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Physical and genetic interaction of filamin with presenilin in *Drosophila*

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SUMMARY

Presenilins were first identified as causative factors in early onset, familial Alzheimer's Disease (FAD). They are predicted to encode a highly conserved novel family of eight transmembrane domain proteins with a large hydrophilic loop between TM6 and TM7 that is the site of numerous FAD mutations. Here, we show that the loop region of *Drosophila* and human presenilins interacts with the C-terminal domain of *Drosophila* filamin. Furthermore, we show that *Drosophila* has at least two major filamin forms generated by alternative splicing from a gene that maps to position 89E10-89F4 on chromosome 3. The longest form is enriched in the central nervous system and ovaries, shares 41.7% overall amino acid identity with human filamin (ABP-280) and contains an N-terminal actin-binding domain. The shorter form is broadly expressed and encodes an alternatively spliced form of the protein lacking the actin-binding domain. Finally, we show that presenilin and filamin are expressed in overlapping patterns in *Drosophila* and that dominant adult phenotypes produced by overexpression of presenilin can be suppressed by overexpression of filamin in the same tissue. Taken together, these results suggest that presenilin and filamin functionally interact during development.

Key words: *Drosophila*, filamin, presenilin, Familial Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system that causes progressive memory and cognitive decline during mid to late adult life. Insight into the mechanisms underlying AD has come from the identification of mutations in three genes, *Beta Amyloid Precursor Protein (APP)*, *presenilin 1* (*PS1*) and *presenilin 2 (PS2)*, which have been linked with early-onset familial AD (FAD) (Hardy, 1997). Mutations in the *APP* gene have been found in a small number of families with disease onset before 65 years of age. In contrast, mutations within the *presenilin* genes are much more common and account for up to 50% of FAD cases.

Presenilins are a highly conserved family of proteins predicted to have eight transmembrane (TM) domains and a large hydrophilic loop between TM6 and TM7 facing the cytosolic compartment (Doan et al., 1996; Li and Greenwald, 1996, 1998). In vertebrates, presenilins are broadly expressed (Lee et al., 1996) and primarily localized to the endoplasmic reticulum and Golgi apparatus (Cook et al., 1996; De Strooper et al., 1997; Kovacks et al., 1996; Walter et al., 1996). Although the function of presenilins is not fully understood a role in protein processing was first proposed based, in part, on their subcellular distribution and their ability to affect processing of APP and Notch, a protein that regulates cell-fate decisions in virtually all species (Borchelt et al., 1996; Citron et al., 1997; Scheuner et al., 1996; Tomita et al., 1997; De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). Recently, Wolfe et al. (1999) have suggested that presenilin may itself have γ-secretase activity and be directly responsible for cleavage. This is based on the observation that mutations in two transmembrane aspartate residues of presenilin eliminate endoproteolysis of presenilin and result in a loss of Aβ production. Consistent with this model, recent data have shown that presenilins are tightly associated with a high molecular weight complex that maintains γ-secretase activity, suggesting that presenilins are either cofactors for γ-secretase or the γ-secretase themselves (Esler et al., 2000; Li et al., 2000a,b). The identities of the additional components of the complex, however, are still unknown. In addition, it remains unclear if cleavage of APP and Notch by the γ-secretase occurs at the plasma membrane or within endosomal compartments.

To gain further insight into the function of presenilin we used the yeast two-hybrid approach to identify presenilin-binding proteins in *Drosophila*. Specifically, we used the large cytoplasmic loop domain between TM6 and TM7 as bait to screen an embryonic library. Although the cytoplasmic loop of presenilin is poorly conserved between species, its functional importance is suggested by the numerous FAD mutations located within it (Cruts et al., 1996; Lendon et al., 1997; Schellenberg, 1995). In addition, this domain is the site of an evolutionarily conserved proteolytic cleavage that gives rise to N- and C-terminal fragments, although the functional

significance of this cleavage is not known (Guo et al., 1999; Podlisny et al., 1997; Thinakaran et al., 1996).

Here we report the identification and cloning of the gene encoding *Drosophila* filamin as a Dps loop-binding protein. We also show that *Drosophila* expresses two major forms of filamin, which arise from differential splicing of a single gene. One form, filamin A, is 240 kDa and is 41.7% identical to human ABP-280 and highly enriched within the central nervous system and ovaries. The second form, filamin B, is a 90 kDa truncated form of the protein that does not contain the N-terminal actin binding domain. However, both contain the C-terminal domain that mediates its association with membrane proteins and binds to presenilin. We also show that *Drosophila* filamin can bind to the cytoplasmic loop of both *Drosophila* and human presenilins, indicating that this interaction is functionally conserved. Finally, we find that Dps and filamin are coexpressed during *Drosophila* development and that overexpression of filamin can suppress dominant adult phenotypes generated by overexpression of *Dps* in third larval instar imaginal discs. Taken together this demonstrates that Dps and filamin can functionally interact during development.

