Developmental Cell, Vol. 3, 85–97, July, 2002, Copyright ©2002 by Cell Press

# *aph-1* and *pen-2* Are Required for Notch Pathway Signaling, γ-Secretase Cleavage of βAPP, and Presenilin Protein Accumulation

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## Summary

**Presenilins are components of the γ-secretase protein complex that mediates intramembranous cleavage of βAPP and Notch proteins. A *C. elegans* genetic screen revealed two genes, *aph-1* and *pen-2*, encoding multipass transmembrane proteins, that interact strongly with *sel-12*/presenilin and *aph-2*/nicastrin. Human *aph-1* and *pen-2* partially rescue the *C. elegans* mutant phenotypes, demonstrating conserved functions. The human genes must be provided together to rescue the mutant phenotypes, and the inclusion of presenilin-1 improves rescue, suggesting that they interact closely with each other and with presenilin. RNAi-mediated inactivation of *aph-1*, *pen-2*, or *nicastrin* in cultured *Drosophila* cells reduces γ-secretase cleavage of βAPP and Notch substrates and reduces the levels of processed presenilin. *aph-1* and *pen-2*, like nicastrin, are required for the activity and accumulation of γ-secretase.**

## Introduction

Presenilins are required for the intramembranous processing of certain type I transmembrane proteins, including the β-amyloid precursor protein (βAPP) and the LIN-12/GLP-1/Notch family of receptors (reviewed by Kopan and Goate, 2000; DeStrooper and Annaert, 2000; Esler and Wolfe, 2001). Processing of βAPP in the extracellular domain by β-secretase followed by γ-secretase cleavage within the transmembrane domain generates neurotoxic amyloid β (Aβ) peptides, the major constituents of senile plaques associated with Alzheimer's disease. Presenilins have been proposed to be the catalytic component of γ-secretase and contain two conserved, essential aspartate residues in adjacent transmembrane domains that may define a novel aspartyl protease active site (Wolfe et al., 1999). This model is supported by the finding that high-affinity γ-secretase inhibitors, including transition state analogs, selectively cross-link to presenilins (reviewed in Wolfe, 2001). Several observations, however, suggest that additional proteins are required for presenilin regulation and γ-secretase activity. First, presenilins are processed to an active, stable form by endoproteolysis, and the abundance of processed presenilin is tightly limited by unknown cellular factors (Ratovitski et al., 1997; Thinakaran et al., 1997). Second, presenilin and γ-secretase cofractionate as a detergent-sensitive, high-molecular weight protein complex (Yu et al., 1998; Li et al., 2000). Finally, one candidate member of the complex, nicastrin, has been identified based on its physical association with presenilins and with the γ-secretase substrates Notch and βAPP, as well as by its functional requirement in Notch signaling (Yu et al., 2000; Goutte et al., 2000; Chen et al., 2001).

Signaling by LIN-12/Notch receptors is dependent on presenilin function and mediates a wide range of developmental cell fate decisions in multicellular organisms (reviewed in Artavanis-Tsakonas et al., 1999). Ligand binding by Notch stimulates sequential cleavages of the receptor analogous to those that occur in βAPP. The first cleavage occurs at an extracellular site (S2) near the membrane and generates a membrane-tethered C-terminal domain. Subsequent cleavage at a site in the transmembrane domain (S3) releases the Notch intracellular domain (NICD) from the membrane, allowing it to translocate to the nucleus, where it can regulate transcription (Schroeter et al., 1998; Struhl and Adachi, 1998). S3 cleavage and NICD generation are inhibited by γ-secretase inhibitors and are abolished in PS-deficient mouse cells (Herreman et al., 2000; Zhang et al., 2000) and in *presenilin* (*psn*) mutant *Drosophila* embryos (Struhl and Greenwald, 1999; 2001). Loss-of-function presenilin mutations in mice (Donoviel et al., 1999, Herreman et al., 1999), *Drosophila* (Struhl and Greenwald, 1999; Ye and Fortini, 1999), and *C. elegans* (Li and Greenwald, 1997; Westlund et al., 1999) result in an array of LIN-12/Notch phenotypes that, for each organism, are indicative of an essential role for presenilins in LIN-12/Notch signaling. Notch S3 cleavage and βAPP γ site cleavages are thus mediated by a common presenilin-dependent γ-secretase activity, although there are some pharmacological differences in the enzyme activities that cleave the two substrates (Petit et al., 2001).

*C. elegans* contains two Notch receptors, *glp-1* and *lin-12*, that function in several well-characterized cell fate decisions (Kimble and Simpson, 1997). *glp-1* signaling in the early embryo is required at the 4-cell stage for specification of the ABp blastomere and proper body morphogenesis, at the 12-cell stage for specification of the anterior pharynx, and in the postembryonic germline for germ cell proliferation and fertility. *lin-12* is required for the postembryonic development of many tissues, including the somatic gonad and egg-laying structures (Greenwald et al., 1983). The two *C. elegans* presenilin genes, *sel-12* and *hop-1*, have mutant phenotypes that reflect partially redundant roles in Notch pathway signaling. *sel-12* mutants have a fully penetrant egg-laying defect and a partially penetrant protruding vulva defect

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(Levitan and Greenwald, 1995; Cinar et al., 2001). *hop-1* mutants have no overt phenotypes, but *sel-12; hop-1* double mutant progeny from *hop-1*$^{+/-}$*; sel-12*$^{-/-}$ mutant mothers (referred to here as *hop-1; sel-12* double mutants) display a *glp-1*-like germline proliferation defect that results in sterility and a *lin-12*-like cell fate defect that results in two anchor cells (Li and Greenwald, 1997; Westlund et al., 1999). The single pass transmembrane protein nicastrin has also been shown to have an essential, conserved function in Notch signaling. *C. elegans aph-2*/nicastrin mutants have an egg-laying-defective (Egl) phenotype, like that of *sel-12* mutants, and maternal-effect lethal defects, including an anterior pharynx-defective (Aph) phenotype, similar to those of *glp-1* mutants (Goutte et al., 2000; Levitan et al., 2001). Analysis of *nicastrin* mutant phenotypes in *Drosophila* has demonstrated an absolute requirement for nicastrin function in Notch signaling, specifically in the presenilin-mediated S3 cleavage step (Chung and Struhl, 2001; López-Schier and St Johnston, 2002; Hu et al., 2002).

Because of the dual function of γ-secretase in mediating both Notch and βAPP processing, genetic screens for Notch pathway components in invertebrates may reveal additional components and regulators of γ-secretase that will be relevant to human Alzheimer's disease. We carried out genetic screens in *C. elegans* and identified two *p*resenilin *en*hancer genes, *pen-1* and *pen-2,* that are essential for *glp-1* and *lin-12* signaling. *pen-1* is identical to the recently cloned gene *aph-1* (Goutte et al., 2002), and we use the *aph-1* name hereafter. We show that *aph-1* and *pen-2* are essential for γ-secretase activity and that this function is phylogenetically conserved.

## Results

### Isolation of Two New Presenilin Enhancers, *aph-1* and *pen-2*

In order to focus our genetic screens on the presenilin-dependent step in Notch signaling, we used sensitized strains that are partially compromised for presenilin function and screened for interacting genes. We mutagenized *sel-12(ep6)* animals and screened 128,000 genomes for mutants that display the *glp-1*-like sterility seen in *hop-1; sel-12* double mutants. The screen yielded 27 new alleles of *glp-1*, 1 allele of *lag-1* (a CSL nuclear transcription factor which mediates *lin-12* and *glp-1* signaling), and 2 alleles of *lag-2* (a *glp-1* ligand), each of which causes presenilin-independent *glp-1*-like sterile phenotypes. In addition, 15 mutants that cause *glp-1*-like sterility only in combination with a *sel-12* mutation were isolated. Eight of these mutants are alleles of *hop-1*, and the remaining mutants fall into two complementation groups of four and three alleles, respectively, corresponding to *aph-1* and a previously undescribed gene, *pen-2*. In separate screens for enhancers of a *hop-1* mutation or a partially suppressed *sel-12* mutation, we isolated two additional alleles of *aph-1* and three alleles of *pen-2,* as well as strong loss-of-function alleles of *aph-2*.

### *aph-1* and *pen-2* Encode Novel, Conserved Transmembrane Proteins

*aph-1* was meiotically mapped to a 50 kb interval between *unc-29* and *fog-3* on chromosome I, and *pen-2* was mapped to a 260 kb interval between *pha-1* and *dpy-18* on chromosome III. We cloned *aph-1* and *pen-2* by screening predicted genes in the intervals with RNA interference (RNAi), mutation detection, and transgenic rescue. *aph-1* corresponds to the predicted gene VF36H2L.1, and *pen-2* corresponds to T28D6.9 (Wormpep V71; Figure 1). Analysis of full-length cDNAs confirmed the intron/exon structures predicted for each gene.

