these observations indicate that the mechanisms by which APP cleavage occurs in flies, as well as the pathways by which this activity is regulated, are conserved with those of mammals.

Genes that regulate γ-cleavage of APP have been isolated successfully through several approaches. Positional cloning of familial AD genes resulted in identification of PS1 and PS2. Biochemical purification of PS1-associated proteins led to the identification of Nicastrin (reviewed in 3–5). Function-based *in vivo* genetic screens conducted in model systems such as *C. elegans* and *Drosophila* for phenotypes that are suggestive of an alteration in γ-secretase activity provide an important complementary strategy for identifying new genes regulating γ-secretase. For example, screens for *C. elegans* mutants with *Notch*-like or *presenilin*-like developmental defects identified the γ-secretase regulators *aph-2* (*nicastrin*) (16), *aph-1* and *pen-2* (17,18). The advantage of the *in vivo* genetic approach is

that it does not rely on direct physical binding to existing proteins such as Presenilins, and thus will not be limited by the low affinity or abundance of important interacting components. In addition, it has the potential to identify components of the regulatory pathways of γ-secretase activity that may not bind to Presenilin. Below, we describe a reporter that allows determination of the endogenous levels of γ-secretase activity in an easy to visualize, nonessential neuronal tissue, the adult fly eye. This reporter is designed to identify changes in γ-cleavage of APP. This is in contrast to previous *C. elegans* and *Drosophila*-based screens which used *presenilin* and *Notch* mutant phenotypes during development as surrogate markers (16–18). In addition, we describe results from a screen utilizing these flies that identified a small region on the second chromosome containing one or more genes whose products may promote APP γ-secretase activity.

RESULTS

A reporter for APP γ-secretase activity in living flies

As outlined in Figure 1B, we generated transgenic flies expressing a chimeric type-1 transmembrane protein that serves as a γ-secretase substrate. The N-terminus of the protein has a cleavable signal sequence which is followed by a fragment of human APP just C-terminal to the β-secretase cleavage site. The yeast transcription factor GAL4 is appended to the APP C-terminus. This protein, known as APP–GAL4, is targeted to the secretory pathway by the N-terminal signal sequence. Cotranslational cleavage of this signal sequence as APP is inserted into the membrane generates a protein that is C-terminal to the β-secretase cleavage site. We specifically expressed APP–GAL4 in the eye under the control of the eye-specific GMR promoter (38). The reporter flies also carry a γ-secretase reporter output construct that consists of a GAL4-responsive transcriptional cassette driving the expression of the *Drosophila* cell death activator GRIM (39). In the absence of γ-secretase, GAL4 remains tethered at the membrane and therefore is unable to enter the nucleus and activate transcription. In the presence of γ-secretase activity, cleavage of APP releases from the membrane a fragment consisting of the intracellular domain of APP (AICD) and GAL4. This fragment migrates to the nucleus and activates GRIM transcription, thereby promoting cell death in the eye (Fig. 1B). Cells and organisms that act as reporters for caspase family proteases, as well as Notch or APP γ-secretase activity, have been generated previously (40–43). In these reporters a proteolytic cleavage event is linked to the transcriptional activation of a reporter such as LacZ or luciferase. Our strategy utilizes retinal cell death as a readout to generate a visible and quantifiable eye phenotype in living animals, and hence facilitates *in vivo* genetic screens.

Flies expressing GMR–APP–GAL4 alone have wildtype-appearing eyes (Fig. 1C), as do flies carrying only UAS–GRIM (Fig. 1D). In contrast, flies expressing both GMR–APP–GAL4 and UAS–GRIM (referred to hereafter as the reporter flies) have small eyes (Fig. 1E). Importantly, the reporter small eye phenotype was completely suppressed when reporter flies also expressed under GMR control the potent cell death and caspase inhibitor baculovirus p35 (38) (Fig. 1F), or the *Drosophila* cell death and caspase inhibitor DIAP1 (44) (data not shown). These observations indicate that the reporter small eye phenotype is strictly due to GRIM-induced caspase-dependent cell death, and not the result of developmental defects or the activation of other signal transduction pathways by the cleaved APP–GAL4 reporter protein.