MATERIALS AND METHODS

Yeast two-hybrid screen

cDNA fragments containing residues 294-481 of *Drosophila* presenilin were subcloned into the yeast two-hybrid vector PAS2-1 (Clonetech, Palo, CA, USA), creating a fusion between the GAL4-DNA binding domain and the presenilin hydrophilic loop. To confirm that the interactions are specific to the Dps loop region we also generated several additional constructs in PAS-1, including one corresponding to the N-terminal domain of Dps (N) and three to the C-terminal domain (C1,C2,C3). These bait constructs were then transformed into the yeast strain Y-190. The resulting Trp^+ strains were then used to screen a *Drosophila melanogaster* embryonic cDNA library (MATCHMAKER, Clonetech) in which cDNAs pooled from 21 hour Canton-S embryos were cloned into the PACT2 vector (Clonetech), which generates a hybrid protein with the GAL4 activation domain. Interaction of the cDNA hybrid protein with the bait fusion protein led to reconstitution of a functional GAL4 transcriptional activator and expression of the *His 3* reporter gene. For further confirmation, transformants with the His^+ phenotype were tested for expression of a second reporter gene, *lacZ*, using a filter assay for β-galactosidase activity as recommended by the supplier (Clonetech). cDNAs were amplified by polymerase chain reaction (PCR) from positive yeast clones with primers flanking the two ends of the inserts.

To determine the ability of positive clones to also interact with the human presenilin 1 and 2 loop domains, fusion constructs were generated in PAS2-1 between the GAL4 DNA-binding domain and amino acids (aa) 351-487 of human presenilin 1 and aa 276-388 of human presenilin 2. These fusion constructs were then transformed into the yeast strain Y-190, where their interaction with an identified prey could be directly tested.

To quantitate the strength of the interaction of the various loop regions of human and *Drosophila* presenilins with filamin we used a liquid β-galactosidase assay using ONPG (o-nitrophenyl-β-D-galactopyranoside) as substrate. Briefly, five yeast colonies from each interaction were grown in liquid SD selection at 30°C overnight. The next day, 8 ml of YPD was added to the 2 ml overnight culture and the fresh culture was incubated at 30°C with shaking for 3-4 hours until the cells were in mid-log phase (OD_{600} of 1 ml=0.5-0.8). 1.5 ml of the sample was then centrifuged and the pellet was washed in 1.5 ml of Z buffer ($Na_2HPO_4.7H_2O$ 16.1 g/l, $NaH_2PO_4.H_2O$ 5.50 g/l, KCl 0.75 g/l, $MgSO_4.7H_2O$ 0.246 g/l, pH 7.0) and resuspended in 100 μl of Z buffer. The tubes containing the sample were then placed in liquid nitrogen for 1 minute and then transferred to a 37°C water bath for 1 minute to thaw. The freeze/thaw cycle was repeated twice to ensure that the cells had broken open. After adding 0.7 ml of Z buffer, 0.27% of β-mercaptoethanol and 160 μl of 4 mg/ml ONPG, the reaction and blank tubes were placed in a 30°C incubator and the time required for the yellow color to develop was recorded in minutes. After the yellow color was detected, 0.4 ml of 1 M Na_2CO_3 were added to the reaction and blank tubes. The samples were then centrifuged and the OD_{420} of the supernatants was recorded. β-galactosidase units were quantified by taking number of β-galactosidase units=$1{,}000 \times OD_{420}/(t \times V \times OD_{600})$, where t=elapsed time (in minutes) of incubation and V=0.1 ml × concentration factor. 1 unit of β-galactosidase is defined as the amount that hydrolyzes 1 μmole of ONPG to O-nitrophenol and D-galactose per minute per cell.

Isolation of filamin genomic and cDNA clones and chromosomal localization

A P1 *Drosophila* high density filter (Genome System, St Louis, Missouri, USA) was screened according to the manufacturer's recommendation with a probe consisting of a partial filamin cDNA identified by yeast two-hybrid. The P1 positive clones, DS01567 and DS04062, were obtained from the Berkeley *Drosophila* Genome Project (BDGP) and their identity was confirmed by Southern blot hybridization and sequencing. Similarly, EST clones (GH12209, LP4904) were obtained from BDGP and sequenced. GenBank Accession Numbers were AF183178, AF183179, AF183180, AF183181 and AF183182.

Pull-down experiments with immobilized GST fusion proteins and affinity chromatography

Pull-down experiments

GST-fusion proteins were generated by subcloning a filamin cDNA isolated from the prey vector or a cDNA encoding the Dps loop (aa 294-481) into the pGEX-KG vector. ^{35}S-labeled C-terminal filamin and luciferase were synthesized by in vitro transcription-translation (TNT Coupled Reticulocyte Lysate Kit; Promega, Madison, WI, USA). In pull-down experiments, GST-fusion proteins were incubated with GST-beads at 4°C for 3 hours. The beads were then washed four times with PBST binding buffer (1×PBS + 2% Triton X-100) and then incubated with 5 μl of reticulocyte lysate containing ^{35}S-labeled filamin or luciferase in 200 μl of binding buffer for 2 hours at 4°C. The beads were then washed in 1 ml of binding buffer four times and the retained polypeptides were eluted directly into 30 μl of 2× SDS-PAGE sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 0.001% Bromophenol Blue). 1 μl of labeled reticulocyte lysate and 10 μl of each eluate were separated by SDS-PAGE and analyzed by phosphoimaging.