*aph-1* encodes a predicted protein of 308 amino acids (Figure 1A). The six *aph-1* alleles introduce stop codons or a predicted splicing defect. They are likely to be null alleles, as they each confer equally strong phenotypes, and these are also shared with other loss-of-function alleles (Goutte et al., 2002) and with reduction of *aph-1* activity by RNAi (see below). *C. elegans* APH-1 defines a new protein family that includes two human members, referred to here as hAPH-1a and hAPH-1b. We identified corresponding mouse APH-1a and APH-1b sequences but only a single related sequence in zebrafish and *Drosophila*. Like *C. elegan*s APH-1, the predicted zebrafish and *Drosophila* proteins are equally related to hAPH-1a and hAPH-1b (Figure 1C). APH-1 proteins contain seven predicted transmembrane domains and share sequence similarity throughout their predicted TM domains and the short regions linking them. The first predicted transmembrane segment lies near the N terminus, but the high sequence conservation in this segment suggests that it is likely to be a transmembrane domain rather than a cleaved signal peptide.

*pen-2* encodes a predicted protein of 101 amino acids (Figures 1B and 1C). The six alleles all introduce stop codons and are likely to be null alleles, as they cause equally strong phenotypes that are also shared with *pen-2* RNAi. Thus, *pen-2*, like *aph-1*, acts positively to promote presenilin function. *C. elegans pen-2* defines a new protein family which includes single predicted *pen-2* orthologs in human, mouse, zebrafish, *Drosophila*, and *Arabidopsis*. The PEN-2 proteins show high sequence similarity throughout their lengths, contain two predicted transmembrane domains, and lack a signal peptide or any previously described protein motifs.

The *haph-1a*, *haph-1b*, and *hpen-2* sequences were mapped to the human genome using the Genebridge and G3 radiation hybrid panels. *Haph-1a* is linked with sequence marker NIB288 on chromosome 1 between the D1S1675 and D1S1595 microsatellite markers. This genomic region has been implicated as a locus associated with late onset Alzheimer's disease (Kehoe et al., 1999). *Haph-1b* is associated with three markers: MR14732, RH119833, and RH39521 on chromosome 15. *Hpen-2* is linked to three markers: RH91910, RH99245, and RH99629 on chromosome 19, near the *ApoE* gene. These chromosomal positions are supported by sequence alignments to the whole genome assemblies.

### *aph-1* and *pen-2* Are Required for *glp-1* Signaling

We found that, although they were isolated as *sel-12* enhancers, *aph-1* and *pen-2* mutations on their own confer a subset of well-characterized Notch pathway phenotypes. Heterozygous *aph-1*/+ and *pen-2*/+ animals produce homozygous mutant progeny that, like *sel-12* and *aph-2* mutants, are egg-laying defective (Egl). In addition, *aph-1* and *pen-2* mutants exhibit strict maternal-effect embryonic lethality (Mel), with two defects

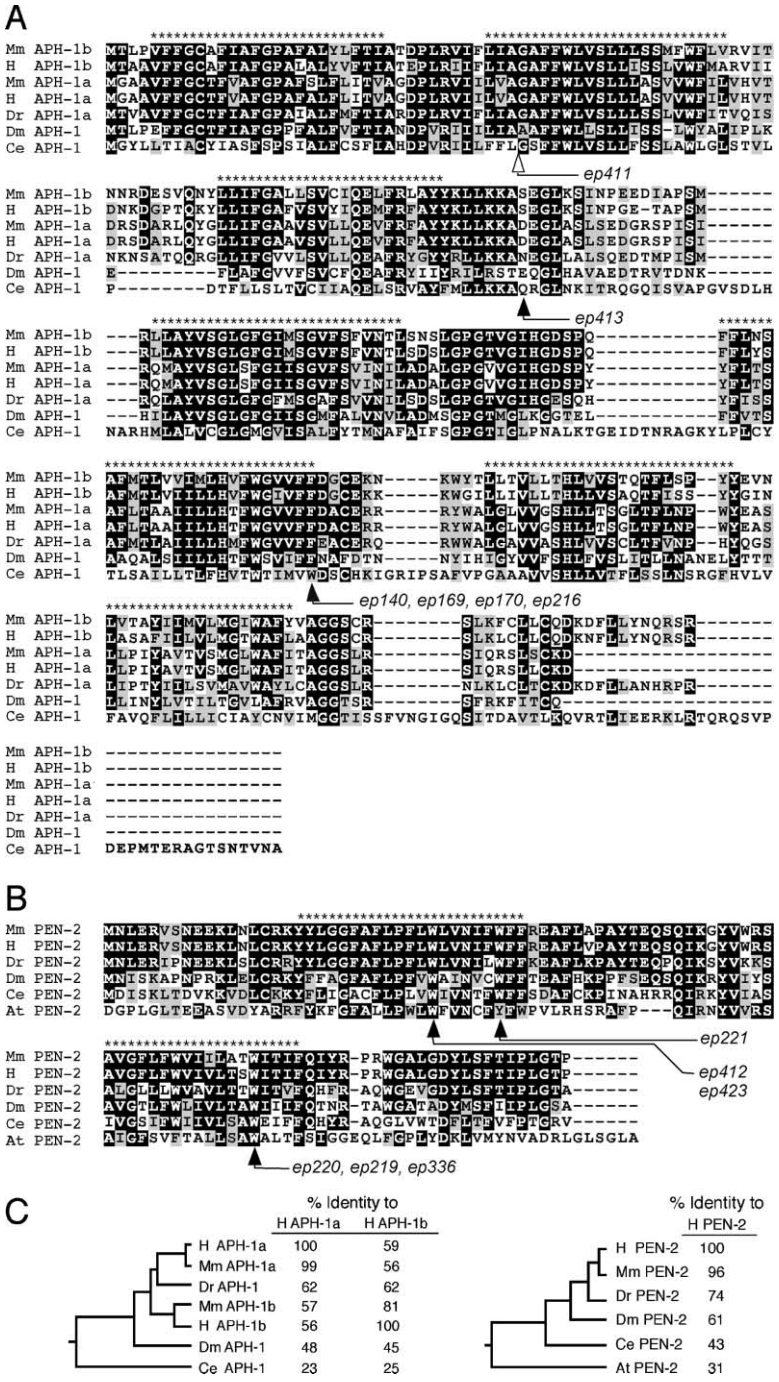


Figure 1. *aph-1* and *pen-2* Encode Conserved Transmembrane Proteins

(A) CLUSTALW alignment of predicted *C. elegans* (Ce) APH-1 (NP_492469.1) and related proteins. Sequences were predicted from cDNAs (GenBank accession numbers in parentheses), as follows: human (H) APH-1a (AAH08732); H APH-1b (AAH20905); mouse (Mm) APH-1a (AAH12406); Mm APH-1b (BAB22004); zebrafish (Dr) APH-1 (this work); *Drosophila* (Dm) APH-1 (AAF51212). Human and mouse *aph-1a* transcripts have alternative splice forms in which the last two amino acids (KD) of the sequence shown here are replaced by the sequence RRQEDSRVMVY SALRIPPED (human: AAH01230, mouse: AAH24111.1). Asterisks indicate approximate positions of transmembrane domains predicted by TOPPRED; the positions vary slightly for the different proteins. Arrowheads indicate locations of mutations: alleles *ep140*, *ep169*, and *ep170* mutate Trp191 UGG to UGA stop, and *ep216* is Trp191 UAG stop; *ep413* is Gln90 TAA stop. The open arrowhead indicates the site of a 48-nucleotide intron containing a 15-nucleotide deletion in *ep411*, which likely causes defects in splicing.

(B) CLUSTALW alignment of *C. elegans* PEN-2 (NP_499459.1) and related proteins. Sequences were predicted from cDNAs as follows: H PEN-2 (NP_060938); Mm PEN-2 (BAB25141); Dr PEN-2 (this work); Dm PEN-2 (this work); *Arabidopsis thaliana* (At) PEN-2 (CAC05457). The cDNA and genomic predictions for At PEN-2 indicate that it contains a 41-amino acid N terminus extension, which was trimmed for this alignment. Predicted transmembrane domains are overlain by asterisks, and the positions of *pen-2* stop mutations are indicated by arrowheads: *ep412* and *ep423* are Trp30 UGA stop, *ep221* is Trp36 UGA stop, *ep219* and *ep336* are Trp74 UGA stop, and *ep220* is Trp74 UAG stop.

(C) Phylogenetic relationships of the *aph-1* and *pen-2* gene sequences and percent amino acid identity.

indicative of loss of *glp-1* signaling (Figure 2A–2F). First, maternally deficient *aph-1* and *pen-2* embryos make most differentiated tissues, including the posterior pharynx, but fail to make anterior pharynx (Aph phenotype). Second, the *aph-1* and *pen-2* embryos arrest with severe morphogenesis defects. Hypodermal cells fail to enclose the embryo ventrally, and some tissues, including intestine, are found at the surface of the embryo. These embryonic defects are, at the level of our analysis, indistinguishable from those caused by removal of maternal *glp-1*, *aph-2*, or presenilin function.