The γ-secretase reporter flies act as a useful genetic background in which to carry out screens for γ-secretase regulators (see below). Any screen, genetic or chemical, generates false positives, i.e. mutations that act as modifiers but that are not interesting for the project at hand. In our case sources of such modifiers include mutations that affect the efficiency of GMR- or GAL4-dependent transcriptional activation, or the efficacy of GRIM-dependent cell death signaling. We intended to use GMR–GAL4, UAS–GRIM to eliminate these false positives. However, since GMR–GAL4, UAS–GRIM flies are lethal, we chose to utilize two strains of flies that act as reporters for false positives. The first strain is GMR–GRIM which expresses GRIM directly under GMR control and has small eyes (Fig. 2D). The second strain is GMR-GAL4, UAS-GRIM G/REAPER (45). This chimeric protein, G/REAPER, has the N-terminus of Grim, and the C-terminus of a second cell death activator, Reaper (Rpr) (46). GMR–GAL4, UAS–G/RPR flies also have a small eye phenotype, due to induction of caspase-dependent cell death (45) (Fig. 2G). True modifiers of γ-secretase activity should alter the eye size of reporter flies, but not those of the false positive reporter flies. False positives, on the other hand, should modify eye size similarly in the presence of both the reporter and false positive reporters. As an example of the latter, reduction in the levels of *glass*, which encodes a transcription factor that drives GMR-dependent expression (47), results in suppression of the small eye phenotype of reporter flies, as well as those of the false positive reporters (data not shown).

Activity of the reporter depends on Presenilin, Nicastrin and Aph-1

Presenilin, Nicastrin and Aph-1 are required for γ-secretase-dependent cleavage of APP in mammals and *Drosophila* (reviewed in 10,14). Thus an important prediction is that the eye phenotype of reporter flies, but not those of the false positive reporter flies (GMR–GRIM or GMR–GAL4, UAS–G/RPR), should be sensitive to the levels of Psn, Nct and Aph-1. We used two approaches to decrease Psn levels: in the first we made reporter flies heterozygous for a *psn* null mutation, psn^{C1} (35), thereby decreasing the *psn* genetic dose by 50%; in the second we generated and expressed a dominant negative version of *Drosophila* Psn under GMR control (GMR–PSNDN flies; see Materials and Methods for details). Heterozygous flies for psn^{C1} or GMR–PSNDN alone had wild-type-appearing eyes (data not shown). In contrast, decreasing Psn levels suppressed the small eye phenotype of the reporter (Fig. 2B and C; cf. A), but not the small eye phenotypes associated with GMR-GRIM (Fig. 2E and F; cf. D) or GMR–GAL4, UAS–G/RPR (Fig. 2H and I; cf. G).

Results of a similar series of experiments showed that the reporter eye phenotype was also sensitive to levels of a second