Affinity chromatography

3 g of adult flies that overexpressed *Dps* from a heat-shock-inducible construct were homogenized in 15 ml of extraction buffer (1× PBS with 100 μg/ml phenylmethylsulfonyl fluoride, 2 mM EDTA and 2% Triton X-100). Homogenates were extracted by end-over-end rotation for 2 hours at 4°C and centrifuged at 15000 rpm for 10 minutes in a Beckman JA-25.50 rotor. The pellet was discarded and the supernatant was then further centrifuged for 30 minutes at 100,000 ***g*** in a Beckman Ti-70 rotor. Glutathione-agarose affinity columns (1 ml bed volume) with about 1 mg of GST-filamin or 1 mg of GST were pre-equilibrated with extraction buffer. The protein extract was precleared by incubation for 4 hours at 4°C with glutathione-agarose beads followed by centrifugation (500 ***g*** for 2 minutes). 5 ml of the recovered extract was then loaded onto a column by recirculation 5 times under gravity flow. Columns were washed with 20 ml of PBS containing 0.25% Triton X-100 and then eluted with 500 μl of 1× PBS containing 0.24%

Triton X-100, 1 mM NaCl and 5mM EDTA. The samples were then eluted with 500 μl of SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

Immunoblots and immunocytochemistry

Protein was extracted in either 1× PBS with 2% Triton X-100 or directly extracted in 2× SDS sample buffer. The amount of total protein was quantified using a BCA assay or probed with an antibody generated to dNSF-2, which is broadly expressed in all tissues during development (G.L.B., manuscript in preparation). Immunoblots and immunocytochemistry were performed using either a filamin-specific antibody (1:4000) generated to the last 810 amino acids of *Drosophila* filamin that recognizes both forms or a Dps-specific antibody (1: 2000) generated to an N-terminal peptide corresponding to amino acids 28-43 (Guo et al., 1999). Primary antibodies were detected with either HRP-conjugated secondary antibodies for immunoblots or fluoroscein-conjugated secondary antibodies for immunocytochemistry.

Northern blot analysis

Poly(A)$^+$ mRNA from embryos, larvae and adults was obtained from Clonetech. Electrophoresis of RNA was carried out in 1% formaldehyde-agarose gels in 1× formaldehyde gel-running buffer (50 mM Hepes, 1 mM EDTA, 5 mM sodium acetate, pH 7.0, 6.67% formaldehyde), followed by transfer to a nylon filter. The filters were then probed with a 3 kb cDNA clone corresponding to the 3′ end of filamin and a full-length clone of *Rp1* (loading control), each labeled with ^{32}P by random priming. Blots were prehybridized in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2) at 50°C for 2-3 hours and hybridized in Church buffer overnight. The blots were then washed at 50°C with 2× SSC, 0.1% SDS for 30 minutes, followed by 0.5× SSC, 0.1% SDS for 30 minutes and a final wash in 0.1× SSC, 0.1% SDS for 30 minutes. The filters were then exposed to Fuji X-ray film for 4 hours to 3 days.

Fly stocks and genetic interaction studies

All fly stocks were maintained at room temperature on standard corn meal agar medium with the exception of crosses between GAL4 lines and various UAS transgenes, which were maintained at 29°C to achieve high levels of GAL4 activity. Transgenic lines carrying a UAS-*Dps* transgene were generated by subcloning a full-length *Dps* cDNA into the polycloning site of the pUAST vector that contains a miniwhite$^+$ reporter gene. Transformants were generated using *white^1* recipients and standard P-transformation methodology (Spradling and Rubin, 1982). Three recombinant lines carrying a UAS-*Dps* transgene and either the *pannier-Gal4* (*pnr*-GAL4), *cut-Gal4* or *daughterless-Gal4* (*da*-GAL4) drivers on the same chromosome were established using standard *Drosophila* genetics. The line EP(3)3715 was obtained from the Berkeley *Drosophila* Genome Project and contains an insertion of a target P-element vector that carries GAL4 binding sites and a basal promoter (Rorth, 1996; Rorth et al., 1998) in the 5′ regulatory region of the *Drosophila* gene encoding filamin. This line can be used to drive overexpression of *filamin* after crossing to various GAL4 drivers.

To detect functional interactions between filamin and Dps we crossed our recombinant lines that carry a GAL4 driver and UAS-Dps transgene to the EP line, EP(3)3175. The resulting F_1 progeny would then overexpress both *filamin* and *Dps* in the same cell/tissue. We then examined the F_1 progeny to determine if overexpression of *filamin* could modify the dominant adult phenotype generated by overexpression of *Dps*.

RESULTS

Drosophila filamin is a presenilin-binding protein

To gain insight into the possible functions of presenilin during normal development and in AD, we performed yeast two-hybrid screens to identify presenilin-binding proteins. The intracellular loop was chosen as bait because it is the site of many presenilin mutations that are causative in FAD. *Drosophila*, unlike most other species, has a single presenilin gene that gives rise to two isoforms, DLA-a and DLA-b, by alternative splicing of exon 7 (Guo et al., 1999). DLA-b differs from DLA-a by an addition of 14 aa (GMPLVTFKSNLRGN), located within the intracellular loop region between TM6 and TM7 (Fig. 1A). We used cDNA fragments encompassing different portions of the Dps intracellular loop as bait, including one fragment that encompasses the entire intracellular loop (DLA-a) and another that corresponds to the loop region from DLA-b that includes the additional 14 amino acids (Fig. 1A,B). These baits were then used to screen approx. 1×10^6 independent clones of a *Drosophila* embryonic library and 14 positively interacting clones were identified. The individual cDNAs were isolated and retransformed into yeast to confirm that these genes encode proteins interacting specifically with the Dps loop region, but not with other unrelated proteins including the N-terminal domain of presenilin itself. DNA sequence analyses of these Dps interacting clones revealed the presence of four distinct groups of cDNAs. Two of these cDNAs encode the C-terminal domain of a *Drosophila* filamin homologue. Other clones are currently being analyzed and will be described elsewhere.