To assess the contribution of maternal *aph-1* and *pen-2* gene activity to *glp-1/lin-12* signaling at later developmental stages, we took advantage of transgenes that only partially restore maternal activity. We obtained rescue of the *aph-1* maternal embryonic lethality using an *aph-1* genomic DNA fragment under the control of *sel-12* regulatory elements and of *pen-2* maternal embryonic lethality using genomic *pen-2* DNA under control of its endogenous regulatory elements. Progeny of *aph-1* or *pen-2* homozygotes that carry a rescuing transgene were fully rescued for embryonic lethality (Table 3). In general, transgene arrays are expressed poorly in the *C. elegans* germline, an effect enhanced by growth

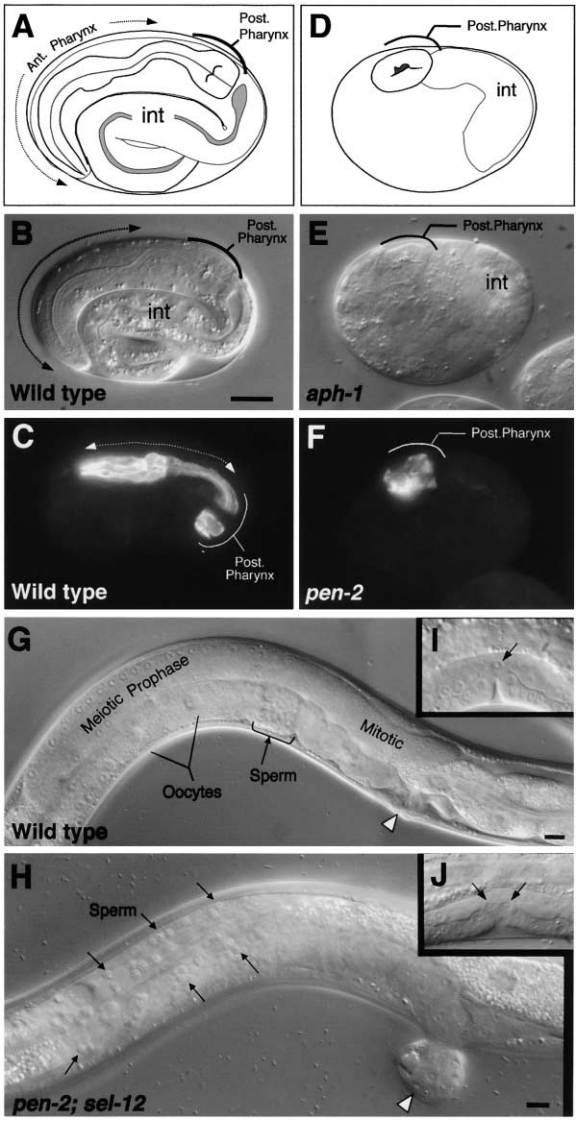


Figure 2. *aph-1* and *pen-2* Mutant Phenotypes

*aph-1* and *pen-2* confer *glp-1*-like maternal embryonic lethality and interact with a *sel-12* mutation to confer zygotic *glp-1* and *lin-12* pathway phenotypes. Line drawings (A and D) depict wild-type (B) and *aph-1(ep140)* (E) embryos visualized by Nomarski DIC optics. Wild-type (C) and *pen-2(ep220)* (F) embryos stained with the pharyngeal-specific antibody 3NB12. Anterior (ant) and posterior (post) lobes of the pharynx and intestine (int) are indicated.

(G) Portion of an adult wild-type hermaphrodite showing the vulva (arrowhead) and one U-shaped gonad arm that contains mitotic germ cells distally and oocytes and sperm proximally.

(H) Portion of an adult *pen-2(ep220); sel-12(ep6)* hermaphrodite showing protruding vulva (arrowhead) and a gonad arm that contains sperm (arrows) but no mitotic or undifferentiated germ cells.

(I) Wild-type L4 larva with one gonadal AC (arrow).

(J) *aph-1(ep140); sel-12(ep6)* L4 larva with two ACs (arrows). Bars equal 10 μm. Specific phenotypes illustrated here for *aph-1* or *pen-2* were identical for both *aph-1* and *pen-2* mutants.

at 15°C, and arrays are not transmitted to all progeny. We found that, when reared at 15°C, *aph-1* or *pen-2* homozygous mutant progeny that fail to inherit a transgene array grow to adulthood but show fully penetrant *glp-1*-like sterility (data not shown). These results suggest that transgene-supplied maternal *aph-1* and *pen-2* gene activity is sufficient for embryonic, but not for postembryonic, *glp-1* signaling. Thus, *aph-1* and *pen-2* are required for *glp-1*-mediated signaling, both in early embryonic patterning and in postembryonic germline proliferation.

### *aph-1* and *pen-2* Interact Genetically with Presenilins and *aph-2*/Nicastrin

*aph-1* and *pen-2* single mutants do not exhibit *glp-1* signaling defects in the germline due to the presence of maternal *aph-1* and *pen-2* gene activity. In contrast, *aph-1; sel-12* and *pen-2; sel-12* double mutants are sterile and exhibit *glp-1*-like defects in germ cell proliferation (Figure 2H). The *aph-1; sel-12* and *pen-2; sel-12* double mutants display two additional phenotypes that are characteristic of reduced *lin-12* signaling during development of the somatic gonad and vulva. First, they contain two anchor cells (2 AC phenotype; Figures 2I and 2J), and, second, they display a protruding vulva (Pvl) phenotype that, by both expressivity and penetrance, is stronger than that of *sel-12* mutants (Figure 2H). Thus, removal of *aph-1* or *pen-2* zygotic activity in *sel-12* mutants results in a loss of both *glp-1*- and *lin-12*-mediated signaling events. These specific phenotypic interactions provide genetic evidence that *aph-1* and *pen-2* act in the same process as presenilins.

We next analyzed the phenotypes of double mutants that lacked combinations of *aph-1*, *pen-2*, and *aph-2* gene function (Table 1). *aph-1*; *pen-2* double mutants made with chromosomal mutations or RNAi did not reveal any additive effects and resemble the single mutants in all respects. No interaction was observed between *pen-2* and the strong *aph-2* chromosomal allele *ep335* in a *hop-1 aph-2; pen-2* triple mutant combination (an *aph-1 aph-2* double mutant was not constructed). However, RNAi of *aph-2,* which should deplete maternal as well as zygotic *aph-2* RNA, interacts strongly with *aph-1* or *pen-2* to give highly penetrant *glp-1*-like sterility. Thus, *aph-1* and *pen-2* interact strongly with *aph-2* but show no comparable interaction with one another.

We also observed differences between *aph-1* and *pen-2* versus *aph-2* in interactions with the presenilins (Table 1). First, *aph-2* hypomorphic alleles (Levitan et al., 2001), strong alleles *(ep335)*, or RNAi in combination with *sel-12* never exhibits the *glp-1*-like sterility seen in *aph-1; sel-12* or *pen-2; sel-12* double mutants, although all of the double mutants share the *lin-12*-like 2 AC phenotype. Second, *aph-2(RNAi)*, but not *aph-1* or *pen-2* mutants or RNAi, in combination with *hop-1,* confers penetrant *glp-1*-like sterility. Therefore, *aph-2* interacts more strongly with *hop-1* loss than *sel-12* loss in the germline, whereas *aph-1* and *pen-2* interact more strongly with *sel-12* loss. These different genetic interactions may reflect different underlying biochemical roles between the *aph-1* and *pen-2* genes versus *aph-2*. Alternatively, they may reflect differences in gene expression, perdurance of maternal gene products, or gene-specific differences in sensitivity to RNAi. Although these genetic data do not distinguish these possibilities, the overlapping specific interaction phenotypes indicate that *aph-1*, *pen-2*, and *aph-2* all function in concert with presenilins in Notch pathway signaling.

Table 1. *aph-1* and *pen-2* Genetic Interaction Phenotypes

| Strain | Genotype[a] | Percentage of Animals with *glp-1*-like Sterility[b] | Other Penetrant Phenotypes[c] |
|---|---|---|---|
| a | *aph-1* | 0 | Egl, Mel/Aph |
| b | *aph-1(RNAi)* | 10 | Mel/Aph |
| c | *pen-2* | 0 | Egl, Mel/Aph |
| d | *pen-2(RNAi)* | 0 | Mel/Aph |
| e | *aph-2* | 0 | Egl, Mel/Aph |
| f | *aph-2(RNAi)* | 0 | Mel/Aph |
| g | *sel-12* | 0 | Egl, Pvl (37%) |
| h | *hop-1* | 0 | — |
| i | *hop-1; sel-12* | 100 | 2 AC, Pvl (100%) |
| j | *aph-1; sel-12* | 100 | 2 AC, Pvl (100%) |
| k | *pen-2; sel-12* | 100 | 2 AC, Pvl (100%) |
| l | *aph-2; sel-12* | 0 | 2 AC, Egl, Pvl (100%) |
| m | *aph-2(RNAi); sel-12* | 0 | Egl, Mel/Aph |
| n | *hop-1(ep168 or ep90) aph-1* | 0 | Egl, Mel/Aph |
| o | *hop-1(ep168); pen-2* | 0 | Egl, Mel/Aph |
| p | *hop-1; pen-2(RNAi)* | 0 | Mel/Aph |
| q | *hop-1(ep171) aph-2* | 0 | Mel/Aph |
| r | *hop-1(ep168) aph-2(RNAi)* | 37 | Mel/Aph |
| s | *hop-1(ep171) aph-2(RNAi)* | 48 | Mel/Aph |
| t | *aph-1; pen-2* | 0 | Egl, Mel/Aph |
| u | *aph-1; pen-2(RNAi)* | 0 | Egl, Mel/Aph |
| v | *aph-1 aph-2(RNAi)* | 43 | Egl, Mel/Aph |
| w | *aph-2(RNAi); pen-2* | 54 | Egl, Mel/Aph |
| x | *hop-1 aph-2; pen-2* | 0 | Egl, Mel/Aph |