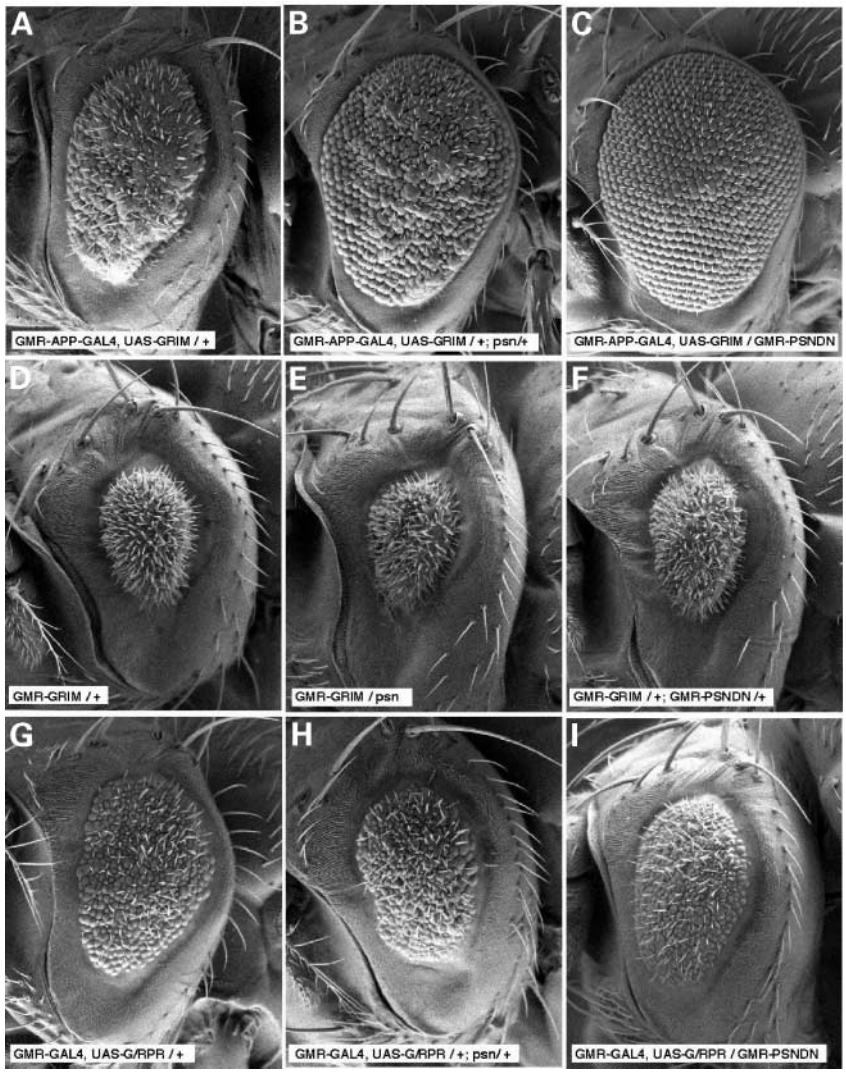

Figure 2. γ-Secretase reporter flies are sensitive to the levels of Psn. Scanning electron micrographs of various genotypes are shown. The genotypes are as follows: (**A**) GMR–APP–GAL4, UAS–GRIM/+; (**B**) GMR–APP–GAL4, UAS–GRIM/+; psn^{C1}/+; (**C**) GMR–APP–GAL4, UAS–GRIM/+; GMR–PSNDN/+; (**D**) GMR–GRIM/+; (**E**) GMR–GRIM/+; psn^{C1}/+; (**F**) GMR–GRIM/+; GMR–PSNDN/+; (**G**) GMR–GAL4, UAS–G/RPR/+; (**H**) GMR–GAL4, UAS–G/RPR/+; psn^{C1}/+; (**I**) GMR–GAL4, UAS–G/RPR/+; GMR–PSNDN/+. (A) is identical to Figure 1E, as the crosses for Figures 1–3 were set up in parallel. Decreasing Psn levels suppress the GMR–APP–GAL4, UAS–GRIM-dependent small eye phenotype (B, C; cf. A), but not the small eye phenotypes associated with GMR-GRIM (E, F; cf. D) or GMR–GAL4, UAS–G/RPR (H, I; cf. G).

component of the γ-secretase complex, Nct (Fig. 3). Again, we used two approaches to reduce the levels of Nct. In the first we made reporter flies heterozygous for a *nct* null allele, nct^{J2} (33), thereby decreasing the *nct* genetic dose by 50%. In the second we generated flies that expressed an *nct* RNA interference construct under GMR control (GMR–RNAi–*nct* flies, see Materials and Methods for details), and introduced these into the reporter background. Heterozygous flies for nct^{J2} or GMR–RNAi–*nct*, in isolation, had wild-type-appearing eyes (data not shown). However, they both led to a strong suppression of the reporter-dependent small eye phenotype (Fig. 3B and C; cf. A). As with *psn* above, reduction of *nct* dose had no effect on the eye size of the false positive reporter flies, GMR–GRIM (Fig. 3E and F; cf. D) and GMR–GAL4, UAS–G/RPR (Fig. 3H and I; cf. G).

Aph-1 is a third conserved protein that is required for γ-secretase activity. Mutations in *Drosophila aph-1* are not available. Therefore, to determine if loss of *aph-1* function suppressed the reporter small eye phenotype we generated flies that expressed an *aph-1* RNAi construct under GMR control (GMR-RNAi-*aph-1* flies). These flies were wild-type in appearance (data not shown). They acted as strong suppressors of the small eye phenotype of the reporter (Fig. 4B), but not that of the false positive reporters, GMR–GRIM (Fig. 4D) and GMR–GAL4, UAS–G/RPR (Fig. 4F). Thus, the γ-secretase reporter fly eye phenotype is sensitive to small changes (roughly 2-fold) in the levels of three known components or regulators of the γ-secretase complex, Psn, Nct and Aph-1. These observations argue that GMR–APP–GAL4/UAS–GRIM flies function as reporters of endogenous γ-secretase activity