To confirm that the interaction between presenilin and filamin is conserved and functionally important we also tested the interaction between *Drosophila* filamin and the loop domain of human presenilin 1 and 2. Although the overall amino acid sequence between *Drosophila* and human presenilins is poorly conserved within this region, the C-terminal domain of *Drosophila* filamin was able to bind both human loop domains in a yeast two-hybrid assay. To quantitate the strength of the interaction between *Drosophila* filamin and the various *Drosophila* and human loop constructs we then measured β-galactosidase activity (Fig. 1B). We found that the shorter Dps isoform (DLA-a) is better able to bind to *Drosophila* filamin than the longer (DLA-b) isoform (0.5386±0.0816 β-gal units compared to 0.3537±0.0499). The human PS1 loop region binds equally as well as the longer Dps isoform to *Drosophila* filamin (human PS1=0.3486±0.06 β-gal units; DLA-b=0.3537±0.0499) whereas the human PS2 loop region binds equally as well as the shorter Dps isoform (human PS2=0.5365±0.0818; DLA-a=0.5386±0.0816). In contrast, we do not detect any significant binding between filamin and an N-terminal Dps bait or the bait vector itself (Fig. 1B). Finally, to further define the minimal domain of presenilin required for the interaction with filamin we tested the ability of different loop domain constructs to bind to filamin using the yeast two-hybrid system. We found that although all three constructs could bind to the C-terminal domain of *Drosophila* filamin, LB and LD exhibited the strongest interaction, suggesting that a region bounded by residues 320-440 in Dps is essential but not necessarily sufficient for binding (Fig. 1B).

Interaction of Dps with filamin in vitro

To confirm that *Drosophila* filamin interacts directly with Dps we used two additional approaches. First we prepared GST fusion proteins containing the loop domain of Dps in the vector pGEX-KG and used this together with GST control proteins in

pull-down experiments. To monitor filamin binding, the cDNA fragment encoding the C-terminal fragment of filamin identified from the yeast two-hybrid screening was subcloned into a plasmid vector under the control of a T7 promoter, and the corresponding proteins were radiolabeled by in vitro translation in the presence of [^{35}S]methionine. An irrelevant protein, luciferase, was labelled as a negative control, and both labelled proteins were detected by electrophoresis and autoradiography. These pull-down experiments confirmed that filamin could bind to the Dps loop domain produced as a GST-fusion protein but not to GST alone (Fig. 2A).

In addition, we used affinity chromatography assays to demonstrate the interaction between filamin and Dps. For these experiments, extracts were prepared from *Drosophila* third instar larvae that overexpressed Dps using a heat-shock construct. We have previously observed that under these conditions, full-length rather than processed Dps protein is produced (Guo et al., 1999). The homogenates were solubilized with Triton X-100 and applied to columns containing GST-filamin or GST alone. The bound proteins were then washed extensively, pre-eluted with 1 M NaCl and dissolved in SDS-sample buffer. Analysis of the eluates by SDS-PAGE, followed by immunoblotting, revealed that the full-length Dps protein was retained on the column to which GST-filamin was bound but not the control column containing GST alone. Furthermore, we find that the Dps bound to the immoblized filamin could be eluted by SDS-sample buffer but not by PBS containing 0.25% Triton X-100 and 1 M NaCl, suggesting that the interaction between Dps and filamin is strong and specific (Fig. 2B).

Alternative splicing gives rise to two distinct filamin forms in *Drosophila*

Filamin, also known as ABP280, was initially identified as an actin-binding protein that consists of a homodimer of 280 kDa subunits arranged in a tail-to-tail conformation (Gorlin et al., 1990; Hartwig, 1992; Hartwig and Shevlin, 1986; Hartwig et al., 1980). In vertebrates, filamin contains an N-terminal actin-binding domain, followed by a semiflexible rod-like domain consisting of 24 tandem repeats, each approximately 96 amino acids in length. The repeats are interrupted by two hinge insertions located between repeats 15 and 16 and between repeats 23 and 24. A C-terminal region has also been mapped that is required for membrane association via integral membrane proteins, suggesting that filamin may function as a direct link between actin filaments and membranes by binding to specific membrane proteins (Fox, 1985; Okita et al., 1985). The two *Drosophila* cDNA clones identified in our yeast two-hybrid screen encode the last 5 (clone 1) or 6 (clone 2) internal repeats, the second hinge region and the C-terminal domain.

The yeast two-hybrid clones were then used to identify genomic and full-length cDNA clones from the Berkeley *Drosophila* Genome Project (Fig. 3A). A single filamin gene was identified that maps to chromosome 3 at position 89E10-89F4 and appears to give rise to two major splice products. EST clones corresponding to both splice forms were obtained and sequenced. The longest clone is 7.5 kb (GH12209) and encodes a predicted 2211 aa (240 kDa) protein that shares 41.7% overall amino acid identity with human filamin (ABP280). Like its vertebrate counterpart, this *Drosophila* filamin has an N-terminal actin-binding domain, internal rod-like repeats, two hinge regions and a C-terminal membrane attachment domain (Fig. 3B). However, unlike vertebrates, *Drosophila* filamin contains only 20 internal repeats instead of 24. Based on the amino acid sequence comparison, it appears that *Drosophila* has only two repeats, which correspond to repeats 6-11 in humans. In