[a] Adult hermaphrodites of the indicated genotype. Alleles used in these tests were: *aph-1(ep140)*, *pen-2(ep220)*, *sel-12(ep6)*, and *aph-2(ep335)*. *aph-2(ep335)*, which carries a R51 stop mutation, confers 100% penetrant Egl and Mel/Aph phenotypes. Based on penetrance of the Egl phenotype and expressivity of the Mel/Aph phenotype, *aph-2(ep335)* is a stronger allele than the transposon-insertion *aph-2* alleles characterized by Goutte et al. (2000). Complete genotypes were as follows: a, *unc-29 aph-1*; b, *aph-1(RNAi)*; c, *pen-2* and *pen-2 dpy-18*; d, *pen-2(RNAi)*; e, *unc-29 aph-2*; f, *aph-2(RNAi)* and *unc74 aph-2(RNAi)*; g, *sel-12*; h, *hop-1(ep90*, *ep168*, or *ep171)*; i, *hop-1(ep168* or *ep171) unc-74*; *sel-12*; j, *unc-29 aph-1*; *sel-12*; k, *pen-2 dpy-18*; *sel-12*; l, *unc-29 aph-2*; *sel-12*; m, *aph-2(RNAi)*; *sel-12*; n, *hop-1(ep90* or *ep168) unc-74 aph-1*; o, *hop-1(ep168) unc-74*; *pen-2*; p, *hop-1(ep171) unc-74*; *pen-2(RNAi)*; q, *hop-1(ep171) unc-74 aph-2*; r, *hop-1(ep168) unc-74 aph-2(RNAi)*; s, *hop-1(ep171) unc-74 aph-2(RNAi)*; t, *unc-29 aph-1*; *pen-2*; u, *unc-29 aph-1*; *pen-2(RNAi)*; v, *unc-29 aph-1 aph-2(RNAi)*; w, *aph-2(RNAi)*; *pen-2 dpy-18*; x, *hop-1(ep171) unc-74 aph-2*; *pen-2 dpy-18*. All *sel-12* genotypes are self-progeny of homozygous *sel-12* hermaphrodites. The various *pen-2*, *aph-1*, or *aph-2* mutant genotypes are progeny of balanced heterozygous stocks (e.g., *aph-1*; *pen-2* worms were obtained from *unc-29 aph-1/hT2*; *pen-2/hT2* stocks). For RNAi, dsRNA was delivered by germline microinjection, and the F1 embryos, larvae, and adults were phenotyped.

[b] In the case of RNAi experiments, the percentage of viable F1 progeny that were sterile is indicated.

[c] All genotypes with an "Egl" entry have 100% penetrant Egl phenotypes. A "Pvl" entry indicates a strong protruding vulva phenotype. Homozygous *pen-2*, *aph-1*, and *aph-2(ep335)* hermaphrodites produce only inviable Aph embryos (designated with "Mel/Aph" entry). RNAi by microinjection produced Mel/Aph embryos with incomplete penetrance.

### *aph-1* and *pen-2* Act at or Upstream of LIN-12/Notch S3 Cleavage

Activated Notch receptors, such as the *lin-12(n950)* allele, that are membrane anchored and ligand independent, cause constitutive signaling that is presenilin dependent (Levitan and Greenwald, 1995), whereas cytosolic Notch intracellular domains, such as *lin-12(intra),* elicit constitutive signaling that is independent of presenilin function (Struhl and Adachi, 1998; Levitan and Greenwald, 1998; De Strooper et al., 1999; Struhl and Greenwald, 1999). We used this genetic assay to ask whether *aph-1* and *pen-2*, like presenilin and nicastrin, act at or upstream of S3 cleavage.

*lin-12(n950)* confers highly penetrant gain-of-function (gf) phenotypes that include formation of ectopic pseudovulvae (Muv phenotype) and absence of an anchor cell (0 AC phenotype). *sel-12* mutations partially suppress both *lin-12(n950)* phenotypes (Levitan and Greenwald, 1995) (Table 2), with residual *lin-12(n950)* activity likely dependent on *hop-1* activity. We find that *aph-1(ep140)* partially suppresses the *lin-12(n950)* Muv and 0 AC phenotypes (Table 2). This suppression is incomplete, likely due to the presence of maternal *aph-1* activity. *lin-12(gf) pen-2* double mutants were constructed using *lin-12(n302)*, a weaker gain-of-function allele that confers a penetrant 0 AC phenotype and a weak Muv phenotype. *pen-2(ep220)* partially suppresses the *lin-12(n302)* 0 AC phenotype (Table 2; Muv suppression was not scored). We constructed a transgene expressing a *lin-12(intra)* fragment similar to one previously described (Levitan and Greenwald, 1998), which causes moderate Muv and 0 AC phenotypes. We found that, in contrast to their ability to suppress *lin-12(gf)* alleles, *aph-1* and *pen-2* do not suppress *lin-12(intra)* phenotypes (Table 2), indicating that *aph-1* and *pen-2* act at or upstream of presenilin-dependent S3 cleavage.

### *pen-2* Is Broadly Expressed and Is Localized in Intracellular Membrane Compartments

To examine the *pen-2* expression pattern, we fused green fluorescent protein (GFP) in-frame with *pen-2* coding sequences in the context of a *pen-2* genomic rescuing fragment. Because we did not obtain rescue with *aph-1* under its endogenous regulatory elements, we

Table 2. *aph-1* and *pen-2* Mutations Suppress *lin-12(gf)* but Not *lin-12(intra)* Phenotypes

| Strain | Genotype[a] | Median No. Vulval Structures/Animal (n) | Percentage of Animals with 0 AC Phenotype (n) |
|---|---|---|---|
| a | N2 (wild-type) | 1 | 0 |
| b | *sel-12* | 1 (>100) | 0 |
| c | *aph-1* | 1 (>100) | 0 |
| d | *lin-12(n950)* | 5.9 (52) | 100 (30) |
| e | *lin-12(n950); sel-12* | 3.8 (60) | ND[b] |
| f | *aph-1; lin-12(n950)* | 3.6 (64) | 76 (41) |
| g | *lin-12(intra)* | 3.2 (38) | 64 (42) |
| h | *aph-1; lin-12(intra)* | 3.0 (34) | 59 (32) |
| i | *pen-2* | 1 (>100) | 0 (32) |
| j | *lin-12(n302)* | ND[b] | 96 (20) |
| k | *lin-12(n302) pen-2* | ND[b] | 38 (43) |
| l | *lin-12(intra)* | ND[b] | 52 (62) |
| m | *pen-2, lin-12(intra)* | ND[b] | 58 (28) |

[a] Complete genotypes were as follows: a, N2 wild-type; b, *sel-12(ep6)*; c, *unc-29 aph-1(ep140)*; d, *lin-12(n950)*; e, *lin-12(n950)*; *sel-12(ep6)*; f, *unc-29 aph-1(ep140)*; *lin-12(n950)*; g, *epEx179[lin-12(intra)*; *rol-6(su1006)]*; h, *unc-29 aph-1(ep140)*; *epEx179[lin-12(intra)*; *rol-6(su1006)]*; i, *pen-2(ep220) dpy-18*; j, *lin-12(n302) dpy-18*; k, *lin-12(n302) pen-2(ep220) dpy-18*; l, *dpy-18*; *epEx180[lin-12(intra)*; *rol-6(su1006)]*; m, *pen-2(ep220) dpy-18*; *epEx180[lin-12(intra)*; *rol-6(su1006)]*.

[b] ND, not determined. Because the *lin-12(n302)* Muv phenotype is much weaker than the *lin-12(intra)* phenotype, suppression of the two by *pen-2* cannot be directly compared.

did not analyze *aph-1*::GFP expression. Transgenes expressing *pen-2* with GFP inserted at the N terminus or within the predicted loop between the two transmembrane domains efficiently rescue the Egl and Mel phenotypes of *pen-2(ep220)*, indicating that both constructs encode functional PEN-2 proteins. In contrast, GFP fused to the C terminus induces a dominant-negative Mel/Aph phenotype in an otherwise wild-type background, similar to the *pen-2* loss of function phenotype, indicating an important function for *pen-2* C-terminal sequences. The N-terminal and loop *pen-2*::GFP fusions gave strong GFP fluorescence in most somatic tissues beginning at the ~100-cell stage of development, including neurons, muscle, intestine, and the developing vulva (Figure 3). *pen-2*::GFP fluorescence was not detectable in early embryos, even though the *pen-2*::GFP transgenes rescue early *pen-2* function. In later stages, most cells show strong perinuclear localization of GFP and a nonuniform cytosolic signal, which likely represents localization to intracellular membranes, such as endoplasmic reticulum (ER) and Golgi. A comparable pattern of broad tissue distribution and subcellular membrane localization has been reported for a *sel-12*::GFP fusion (Levitan and Greenwald, 1998). We examined PEN-2::GFP expression in *aph-1, aph-2*, and *hop-1; sel-12* mutants and observed no significant changes in expression levels or localization, although we have not determined whether transgenic PEN-2::GFP accurately reflects the expression pattern of endogenous PEN-2 or whether overexpression of the transgene masks changes in a functional pool of the protein.