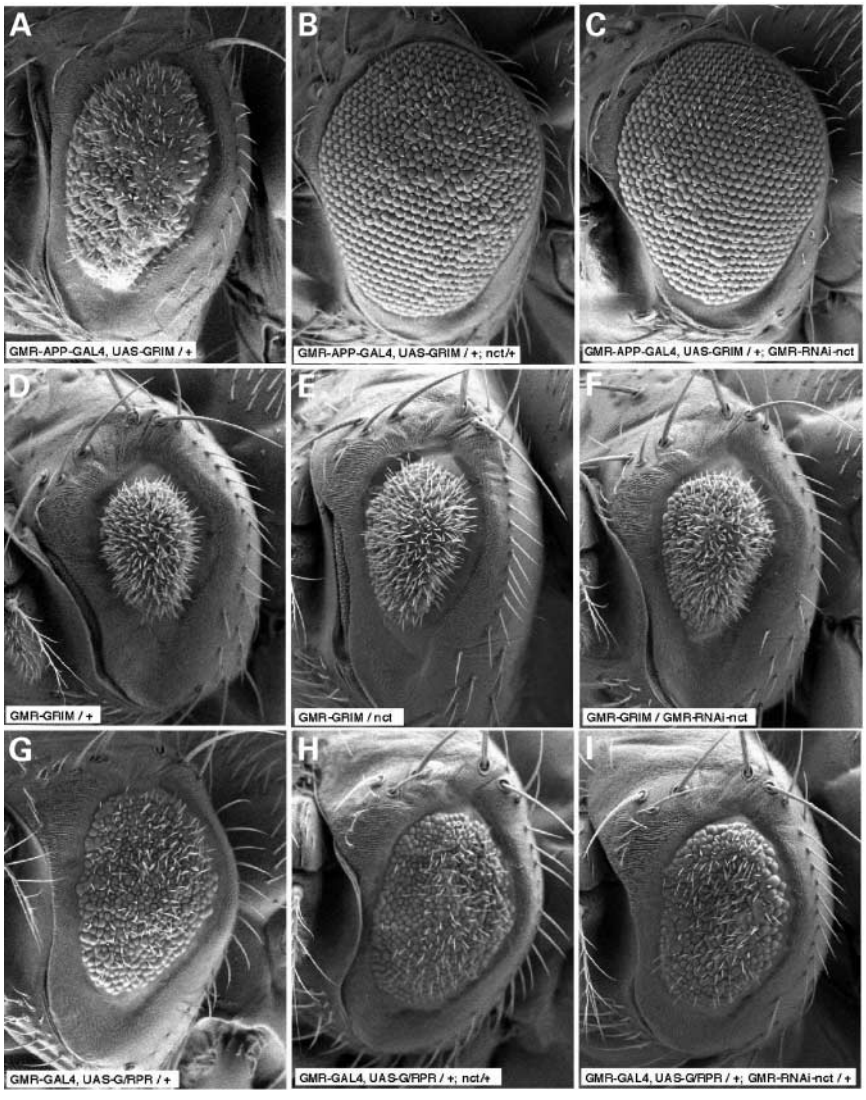

Figure 3. γ-Secretase reporter flies are sensitive to the levels of *nct*. Scanning electron micrographs of various genotypes are shown. The genotypes are as follows: (**A**) GMR–APP–GAL4, UAS–GRIM/+; (**B**) GMR–APP–GAL4, UAS–GRIM/+; nct^{J2}/+; (**C**) GMR–APP–GAL4, UAS–GRIM/+; GMR–RNAi-*nct*/+; (**D**) GMR–GRIM/+; (**E**) GMR–GRIM/+; nct^{J2}/+; (**F**) GMR–GRIM/+; GMR–RNAi-*nct*/+; (**G**) GMR–GAL4, UAS–G/RPR/+; (**H**) GMR–GAL4, UAS–G/RPR/+; nct^{J2}/+; (**I**) GMR–GAL4, UAS–G/RPR/+; GMR–RNAi-*nct*/+. (A) is identical to Figures 1E and 2A, (D) is identical to Figure 2D, and (G) is identical to Figure 2G as the crosses for Figures 1–3 were set up in parallel. Decreasing *nct* levels suppresses the GMR–APP–GAL4, UAS–GRIM-dependent small eye phenotype (B, C; cf. A), but not the small eye phenotypes associated with GMR-GRIM (E, F; cf. D) or GMR–GAL4–UASG/RPR (H, I; cf. G).

and that they constitute a sensitized background in which to screen for γ-secretase regulators and components.

The 23C1-3 region contains potential regulators of γ-secretase activity

We carried out a genetic screen for suppressors of APP γ-secretase activity in *Drosophila*. We screened the second chromosome using a publicly available 'deficiency kit' that contains 85 stocks that in total delete about 80% of this chromosome, which makes up about two-fifths of the *Drosophila* genome. Heterozygous deletion-bearing flies were crossed to the reporter flies and progeny bearing the deletion and reporter identified. These were then scored for suppression as compared to flies carrying the reporter alone. Several regions that contained strong suppressor activity were identified (Table 1). One of these, at 55A-55F, defined by Df(2R)1547, contains the *pen-2* gene (55C1-5) which encodes the fourth component of mammalian and *Drosophila* γ-secretase complex. A second strong suppressor region was identified at 23A1-C5, as defined by the deficiency Df(2L)90 (Fig. 5A and C). This region contains *aph-1* (23A6). Df(2L)3910 (23A6-B1) also removes *aph-1*. This deficiency acted as a reporter suppressor, but a much weaker one than Df(2L)90 (Fig. 5D). This suggested that the region deleted by Df(2L)90 contains additional γ-secretase suppressor loci. We used smaller deficiencies in the region to further map the suppressor activity. Df(2L)97 (23C3–D2), Df(2L)4954 (23D2–23E3) and