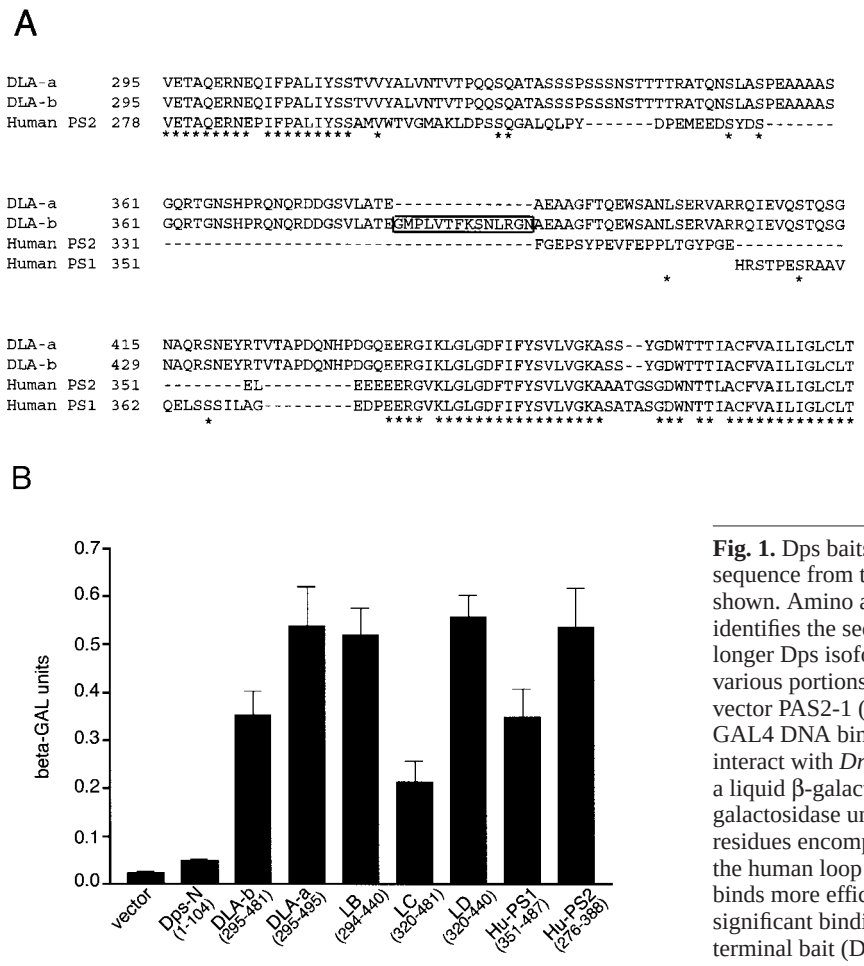


Fig. 1. Dps baits for yeast two-hybrid screening. (A) The amino acid sequence from the loop region of Dps and human PS1 and PS2 is shown. Amino acid identities are shown by an asterisk. A box identifies the sequence of the additional 14 amino acids found in the longer Dps isoform. (B) Dps baits were generated by subcloning various portions of a full-length cDNA into the yeast two-hybrid vector PAS2-1 (Clonetech, Palo, CA, USA), creating a fusion of the GAL4 DNA binding domain. The ability of *Drosophila* filamin to interact with *Drosophila* and human presenilins was measured using a liquid β-galactosidase assay. Shown are the number of β-galactosidase units obtained for each interaction. The amino acid residues encompassed in each bait are indicated in parentheses. Both the human loop domains of PS1 and PS2 bind filamin although PS2 binds more efficiently than PS1. In contrast, we did not detect any significant binding of *Drosophila* filamin to either the Dps N-terminal bait (Dps-N) or to vector alone.

addition to this full-length cDNA clone, we also identified a shorter 3.2 kb clone (LP4904), which encodes a predicted 839 aa (90 kDa) protein that includes only nine of the internal repeats, a single hinge region and the C-terminal Dps-binding domain. To confirm that both splice forms are expressed we then performed northern blots of extracts from embryos, larvae and adults using the conserved C-terminal domain of filamin as a probe. We observed two major transcripts of approximately 7.5 and 3.5 kb (Fig. 4). The two transcripts are likely to correspond to two distinct forms that can be detected on western blots with an antibody derived to the C-terminal domain of filamin. The larger form is 240 kDa and herein referred to as Filamin 240 whereas the smaller form is 90 kDa and referred to as Filamin 90.