We also examined human *aph-1* and *pen-2* gene transcript distribution by Northern blotting and RT/PCR and found that *haph-1a*, *haph-1b*, and *hpen-2* are expressed in all 15 tissues tested, including brain (data not shown). Thus, the *haph-1* and *hpen-2* genes are broadly expressed in human tissues, and *pen-2* is broadly expressed in *C. elegans*. Intracellular membrane localization of the PEN-2::GFP fusion is consistent with its prediction as a transmembrane protein and with a functional interaction with presenilin in membrane compartments.

### Corequirement for Human *aph-1* and *pen-2* for Rescue of *C. elegans aph-1* and *pen-2* Mutants

Human *PS1* and *PS2* transgenes confer strong rescue of *C. elegans sel-12* mutant phenotypes when expressed as cDNAs under the control of *sel-12* promoter elements (Levitan et al., 1996). Similarly, human *nicastrin* transgenes can partially rescue the *C. elegans aph-2* mutant phenotypes (Levitan et al., 2001). We used similar cDNA transgenes regulated by the *sel-12* promoter to compare the human and *C. elegans aph-1* and *pen-2*

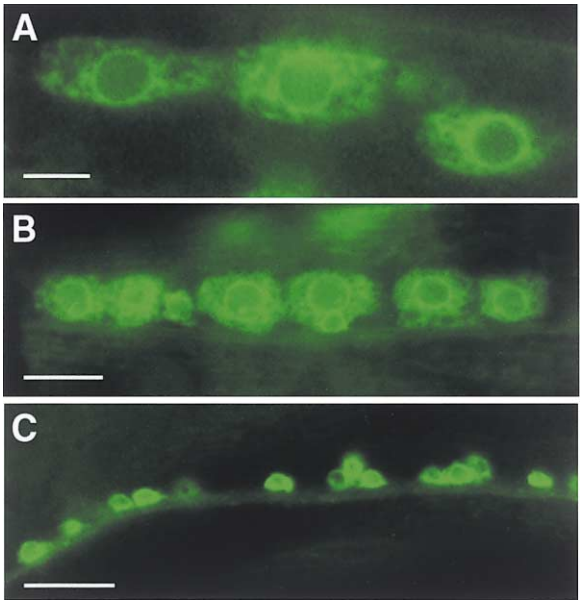


Figure 3. *pen-2*::GFP Expression

*pen-2*::GFP expression is observed in most somatic *C. elegans* tissues. Representative PEN-2::GFP (loop) localization in body-wall muscle cells (A), vulval precursor cells (B), and ventral cord motorneurons (C). Perinuclear and patchy cytoplasmic signal consistent with internal membrane localization is particularly visible in cells with a large cytoplasmic volume. Bars equal 10 μM.

Table 3. Transgenic Rescue of *aph-1* and *pen-2* Mutants by *C. elegans* and Human DNAs

| Injected DNA(s) | | | | Strain Injected[a] | Concentration of Injected DNA (μg/ml)[b] | No. Stable Lines (No. Injected)[c] | No. Lines Rescued to Egl(+)[d] |
|---|---|---|---|---|---|---|---|
| *C. elegans* DNAs | | | | | | | |
| *pen-2* genomic | | | | *pen-2* | 20 | 10 (42) | 10/10 |
| *pen-2* cDNA (*sel-12* pr) | | | | *pen-2* | 18 | 5 (16) | 5/5 |
| *aph-1* genomic | | | | *aph-1* | 20 | 18 (46) | 0/18 |
| *aph-1* genomic (*sel-12* pr) | | | | *aph-1* | 30 | 5 (26) | 3/3 |
| *aph-1* cDNA (*sel-12* pr) | | | | *aph-1* | 18 | 4 (16) | 4/4 |
| Human *pen-2*, *aph-1*, and *PS1* cDNAs | | | | | | | |
| *pen-2* | *aph-1a* | *aph-1b* | *PS1* | | | | |
| + | | | | *pen-2* | 15 or 25 | 0 (76) | — |
| + | | | | *pen-2* | 5 | 4 (48) | 0/4 |
| | + | | | *aph-1* | 90 | 0 (16) | — |
| | + | | | *aph-1* | 15 | 3 (36) | 0/3 |
| | | + | | *aph-1* | 5, 15, or 90 | 0 (73) | — |
| | | + | | *aph-1* | 0.5 | 2 (24) | 0/2 |
| + | + | + | + | *pen-2* | 25 | 4 (61) | 4/4 |
| + | + | + | + | *aph-1* | 25 | 3 (103) | 3/3 |
| | + | + | + | *pen-2* | 25 | 5 (80) | 0/4 |
| + | + | | + | *pen-2* | 25 | 3 (77) | 3/3 |
| + | | + | + | *pen-2* | 25 | 1 (88) | 1/1 |
| + | + | + | | *pen-2* | 25 | 3 (72) | 2/2 |
| + | + | | | *pen-2* | 25 | 0 (75) | — |
| + | | + | | *pen-2* | 25 | 0 (85) | — |
| + | | | + | *pen-2* | 25 | 0 (78) | — |
| | | | + | *pen-2* | 25 | 16 (40) | 0/1 |

[a] Transgenic extrachromosomal arrays were generated in *unc-29 aph-1(ep140)/fog-3* or *pen-2(ep220) dpy-18/unc-119* hermaphrodites.
[b] Concentration per DNA injected. In experiments with multiple cDNAs, each cDNA was injected at the same indicated concentration, together with 25 μg/ml of transformation marker Y47D3B.7::GFP. The transformation marker *rol-6(su1006)* (pRF4) at 100–150 μg/ml was used with genomic rescue fragments.
[c] Number of independent extrachromosomal array lines. Lines transgenic for *C. elegans aph-1*, *pen-2*, or human *PS1* DNAs were obtained at normal frequencies. In contrast, lines transgenic for one or more of the human *aph-1* or *pen-2* cDNAs were obtained at a reduced frequency or not at all. In cases where no lines were obtained, progeny of the injected animals that expressed the GFP cotransformation marker were inviable and arrested early in embryonic development. These embryos did not exhibit an Aph phenotype as would be expected for a dominant-negative effect on *aph-1* or *pen-2* gene function. In cases where viable lines were obtained, inviable embryos were also observed at low frequency.
[d] Number of lines with full or partial rescuing activity out of the number of lines scored (not all lines were scored for some transgenes). See Figure 4 for quantitation of rescue.

genes for their ability to rescue *aph-1* and *pen-2* mutant phenotypes. Transgene lines expressing the *C. elegans aph-1* or *pen-2* cDNAs were readily obtained and fully rescued the *aph-1* or *pen-2* Egl phenotypes (Table 3). Although the *aph-1* or *pen-2* Mel phenotypes were not rescued in these lines, likely due to inefficient transgenic promoter activity in the germline, rescue of the Egl phenotypes provides a context for assaying rescue by the human *aph-1* and *pen-2* genes. Rescue was quantitated by the fraction of transgenic worms which were $Egl^+$ and by the number of eggs laid by each rescued $Egl^+$ animal (Figure 4). Injection of *haph-1a*, *haph-1b*, or *hpen-2* cDNAs at concentrations comparable to those used for the *C. elegans aph-1* and *pen-2* gene cDNAs (25–90 μg/ml) failed to produce transgene lines, but injection at lower concentrations (0.2–15 μg/ml) yielded viable lines at low frequencies. Although RT-PCR analysis showed that the transgenes expressed RNA (data not shown), none of the lines were rescued for the *aph-1* or *pen-2* Egl phenotypes. Individual human *aph-1* and *pen-2* cDNAs are thus unable to substitute for the *C. elegans aph-1* and *pen-2* genes under these conditions.

Because presenilin functions as part of a protein complex, we next tested whether coexpression of human *aph-1*, *pen-2*, and *presenilin-1 (PS1)* genes would rescue the *aph-1* or *pen-2* mutants. We found that a mixture containing *haph-1a*, *haph-1b*, *hpen-2*, and *PS1* injected at 25 μg/ml/cDNA generated transgenic lines that partially rescued the *aph-1* and *pen-2* Egl phenotypes. The human gene mixture, in contrast to the individual genes, is able to functionally rescue both the *aph-1* and *pen-2* mutants.