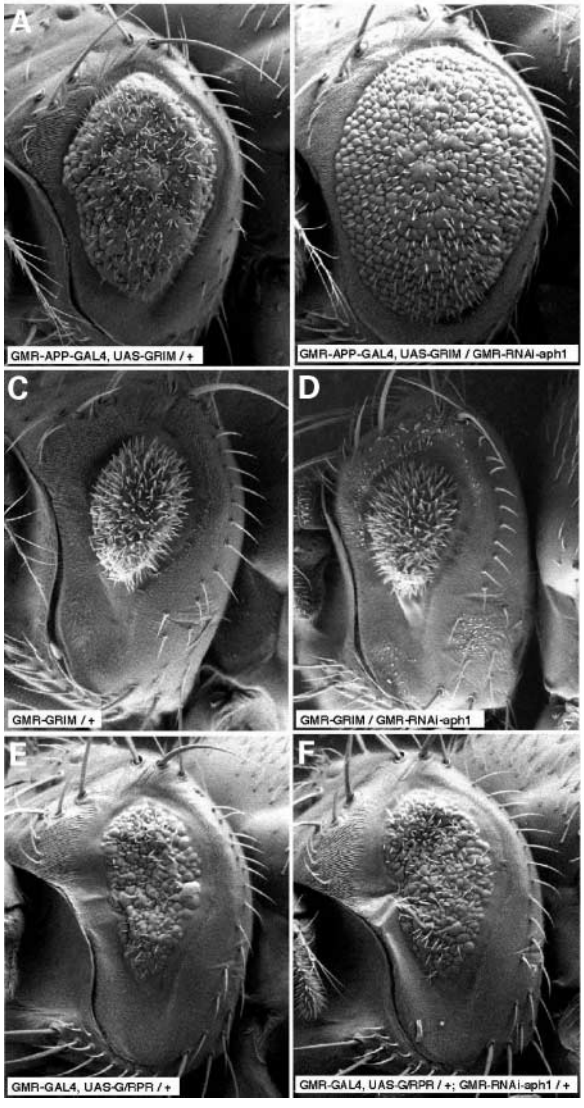

Figure 4. γ-Secretase reporter flies are sensitive to the levels of *aph-1*. Scanning electron micrographs of various genotypes are shown. The genotypes are as follows: (**A**) GMR–APP–GAL4, UAS–GRIM/+; (**B**) GMR–APP–GAL4, UAS–GRIM/+; GMR–RNAi-*aph-1*; (**C**) GMR–GRIM/+; (**D**) GMR–GRIM/+; GMR–RNAi–*aph-1*/+; (**E**) GMR–GAL4, UAS–G/RPR/+; (**F**) GMR–GAL4, UAS–G/RPR/+; GMR–RNAi–*aph-1*/+. Decreasing *aph-1* levels suppress the GMR–APP–GAL4, UAS–GRIM-dependent small eye phenotype (B; cf. A), but not the small eye phenotypes associated with GMR–GRIM (D; cf. C) or GMR–GAL4, UAS–G/RPR (F; cf. E).

Df(2L)6875 (23C5–23E2) did not suppress the reporter phenotype. However, Df(2L)1567, which deletes from 23C1–E2 and does not overlap with Df(2L)3910, acted as a very strong reporter suppressor (Fig. 5E). Df(2L)1567 did not suppress the small eye phenotypes of the two false positive reporter lines, GMR–GRIM and GMR–GAL4, UAS–G/RPR (Fig. 5G and I). Together, these observations place the suppressor in the 23C1–3 region. Based on current annotations from the Berkeley *Drosophila* Genome Project (BDGP), this region spans roughly 150 kb, and contains 10–15 annotated genes.

Table 1. Summary of the suppressors of reporter eye phenotype identified in second chromosome deficiencies. The map positions are based on information provided by Flybase at: http://flystocks.bio.indiana.edu/df-2.htm, updated on August 1, 2003. The strength of the suppression for each deficiency is listed, with the number of '+' indicating the strength (++++ being the strongest).

Deficiency	Map position	Effects on reporter	Strength
Df(2L)3548	21B8–C1; 21C8–D1	Suppression	++
Df(2L)90	23A1–2; 23C3–5	Suppression	++++
Df(2L)3910	23A6; 23B1	Suppression	++
Df(2L)1567	23C1–2; 23E1–2	Suppression	+++
Df(2R)1547	55A; 55F	Suppression	+++

The above observations are consistent with the hypothesis that this region contains a gene(s) that promote γ-secretase activity. However, we cannot exclude the possibility that the modifier functions instead to regulate the fate of the AICD, to which GAL4 is appended (see also the Discussion below). This possibility is not uninteresting. AICD has been shown to interact with a number of proteins, and evidence suggests that it can participate in multiple cell-biological processes (reviewed in 9,48). Once the responsible modifier gene is identified, cell culture-based assays using RNAi interference to follow AICD processing and fate in the absence of this gene (18) will distinguish these possibilities.