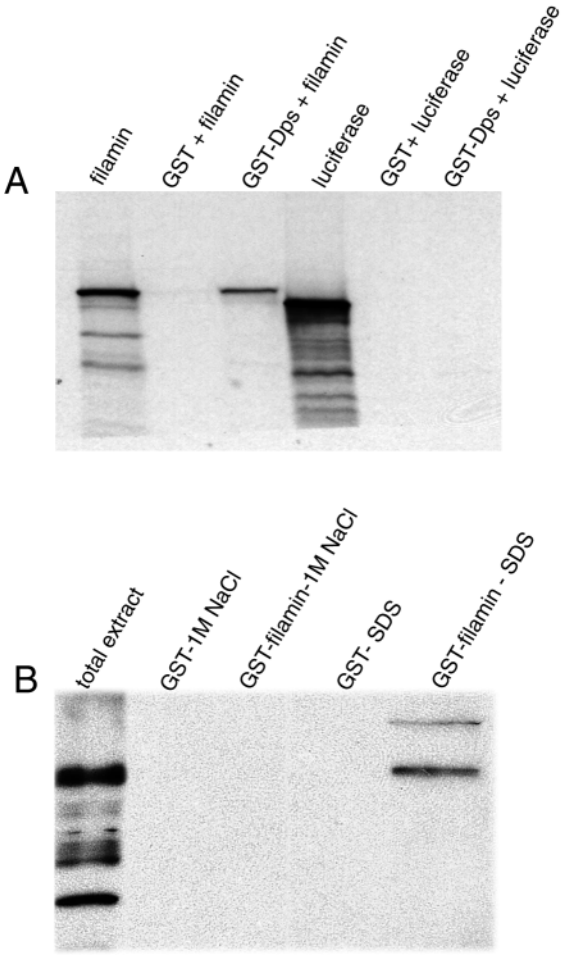


Fig. 2. Interaction of filamin with presenilin. (A) GST-pull down experiments. GST-Dps fusion proteins were incubated with GST-beads at 4°C for 3 hours. The beads were washed four times with PBST binding buffer (1× PBS + 2% Triton X-100) and then incubated with 5 μl reticulocyte lysate containing ^{35}S-labelled filamin or luciferase in 200 μl of binding buffer for 2 hours at 4°C. The beads were washed in excess binding buffer four times and the retained polypeptides were eluted directly into 30 μl of SDS-PAGE sample buffer. 1 μl reticulocyte lysate prior to binding together with 10 μl of each eluate were separated by SDS-PAGE. (B) Affinity chromatography on immobilised GST fusion proteins. Extracts derived from flies that express a heat-shock-inducible Dps transgene were loaded onto GST and GST-filamin affinity columns by recirculation five times under gravity flow. Columns were washed and eluted first in high salt (GST-1 M NaCl) followed by elution in 2× sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

Filamin 240 and Filamin 90 are differentially expressed

We then examined the distribution of both filamin forms during development and in specific tissues using western blot analysis (Fig. 5). We find that the levels of each filamin form increases during embryogenesis with the highest levels reached just before hatching (Fig. 5A). At later developmental stages, we find that Filamin 90 predominates, and in fact, is the only form detected in whole third instar larvae (Fig. 5B, L3). In contrast, Filamin 240 is undetectable during larval stages (L1, L2, L3) but reappears in late larvae, pupae and adults. We have

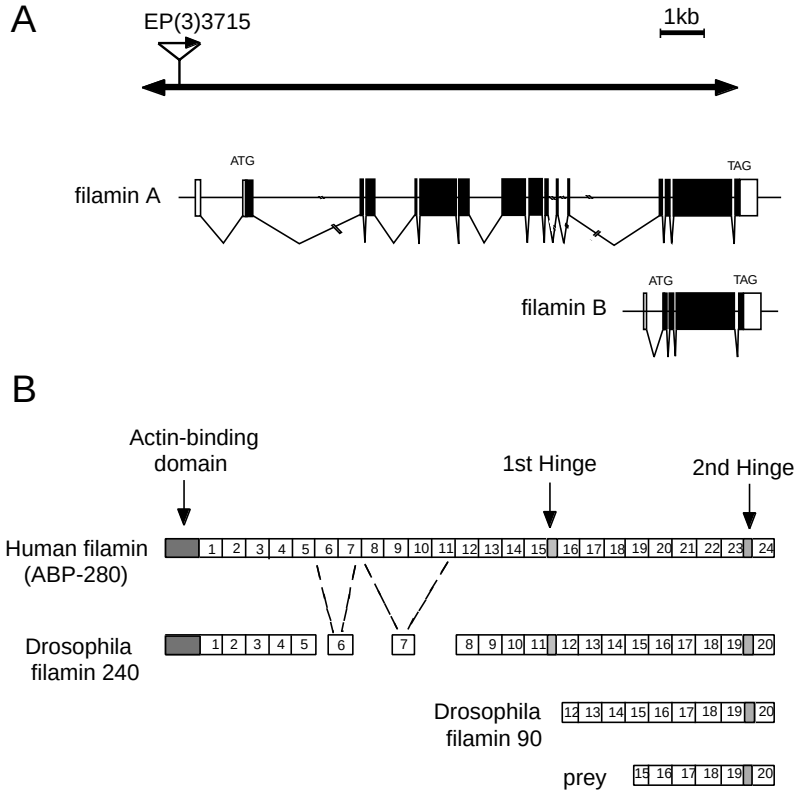


Fig. 3. (A) Genomic organization of the *Drosophila filamin* gene. The intron/exon structure of the *Drosophila filamin* gene is illustrated. The structure was determined by sequencing of the P1 genomic clones, DS01567 and DS04062, and the EST clones, LP4904 and GH12209. The location of the EP insertion at the 5′ end of the gene is indicated. Filamin 240 and Filamin 90 arise from alternative splicing of a single gene, as shown. (B) Structure of filamin proteins. Filamin comprises an N-terminal actin-binding domain followed by 24 repeats that are interrupted by two hinge regions. The C-terminal domain is involved in dimerization and membrane association. *Drosophila* Filamin 240 contains the actin-binding domain but with several of the internal repeats deleted. Both hinge regions and the C-terminal domain are also conserved. In contrast, Filamin 90 appears to represent a truncated form of the protein lacking the actin-binding domain and the first 11 repeats. The region identified in the two hybrid screen is indicated as prey and includes repeats 15-20, the second hinge region and the C-terminal domain.