We next examined the minimal requirements for rescue of *pen-2* mutants by the human gene mixture (Table 3; Figure 4). Omission of *hpen-2* from the rescuing gene mixture abrogated rescue, demonstrating that *hpen-2* can substitute for *C. elegans pen-2* function when provided in the context of *haph-1* and *PS1*. Either *haph-1a* or *haph-1b* could be omitted without reduction of rescuing activity, demonstrating that the two human *aph-1* isoforms are functionally equivalent in this assay when provided in the context of *hpen-2* and *PS1*. When both *haph-1a* and *haph-1b* were omitted, no transgene lines were recovered, suggesting that at least one of the *haph-1* genes must be provided in the context of *hpen-2* and *PS1* for rescue of *pen-2*. Finally, omission of *PS1* from the mixture reduced, but did not eliminate, rescue. Thus, a combination of *hpen-2*, *haph-1a*, and *haph-1b*

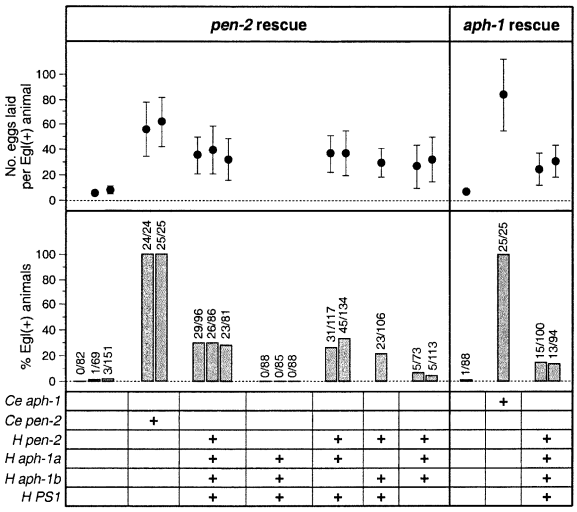


Figure 4. Rescue of *aph-1* and *pen-2* Egl Phenotypes by Human *aph-1* and *pen-2* cDNAs

Transgenic lines bearing extrachromosomal arrays of cDNAs indicated in the bottom panel were scored for rescue of the Egl phenotype of *pen-2 dpy-18* or *unc-29 aph-1* homozygotes. Control *pen-2* and *aph-1* animals are nontransgenic lines established in parallel with transgenic lines after injection. The percentage of worms that laid ≥5 eggs is plotted as a histogram (middle panel), with each bar representing a different transgenic line. Numbers above each bar are number of $Egl^+$ animals per number of animals scored. The mean number of eggs laid per $Egl^+$ animal is plotted in the top panel, plus or minus standard deviation.

are sufficient for rescue of *pen-2*, and *PS1* contributes substantially to the overall rescuing activity. We did not obtain transgene lines with *pen-2* and a single *haph-1a* or *haph-1b* isoform in the absence of *PS1*, providing further evidence that *hpen-2*, *haph-1*, and *PS1* interactions all contribute to a functional rescuing activity. The corequirement for *hpen-2* and *haph-1* is consistent with our genetic results showing similar, nonadditive functions for *aph-1* and *pen-2* and suggests that they cooperate closely in the same process to promote presenilin activity.

### *aph-1* and *pen-2*, Like Nicastrin, Are Required for γ-Secretase Activity and Presenilin Protein Accumulation

To study the roles of *aph-1* and *pen-2* in APP and Notch cleavage, we developed γ-secretase activity assays in *Drosophila* tissue culture cells (Figure 5D), similar to nuclear access assays that accurately report presenilin-dependent processing in *Drosophila* (Struhl and Adachi, 1998; Struhl and Greenwald, 1999). To confirm that the *Drosophila* cell assays accurately measure γ-secretase-like activity, we first tested Dmel2 cells and developing flies for sensitivity to previously described γ-secretase inhibitors. When applied to *Drosophila* embryos and larvae at intermediate concentrations, compound E (Seiffert et al., 2000) causes adult wing notching (Figures 5A and 5B) and rough eye phenotypes characteristic of *Notch* mutants and a presenilin hypomorphic mutant (data not shown). Compound E also induces *glp-1*-like sterility when applied to developing *hop-1* mutant *C. elegans* (Figure 5C). These results demonstrate that this selective γ-secretase inhibitor interferes with Notch signaling in vivo in *Drosophila* and *C. elegans*. In Dmel2 cell assays, compound E inhibited membrane-tethered APP C99-GV reporter activity by 2- to 3-fold but had no effect on the non-membrane-bound APP C59-GV reporter activity (Figure 5E). Similar reporter inhibition was obtained with two additional γ-secretase inhibitors (data not shown). Membrane-tethered $N^{ECN}$-GV reporter activity was inhibited by compound E at very similar concentrations to C99-GV, while $N^{INTRA}$-GV reporter activity was unaffected (data not shown).

To determine whether the γ-secretase activity we observe in Dmel2 cells produces the same cleavage products as the human enzyme, we measured secreted Aβ peptides released from the C99-GV substrate using specific Aβ40 and Aβ42 ELISA assays. Aβ40 and Aβ42 are both detected, and, as in human cell supernatants, Aβ40 is approximately 10-fold more abundant than Aβ42. Production of both peptides is inhibited by compound E to levels below the limit of detection in our ELISA assay conditions (Figures 5G and 5H). The ability of compound E to completely inhibit Aβ production in this assay contrasted with the maximum 2- to 3-fold inhibition of $N^{ECN}$-GV and C99-GV reporter gene activity we observed, suggesting that residual reporter gene activity may be a result of presenilin-independent nuclear access of the GV activator in Dmel2 cells. As described for mammalian presenilins (Beher et al., 2001), high concentrations of compound E cause an increase in ~50 kDa full-length presenilin levels (Figure 5F), but the strong inhibition of Aβ production we observe in this assay cannot be explained simply by reduction in presenilin CTF levels. These data confirm that Dmel2 cells have an endogenous γ-secretase activity with pharmacological and substrate cleavage properties similar to human γ-secretase.

We next used RNAi to determine whether γ-secretase activity in Dmel2 cells is presenilin dependent (Figures 5I–5M). RNAi of *Drosophila psn* strongly reduced endogenous PSN C-terminal fragment (CTF) protein levels (Figure 5K). *psn* RNAi also reduced secreted Aβ40 and Aβ42 peptides by 85% (Figures 5L and 5M) and decreased C99-GV and $N^{ECN}$-GV reporter gene activity by 50%–70% but had no effect on the intracellular domain reporters C59-GV or $N^{INTRA}$-GV (Figures 5I and 5J). RNAi against two control genes, *dack* and *rosy* (xanthine dehydrogenase), had no effect on APP or Notch reporter assays, secreted Aβ, or PSN CTF protein levels. The APP cleavage reporter, Notch cleavage reporter, and Aβ ELISA assays thus each demonstrate presenilin dependence and provide independent measures of γ-secretase function.

We find that RNAi of *Drosophila aph-1*, *pen-2*, or *nicastrin (nct)* results in reduction of secreted Aβ40 and Aβ42 (Figures 5L and 5M) and reduction in reporter gene activity elicited by both C99-GV and $N^{ECN}$-GV, but not by C59-GV or $N^{INTRA}$-GV (Figures 5I and 5J). Inhibition in each case is approximately as strong as that observed for RNAi of presenilin itself. In addition, we observe that RNAi of *aph-1* and *pen-2*, like that of *nct*, leads to strong reductions in PSN CTF protein levels (Figure 5K), with no buildup of detectable full-length PSN. *pen-2* RNAi is slightly less efficient and more variable in these assays than *aph-1* or *nct* RNAi, in contrast to the identical behavior of *pen-2* and *aph-1* in all *C. elegans* in vivo genetic

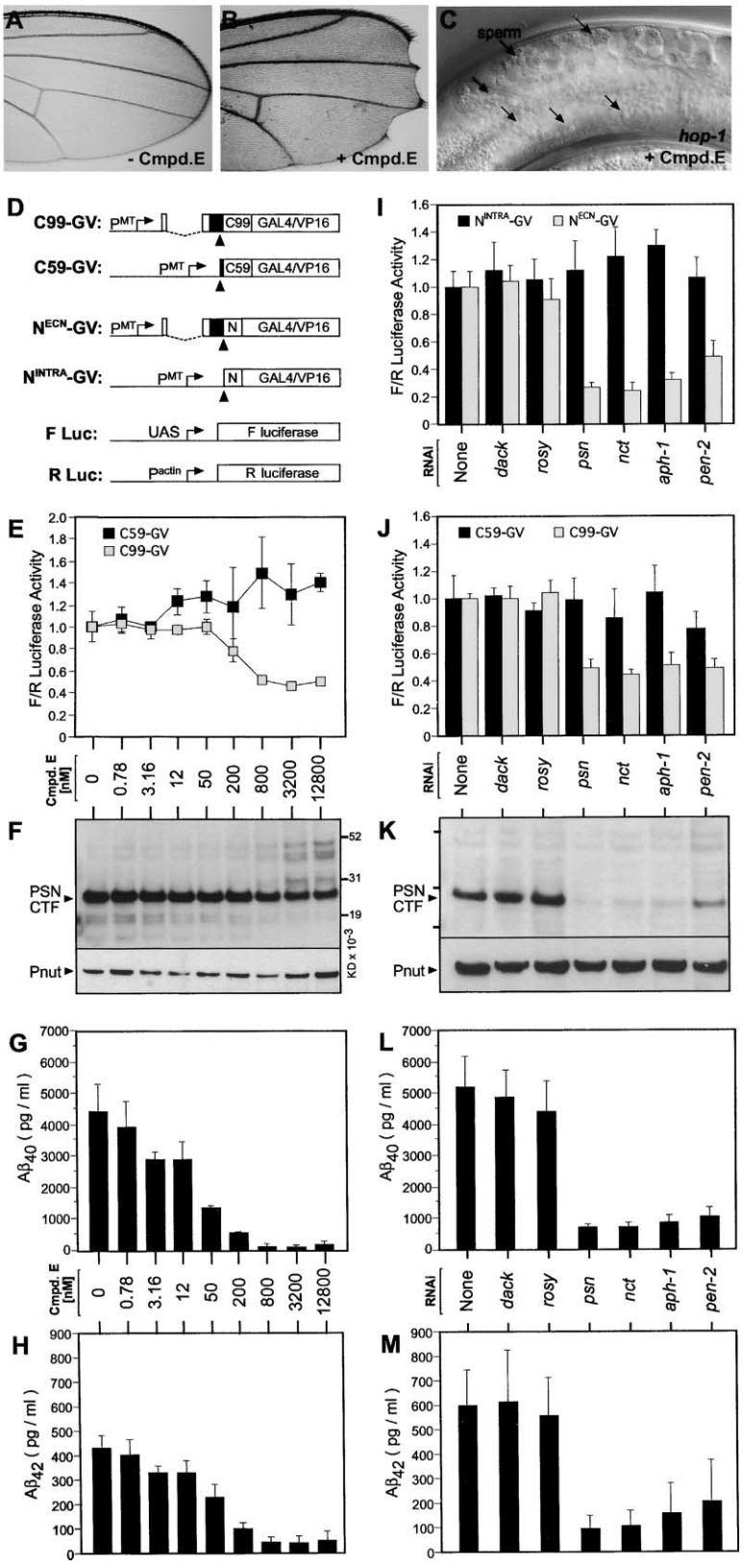


Figure 5. *Drosophila aph-1*, *pen-2*, and *nct* Are Required for γ-Secretase Activity and Presenilin Protein Accumulation

(A–C) γ-secretase inhibitor compound E induces Notch pathway phenotypes in *Drosophila* and *C. elegans*.