DISCUSSION

Mutations in three genes, Presenilin 1, Presenilin 2 and APP result in early-onset AD, while a polymorphism in APOE4 is associated with increased susceptibility to late onset AD. However, these genes are thought to account for as little as 30% of the genetic variance in AD susceptibility (5), indicating important regulators remain to be identified. Because the pathogenesis of AD results from increased deposition of Aβ, it seems likely that components or regulators of γ-secretase activity are among these.

Here we described the generation of flies that function as living reporters for APP γ-secretase activity. In these flies, γ-secretase-dependent cleavage of an APP-tethered transcription factor (a protease substrate), expressed specifically in the developing eye, leads to transcriptional activation of a cell death activator, Grim. Thus, the levels of endogenous γ-secretase activity are read out as a function of Grim-dependent cell death, which manifests itself as flies that have decreased eye size. The *Drosophila* eye is particularly useful as a screening system for regulators of γ-secretase activity since its cell population is dominated by neurons, and the eye is dispensable for viability and fertility. Importantly, the reporter small eye phenotype is easily quantifiable, and is regulated in a dose-dependent manner by modest changes (roughly 2-fold) in the levels of *psn*, *nct* and *aph-1*, which encode known components of the γ-secretase-containing complex. Thus, these flies constitute a very sensitized background in which to carry out genetic screens for other regulators of γ-secretase activity.

It is important to point out that it is not surprising that modest changes in the levels of γ-secretase components (2-fold reductions associated with heterozygosity) alter the reporter eye phenotype, but have no effect on γ-secretase-dependent