previously shown that Dps is ubiquitously expressed during development but accumulates in the CNS of third instar larvae (Guo et al., 1999). We therefore examined the distribution of filamin forms in the larval CNS to determine if both were present and able to interact with Dps. We found that both forms can be detected in isolated CNS from third instar larvae (Fig. 5B, L3-CNS). Therefore, although the levels of Filamin 240 are too low to detect in whole larvae (L3), it is abundantly expressed in the CNS, raising the possibility that Dps may interact with both filamin isoforms within the CNS (Fig. 5B). Finally, we also examined the distribution of filamin forms in various adult tissues (Fig. 5C). We found that Filamin 90 is broadly expressed in all tissues examined with the exception of ovaries, where the levels are significantly lower. In contrast, Filamin 240, which contains the actin-binding domain, is enriched in ovaries and localizes to egg chamber ring canals (Sokol and Cooley, 1999). Finally, female sterile *cheerio* mutations have been shown to correspond to mutations in the gene encoding *Drosophila filamin*. These mutants reduce the levels of Filamin 240 expression in the ovary but have no apparent effect on Filamin 90.

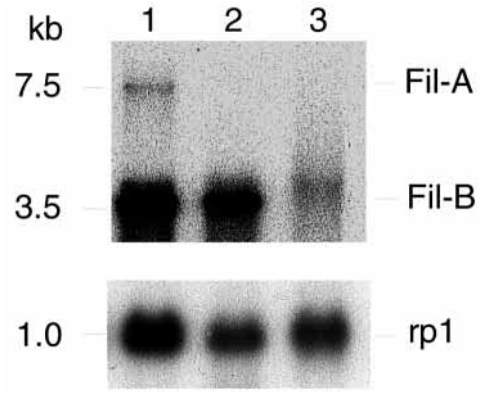
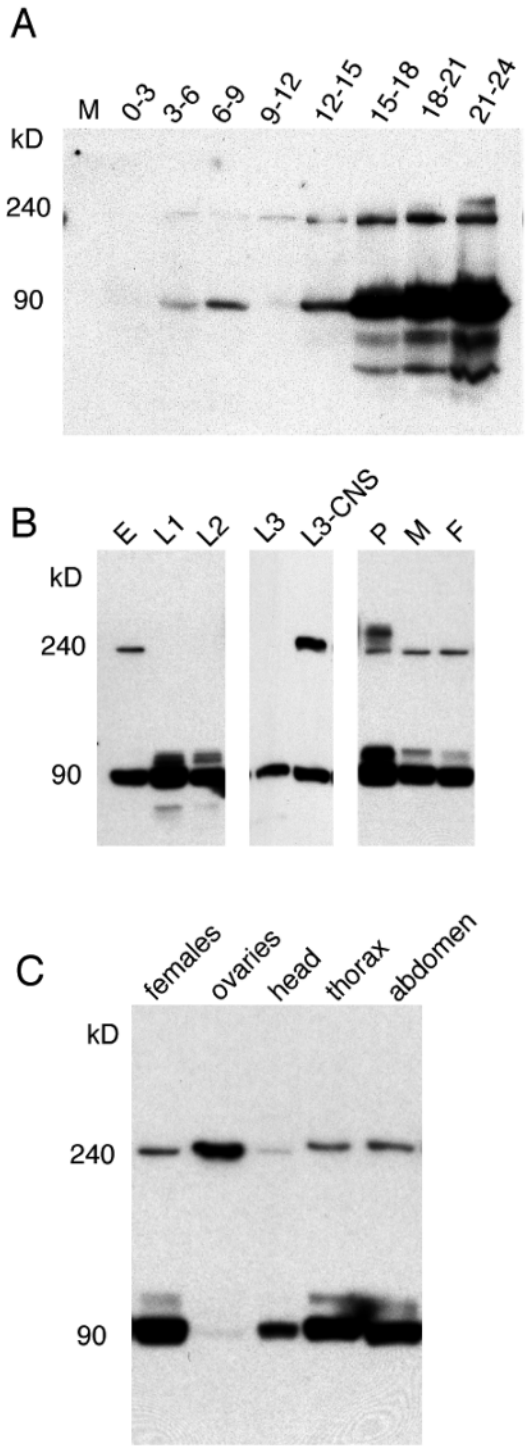


Fig. 4. *Drosophila* filamin is alternatively spliced and gives rise to two distinct forms. Poly(A)$^+$ RNA from embryos (lane 1), larvae (lane 2) and adults (lane 3) were electrophoresed, transferred to nitrocellulose and probed with a cDNA corresponding to the C-terminal domain of *Drosophila filamin*. We find two major transcripts of approximately 7.5 and 3.5 kb in embryos whereas only the 3.5 kb transcript is detectable in larvae and adults.

Fig. 5. *Drosophila* filamins are differentially expressed. We have examined the tissue distribution of the two filamin forms by western blotting using a filamin-specific antibody. (A) Expression of filamin forms during *Drosophila* embryogenesis. Both Filamin 240 and Filamin 90 are expressed at low levels during early embryogenesis (0-9 hours) but increase at later stages (15-24 hours).
(B) Developmental western blot. The distribution of both filamin forms was examined at different development stages. The total protein in each extract was quantitated by BCA and 25 μg was loaded into each lane. E, embryos; L1, first instar larvae; L2, second instar larvae; L3, third instar larvae; L3-CNS, CNS from third instar larvae; P, pupae; M, adult males; F, adult females. (C) Tissue distribution of filamin forms. The distribution of filamin forms in different tissues was determined after loading extracts containing 25 μg of total protein/lane. Filamin 240 is enriched in the CNS and ovarioles while Filamin 90 was more broadly expressed but is almost completely absent from ovaries.