(A) Untreated wild-type *Drosophila* wing.

(B) Wing from an animal raised on compound E (40 μl of 5 mg/ml solution in DMSO placed on food surface).

(C) One gonad arm of a *C. elegans hop-1(ep171)* hermaphrodite raised on compound E (100 μl of 10 μM solution placed on a 10 ml agarose growth plate), showing a *glp-1*-like germline proliferation defect. The adult germline contains sperm (arrows) but no undifferentiated germ cells (compare to wild-type untreated in Figure 2G). Compound E does not induce *glp-1*-like sterility in wild-type or *sel-12* mutants at the same concentration, suggesting that the compound is able to inhibit *sel-12*-dependent γ-secretase activity, but that *hop-1*-dependent γ-secretase might be resistant to inhibition.

(D) Constructs used for detection of γ-secretase activity in *Drosophila* Dmel2 cells. γ-secretase cleavage sites are indicated by closed triangles, and transmembrane domains are shaded. Extracellular deleted regions in $N^{ECN}$ and APPC99 are indicated by dotted lines.

(E) UAS-luciferase reporter gene activity driven by C99-GV, but not C59-GV, is sensitive to γ-secretase inhibitor compound E. Data is expressed as the ratio of F luc/R luc activity normalized to values for untreated cells. Error bars represent the standard deviation for assays done in triplicate. Data is representative of five independent experiments.

(F) Western blots of whole-cell pellets from the compound titration experiment shown in panel E, probed with antibodies to *Drosophila* PSN CTF fragment or a control antibody to the Peanut protein.

(G and H) Secreted Aβ40 (G) and Aβ42 (H) production is inhibited by compound E in Dmel2 cells. Data are normalized for cell density by ProCheck cell viability assay and are the median of eight independent repetitions; error bars represent standard deviations.

(I) RNAi inactivation of *psn*, *nct*, *aph-1*, and *pen-2* inhibits UAS-luciferase reporter gene activity driven by $N^{ECN}$-GV, but not by $N^{INTRA}$-GV. Data are normalized to values for no RNAi control.

(J) RNAi inactivation of *psn*, *nct*, *aph-1*, and *pen-2* inhibits UAS-luciferase reporter gene activity driven by C99-GV, but not by C59-GV. Data are normalized to values for no RNAi control.

(K) Western blots of cell lysates from the experiment shown in panel J probed with antibodies to *Drosophila* presenilin CTF or Peanut.

(L) Secreted Aβ40 and (M) Aβ42 production from C99-GV is dependent on *psn*, *nct*, *aph-1*, and *pen-2* activity. Data are normalized for cell density by ProCheck cell viability assay and are the median of eight independent repetitions; error bars represent standard deviations.

assays. The difference in efficiency of *pen-2* versus *aph-1* RNAi in *Drosophila* cells may thus reflect incomplete inactivation of *pen-2* by RNAi rather than a functional difference between the two genes. These data reveal that *aph-1* and *pen-2,* like *nct* (Figures 5I–5M; Chung and Struhl 2001; López-Schier and St Johnston, 2002; Hu et al., 2002), are necessary for γ-secretase activity and for the accumulation of processed presenilin protein in *Drosophila* cells.

## Discussion

Using a genetic approach in *C. elegans*, we identified *aph-1* and *pen-2*, two new, phylogenetically conserved members of the Notch pathway. *aph-1* and *pen-2* have very similar functions in *C. elegans*, as their individual mutant phenotypes and their genetic interaction phenotypes with *aph-2*, *hop-1*, and *sel-12* are indistinguishable. *aph-1* and *pen-2* are required for all *lin-12*- and *glp-1*-mediated signaling events we have examined, and no phenotypes were observed in the *aph-1* or *pen-2* mutants that would suggest functions other than in *lin-12/glp-1*-dependent processes. We conclude that *aph-1* and *pen-2* define new obligate members of the Notch signaling pathway.

The *sel-12* enhancer screen identified only these two genes with strong effects on germline presenilin activity. We performed three other related genetic screens for presenilin pathway components and isolated additional alleles of *aph-1*, *pen-2* and *aph-2*, but no additional genes with similar strong phenotypes (our unpublished data). If other genes that are essential for presenilin function in *C. elegans* exist, they may be masked by genetic redundancy or may have additional functions and associated phenotypes that obscure their contributions to Notch signaling. These genetic studies suggest that *aph-1, pen-2*, and *aph-2* define a set of genes that is unique in the strength and specificity of their interactions with the presenilin genes.

The *lin-12(gf)* suppression tests and *Drosophila* cell culture experiments on defined γ-secretase substrates presented here demonstrate that *aph-1* and *pen-2* are required for Notch and βAPP cleavages, specifically at the presenilin-mediated γ-secretase/S3 step. We also found that *aph-1* and *pen-2*, like *nct*, are required for accumulation of processed presenilin protein. Although this requirement could be the simple explanation for the loss of γ-secretase activity upon depletion of *aph-1* and *pen-2*, the recent studies of López-Schier and St. Johnston (2002) suggested that there may be separable requirements for nicastrin in Notch signaling and in accumulation or maintenance of presenilin protein. Similarly, our cell culture data suggests that *pen-2* RNAi can have stronger inhibitory effects on γ-secretase activity than on presenilin protein levels (Figures 5J and 5K), suggesting that, as is the case for nicastrin, γ-secretase activity may be lost more rapidly than PSN-CTF stability or accumulation upon *pen-2* depletion. Although the mechanism is not clear, accumulation of processed presenilin in *Drosophila* cells depends on *aph-1*, *pen-2*, and *nct*.

Despite their extensive functional similarities with *aph-2*, *aph-1* and *pen-2* behave distinctly from *aph-2* in certain genetic interaction tests. First, *aph-2* mutants strongly interact with *hop-1*, but not with *sel-12* mutants, to produce the *glp*-sterile phenotype, while *aph-1* and *pen-2* mutants exhibit sterility with *sel-12*, but not with *hop-1* mutants. Second, *aph-1* and *pen-2* mutants or RNAi do not show additive phenotypes with each other, while *aph-2* RNAi interacts with both *aph-1* and *pen-2* mutants to give partially penetrant sterility. Third, rescue of either *aph-1* or *pen-2* was dependent on coexpression of human *aph-1* and *pen-2* genes together, but not on nicastrin, whereas *aph-2* is rescued by human nicastrin alone (Levitan et al., 2001). APH-2/nicastrin is a type 1 glycosylated transmembrane protein, while *aph-1* and *pen-2* are predicted to be polytopic integral membrane proteins, suggesting a basis for functional differences between *aph-2* and the *aph-1* and *pen-2* genes. We suggest that *aph-1* and *pen-2* interact closely with each other in the same process, perhaps at the same step, but in a role somewhat distinct from that of *aph-2*/nicastrin, to facilitate presenilin activity.

In our rescue experiments we found that human *aph-1* and *pen-2* are corequired for rescue. APH-1 and PEN-2 thus cooperate functionally, consistent with a model in which the two proteins associate directly. The APH-1 and PEN-2 proteins are highly conserved, but sequence divergence between the *C. elegans* and human APH-1 and PEN-2 proteins might interfere with functional cross-species APH-1/PEN-2 interactions and account for the corequirement we observe for *aph-1* and *pen-2* mutant rescue. Cross-species interactions between a putative APH-1/PEN-2 unit and presenilins can occur because *haph-1* and *hpen-2* together are able to confer low-level rescue of *pen-2* mutants in the absence of *PS1* and because human *PS1* or *PS2* alone is able to rescue *sel-12* mutants (Levitan et al., 1996). However, coinjection of human *PS1* together with *haph-1* and *hpen-2* improves the efficiency of *pen-2* rescue, suggesting that the putative APH-1/PEN-2 protein complex also interacts with presenilin, and these interactions are more functional with intraspecific forms. APH-1 and PEN-2 are good candidates to be regulators and/or components of the high-molecular weight γ-secretase complex.