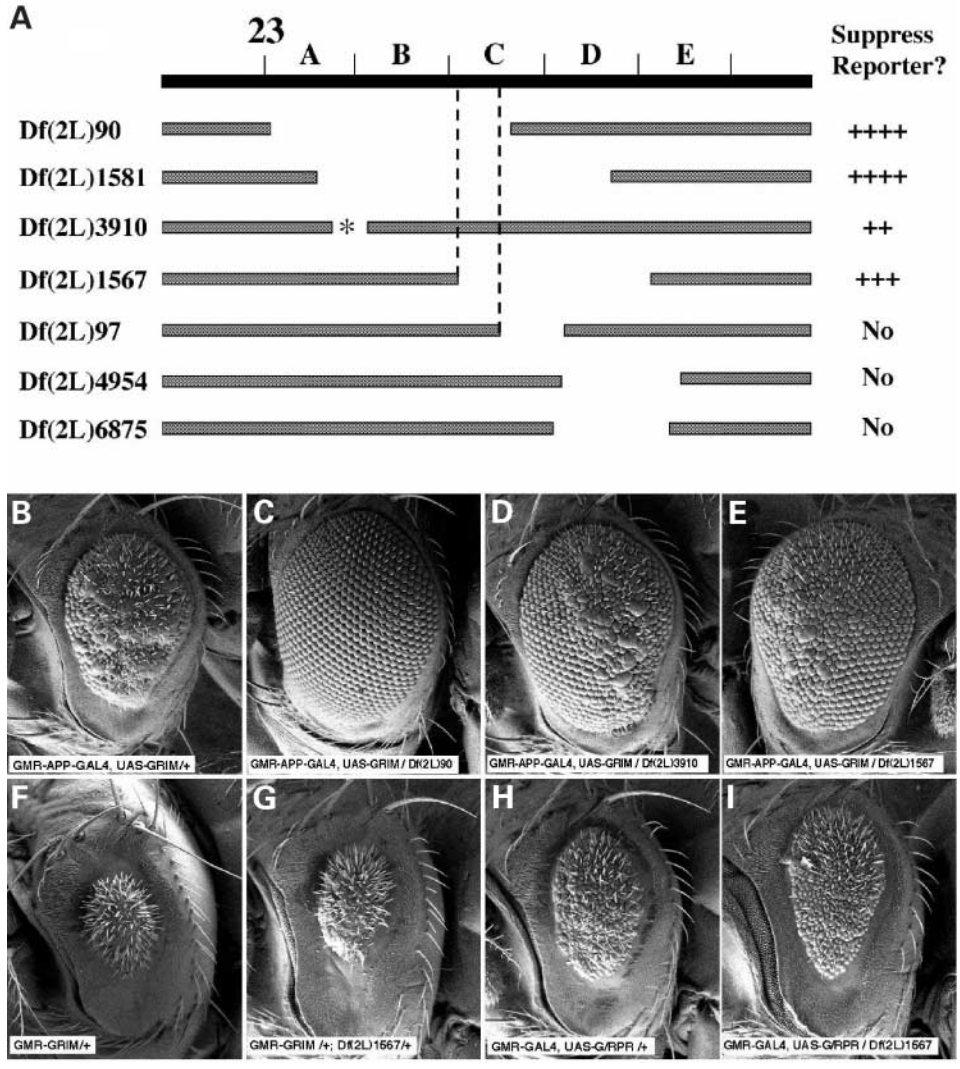

Figure 5. The 23C1-3 region contains a potential regulator of γ-secretase activity. (**A**) Schematic drawings of the chromosomal region 23A–E on the left arm of the second chromosome (2L) (the solid black bar). The deficiencies utilized in crosses are indicated on the left. Regions deleted are indicted by gaps in the bars. The location of Aph-1 is indicated by the asterisk. The strength of reporter suppression for each deficiency is indicated to the right, with (++++) being the strongest (see also C). The boundaries of the 23C1-3 region identified as containing a suppressor are indicated by the dashed vertical lines. (**B**–**E**) Representative scanning electron micrographs of fly eyes of different genotypes. Df(2L)90 results in very strong suppression of the GMR–APP–GAL4, UAS–GRIM small eye phenotype (C, cf. B). Df(2L)3910, a much smaller deficiency that deletes Aph-1, results in weaker suppression (D). Df(2L)1567, which does not include Aph-1, also results in suppression of the GMR–APP–GAL4, UAS–GRIM small eye phenotype (E). Df(2L)1567 does not suppress GMR–GRIM (**G**, cf. **F**) or GMR–GAL4, UAS–G/RPR (**I**, cf. **H**). Together these observations are consistent with the hypothesis that the 23C1–3 region contains a gene(s) that promotes γ-secretase activity.

developmental processes in the eye, such as *Notch* signaling. Our reporter is specifically designed to be sensitive to, and to be able to report on, levels of γ-secretase activity within the normal physiologic range. Thus, the reporter flies have been engineered to display a dominant eye phenotype in response to wild-type levels of γ-secretase activity. This is brought about in several different ways. The reporter protein contains an N-terminal signal sequence that is removed cotranslationally, generating a protein that has the N-terminus of β-secretase-cleaved APP. Therefore, in contrast to endogenous substrates, the reporter protein does not require α-or β-secretase cleavage in order to become a suitable substrate for γ-secretase. To the extent that these prior cleavage events are rate-limiting, the reporter will become the preferred substrate. In addition, in the reporter flies APP–GAL4 is expressed at high levels, probably much higher than those of endogenous γ-secretase substrates such as Notch. Therefore, it is likely that the reporter protein becomes, on a molar basis, the dominant substrate. Finally, the output of our reporter, cell death in the eye, requires GAL4-dependent transcriptional activation from the UAS–GRIM element. Five optimized GAL4 binding sites are present in this construct (49), thereby conferring a significant level of transcription-dependent amplification. In this sensitized background modest changes in the level of γ-secretase activity brought about by changes in the levels of its components would be expected to result in modification of the eye phenotype. In contrast to this engineered situation, heterozygosity for almost all genes in the fly genome (including *psn*, *nct*, and *aph-1*) does

not result in observable phenotypes during normal development because the residual 50% gene activity is sufficient to mediate normal signaling within and between cells. In other words, endogenous cellular signaling pathways such as *Notch* are normally buffered against phenotypic variability in response to modest changes in the activity of genes required for Notch processing (reviewed in 50).

The γ-secretase reporter flies were designed to identify factors that regulate γ-secretase cleavage. However, it is important to note that several other sorts of interesting modifiers may be identified. These include factors that influence the stability and subcellular localization of APP–GAL4, AICD and γ-secretase components. They could also include factors that regulate proteolysis of the reporter outside the γ-secretase target site. In particular, regulators of a recently identified ε-cleavage which is C-terminal to the γ-cleavage site and is also regulated by Psn (51–54) may also be detected. Many of these factors would be of potential relevance to the understanding of Alzheimer's disease mechanisms.

This reporter has several advantages for conducting genome-wide genetic screens. First, the small eye phenotype of the reporter flies acts as a readout of the endogenous, physiological levels of γ-secretase. This stands in contrast to the situation with many eye-based dominant modifier screens, in which the sensitized background results from hyperactivation of a signaling pathway (55). Thus modification of the reporter small eye phenotype by genetic manipulations or chemical compounds directly reflects modifications of the endogenous, physiologically relevant level of γ-secretase activity. Second, this reporter allows us to conduct screens that are focused specifically on identifying genes that regulate cleavage of APP. It makes no assumption about the role of *psn* in γ-secretase function, and thus constitutes an unbiased approach to identifying factors regulating APP processing. Again, this stands in contrast to screens focused on identifying genes that use *Notch* or *psn* loss-of-function phenotypes during development as a surrogate marker. Third, the sensitivity of the reporter allows us to conduct F1-dominant modifier screens. Since a large number of flies can be screened in a short period of time (F1, first generation), this sets the stage for a genome-wide search for mutations affecting γ-secretase. Moreover, disruption of one copy of almost all genes in the fly genome leads to flies that are viable and fertile. Thus, a function of these genes can be tested for even though homozygous deletion of the gene might be lethal earlier in development. Finally, the reporter flies are also useful for exploring the roles of candidate genes in γ-secretase regulation. In particular, the fact that levels of specific genes in the eyes of reporter flies can be selectively decreased using GMR-RNAi constructs, as illustrated here for *nct* and *aph-1*, provides a rapid method to test candidate γ-secretase regulators in a neural tissue *in vivo*.