The role for γ-secretase in proteolysis of Notch, APP, and other cell surface transmembrane proteins suggests a site of action at the cell surface or in endocytic compartments. Some presenilin is detected in these locations (Ray et al., 1999; Lah and Levey, 2000; Kim et al., 2000), although most is found in ER and Golgi compartments (Kovacs et al., 1996; Annaert et al., 1999). PEN-2::GFP expression in adult *C. elegans*, like SEL-12::GFP, is found primarily in internal membrane compartments, consistent with a model in which PEN-2 interacts with APH-1 and presenilin early in the secretory pathway. Endogenous APH-2 protein in early *C. elegans* embryos, in contrast, is localized primarily to the plasma membrane (Goutte et al., 2000). In *aph-1* and *hop-1; sel-12* mutant embryos, APH-2 localizes instead in a perinuclear staining pattern consistent with ER/Golgi localization (Goutte et al. 2002). Transport of APH-2/nicastrin to the cell surface is thus dependent on presenilins and APH-1. One model for APH-1 and PEN-2 function would be to facilitate trafficking of APH-2 to the cell surface, which in turn promotes trafficking of presenilin. However, our genetic data favor a model in which APH-1

and PEN-2 together interact directly with presenilin or a presenilin/nicastrin complex to promote maturation and accumulation of the complex. It will be important to examine expression of endogenous proteins in the same cell types to determine to what extent APH-1 and PEN-2 colocalize with presenilins and nicastrin.

*aph-1*, *pen-2*, and *aph-2*/nicastrin define a core set of genes essential for the activity and accumulation of presenilin-dependent γ-secretase complexes. Several mechanisms could explain the failure to stably accumulate processed presenilin protein in the absence of APH-1, PEN-2, and APH-2/nicastrin proteins, including an inability to assemble, mature, transport, or stabilize the γ-secretase complex. If APH-1 and PEN-2 proteins interact directly with each other and with presenilin, these interactions could be transitory during early steps in γ-secretase complex assembly or may persist in the mature enzyme complex. Further studies of APH-1 and PEN-2 function should enhance our understanding of the γ-secretase enzyme and improve opportunities for the design of selective Alzheimer's disease therapeutics.

## Experimental Procedures

### *C. elegans* Genetics and Enhancer Screens

The following *C. elegans* mutations were used. LG I: *unc-74(x19)*, *dpy-5(e61)*, *unc-29(e1072* and *e193)*, *fog-3(q443)*, and *dpy-24(s71)*; LG III: *dpy-19(e1259)*, *unc-32(e189)*, *glp-1(q231)*, *unc-119(e1498)*, *pha-1(e2123)*, and *dpy-18(e364)*; LG IV: *him-4(e1489)*; LG X: *unc-1(e1598 n1201)* and *lon-2(e678)*. Rearrangements were as follows: *mnDp66(X;I)* and *hT2(I;III)*. Tc1 insertions in *sel-12* and *hop-1* were identified by PCR screening of strain TR679 and then excised imprecisely to generate *sel-12(ep6)* and *hop-1(ep90)*. *sel-12(ep6)* removes amino acids 34–441 and shifts the *sel-12* reading frame. *hop-1(ep90)* removes the C-terminal 144 amino acids and most of the *hop-1* 3′ UTR. *hop-1(ep168)* and *hop-1(ep171)* are strong alleles isolated in the *sel-12* enhancer screen. RNAi assays were performed by microinjection.

For the *sel-12* enhancer screen, *sel-12(ep6)* or *unc-74; sel-12(ep6)* hermaphrodites were treated with ethyl methane sulfonate and single F1 self-progeny picked onto separate plates. Sterile F2 self-progeny with a dark appearance were examined using Nomarski DIC optics to identify mutants with *glp-1*-like sterility. Heterozygous mutants were crossed to *dpy-19; him-4; lon-2* males, and segregation of the sterile phenotype was followed in the F2 and F3 generations to determine *sel-12* dependency.

### Interaction Tests with Activated *lin-12* Alleles

Suppression of *lin-12(e950gf)* phenotypes by *aph-1(ep140)* was scored in the *unc-29 aph-1; lin-12* self-progeny of *unc-29 aph-1/fog-3; lin-12* hermaphrodites. Suppression of the *lin-12(n302)* 0 AC phenotype by *pen-2(ep220)* was scored in the *lin-12 pen-2 dpy-18* self-progeny of *lin-12 pen-2 dpy-18/lin-12* hermaphrodites. The *lin-12(intra)* construct contains the 1.4 kb *sel-12* promoter element fused to a *lin-12* genomic fragment beginning at Asn941 of the intracellular domain and extending 1 kb downstream of the termination codon. Extrachromosomal arrays of this construct and pRF4 were generated by injection into *unc-29 aph-1(ep140)/fog-3* and *pen-2(ep220) dpy-18/unc-119* stocks, and one array in each was chosen for suppression tests.

### Transformation Rescue

Transformation rescue was performed using microinjection of PCR-amplified DNA fragments to generate extrachromosomal arrays in *unc-29 aph-1/fog-3* or *pen-2 dpy-18/unc-119* hermaphrodites. For genomic rescue, an *aph-1* genomic fragment with 2.8 kb of DNA 5′ to the initiator AUG and a *pen-2* fragment with 1.6 kb of DNA 5′ to the AUG were tested. The *aph-1* genomic fragment had no rescuing activity, so a *sel-12* regulatory sequence consisting of the 1.4 kb fragment 5′ to the *sel-12* AUG was used to drive *aph-1* expression. For cDNA rescue constructs, expression was regulated by the 1.4 kb *sel-12* promoter and an *unc-54*-derived 3′UTR, modified to contain introns, from pPD115.116. For human PS1 cDNA expression, a 0.5 kb *sel-12* promoter fragment was used. Primer sequences used to generate these constructs are available upon request. For the *pen-2::GFP* constructs, the GFP coding sequence from pPD114.108 was fused in-frame to the *pen-2* coding region at the N terminus, C terminus, or between the two transmembrane domains after amino acid 48. To score Egl rescue, homozygous *unc-29 aph-1* or *pen-2 dpy-18* hermaphrodites carrying a transgene array (marked with Y47D3B.7::GFP that is expressed in the intestine at all stages; S. Elson and M. Costa, personal communication) were transferred as L4 larvae onto separate plates and scored for number of eggs laid over 4 days.

### *Drosophila* Cell Culture Assays

C99-GV is similar to spA4CT (Fossgreen et al. 1998), C59-GV initiates at the APP γ-secretase (40) cleavage site, $N^{ECN}$-GV is as described in Struhl and Adachi (2000), and $N^{INTRA}$-GV initiates at the S3/γ-secretase cleavage site; all were under control of the copper-inducible metallothionein promoter. The firefly luciferase (F Luc, pGEM-luc; Promega) reporter is under control of the yeast UAS and *Drosophila* minimal hsp70 promoter, and Renilla luciferase (R Luc) from pRL null (Promega) is under control of the *Drosophila* actin5C promoter.

Dmel-2 cells were grown at 25°C in *Drosophila* serum-free medium (DSFM) (Invitrogen) supplemented with 20 mM L-glutamine. Luciferase reporter assays were performed on transiently transfected cells. A total of $2 \times 10^7$ cells were transfected in 10 mm dishes using Effectene (Qiagen) and DNA mixes containing 250 ng each of C99-GVP or C59-GVP, F Luc, and R Luc. Reducing the DNA concentrations increased the ratio of presenilin-dependent signal to presenilin-independent background signal to ≥3×, so, for the Notch reporter assays shown, we transfected 5 ng of $N^{ECN}$-GV or $N^{INTRA}$-GV, 10 ng R Luc, and 250 ng F Luc. After 18 hr, cells were resuspended in DSFM, treated with inhibitor or dsRNA for 72 hr, induced with 70 μM $CuSO_4$ for 24 hr, and assayed in triplicate using the Dual-Luciferase Reporter Assay System (Promega) and a Wallac Victor2 V1420 Multilabel HTS counter (PerkinElmer Life Sciences). *Drosophila* presenilin CTF antibody was generated in rabbits by immunization with a KLH-coupled peptide containing presenilin amino acids 359–376 (CASGQRTGNSHPRQNQRDD). Anti-peanut monoclonal antibody 4C9H4 was obtained from the Developmental Studies Hybridoma Bank. The compound E material used here was a racemic mixture at carbon 3.

For Aβ assays, Dmel-2 cells stably transfected with C99-GV were treated with inhibitor or dsRNA for 72 hr and induced with 70 μM $CuSO_4$ for 24 hr. Half of the supernatants were collected and assayed for specific Aβ forms by sandwich ELISA (Yan et al., 1999) using 6E10 (Senetek) as capture antibody and 208 and 165 (NY Institute Basic Research) as detection antibodies for Aβ40 or Aβ42, respectively. The remaining supernatant and cell pellets were assayed for cell viability by ProCheck (Intergen).

### Acknowledgments

We thank Chris Park and the Exelixis sequencing facility for support, Joel Rothman for providing the 3NB12 antibody, Michael Morrissey for support in synthesis of compound E, Jenny Kopczynski for contributions to the initiation of the genetic screens, and Ismail Kola and members of the Exelixis Genetics department for helpful discussions. Some of the *C. elegans* strains used in this work were provided by the *Caenorhabditis* Genetics Center.

Received: April 1, 2002
Revised: April 29, 2002

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## Accession Numbers

GenBank accession numbers for the new sequences reported in this work are as follows: *Drosophila pen-2*, AF512426; zebrafish *pen-2*, AF512427; zebrafish *aph-1*, AF512428.