MATERIALS AND METHODS

Constructs and transgenes

The APP–GAL4 coding sequence contains a cleavable signal sequence followed by a C-terminal fragment of human APP (56) that initiates immediately downstream of β-cleavage site, residue 671, and that also lacks a stop codon. GAL4 (57), was appended to the C-terminus of APP, generating APP–GAL4.

Residue D278 in *Drosophila* Psn corresponds to one of two evolutionarily conserved aspartate residues located in putative transmembrane domains 6 and 7 (29). Mutation of either of these conserved residues to alanine in mammalian PS1 creates a protein with dominant negative activity (58). We generated a dominant negative version of *Drosophila* Psn (Psn-D278A) from a *psn* cDNA, LD25307, obtained from Research Genetics, using the Stratagene QuikChange Site-Directed Mutagenesis Kit. Psn-D278A was introduced into the GMR vector, generating GMR–Psn–D278A.

GMR-driven *nct* and *aph-1* RNAi constructs were generated following a strategy that results in transcription of sense and antisense strands of a transgene expressed under GMR control (59). A similar strategy has been developed to drive double-stranded RNA production under UAS control (60). A fragment of *Drosophila nct* (coding region residues 291–696) or *aph-1* (coding region amino acid residues 29–174) was introduced between two GMR promoter sequences oriented in opposite directions in the vector PCasper, generating GMR-RNAi-*nct* and GMR-RNAi-*aph-1*, respectively. To ensure that the RNAi effect was specific to the transgene in question, transgenic flies containing GMR–RNAi–*nct*, which suppresses the small eye phenotype of the GMR–APP–GAL4, UAS–GRIM reporter, were crossed to flies with small eyes due to expression of GMR–GRIM, GMR–REAPER (RPR) or GMR–HID (44,61). The small eye phenotypes of these flies was not suppressed (data not shown).

Transformants were generated using standard microinjection (62) procedures in 0–1 h w^{1118} embryos. Multiple transgenic lines were obtained in each microinjections. For each experiment, at least four different insertion lines were tested.

Fly strains and genetics

Drosophila strains were raised on standard cornmeal-yeast agar medium at 25°C, except for the GMR–GAL4, UAS–G/REAPER stock, which was raised at room temperature (20°C). Alleles used in this study were as follows: psn^{C1} and psn^{C2} from Struhl (35); nct^{J2}, nct^{J1} and nct^{A7} from Fortini (33); GMR–G/REAPER (45) from Nambu; GMR–P35 (38) and GMR-DIAP1 (44).

Flies carrying one copy of GMR–APP–GAL4 and one copy of UAS–GRIM on the same chromosome were generated by meiotic recombination. The resulting GMR–APP–GAL4, UAS–GRIM chromosome was balanced over *CyO*. GMR–APP–GAL4, UAS–GRIM flies were crossed into different genetic backgrounds and the appropriate progeny compared with the progeny of GMR–APP–GAL4, UAS–GRIM outcrossed to w^{1118}, the background stock into which the GMR-driven transgenes were introduced. Similar crosses were carried out with the two false positive reporters, GMR–GRIM and GMR–GAL4, UAS–G/RPR. All crosses were carried out at 25°C, except those involving GMR–GAL4, UAS–G/RPR, which were carried out at room temperature (20°C).

A group of stocks (the deficiency kit) that together delete, one piece at a time, a large fraction of the second chromosome, was obtained from the Bloomington Stock Center. These lines were crossed to reporter flies. Reporter progeny carrying one copy of the deficiency chromosome were examined for

suppression of the reporter small eye phenotype. Those deficiencies that tested positive in this assay were further characterized in similar crosses to the false positive reporter strains GMR–GRIM and GMR–GAL4, UAS–G/RPR.

Scanning electron microscopy

For some samples flies were dehydrated in an ethanol series, incubated overnight in hexamethyldisilazane (Sigma), and air dried prior to being analyzed using a Hitachi scanning electron microscopy. For others, flies were quickly frozen at −80°C, then mounted and analyzed directly. Both methods of preparation gave identical results.

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