Functional interaction of filamin with Dps

To determine if *Dps* and *filamin* could genetically interact in *Drosophila* we then examined the ability of filamin overexpression to modify a *Dps* dominant adult phenotype. To overexpress *filamin*, we used the line EP(3) 3715 that has an insertion of an EP element 5′ of *filamin* gene (see Fig. 3A). EP elements are P-elements that contain a GAL-4 responsive UAS element that render downstream genes responsive to GAL4 activation. To prove that this EP insertion could be used to



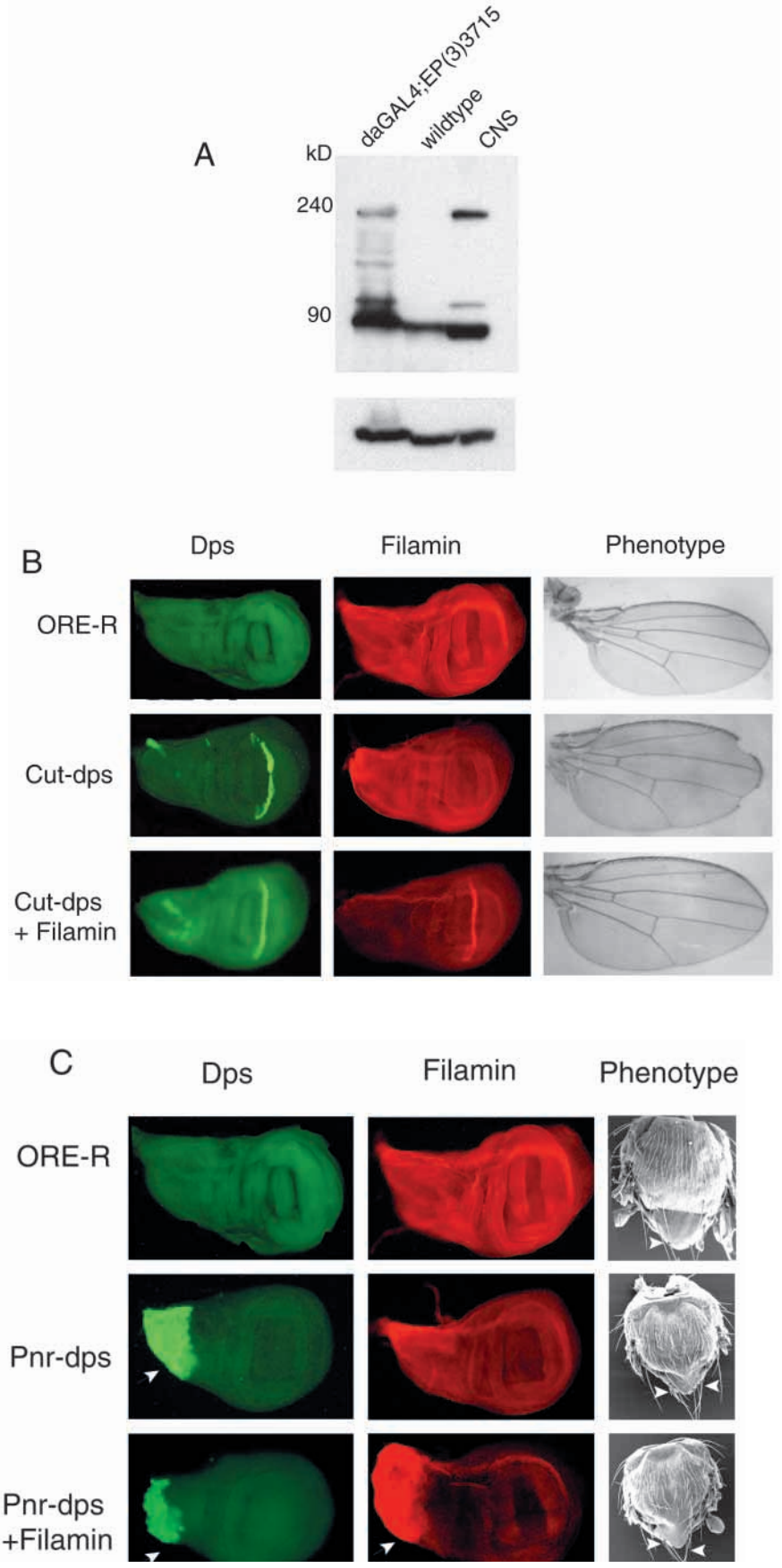


Fig. 6. (A) To demonstrate filamin overexpression we crossed flies that contained a daughterless-GAL4 driver to an EP line inserted into the 5′ end of filamin and examined the levels of filamin protein in extracts from whole third instar larvae on western blots. We find that both forms of filamins can be detected in progeny containing both the GAL4 driver and the EP insertion (daGAL4;EP(3)3715). In contrast, only Filamin 90 can be detected from the control line (wild type). The levels obtained by overexpression are comparable to those observed in the CNS of wild-type larvae (CNS). In the bottom panel the blot was reprobed with dNSF-2 that is expressed at equal levels in all tissues to demonstrate that equal amounts of protein were loaded in each lane. (B) The ability of filamin to interact with Dps was determined by crossing flies that contained a cut-GAL4 driver and UAS-Dps transgene to an EP insertion at the 5′ end of the filamin gene. The F_1 progeny from this cross would overexpress both Dps and filamin at the presumptive wing margin in third larval instar wing imaginal discs. Shown is the expression of Dps and filamin in wild-type (ORE-R), Cut-Dps and Cut-Dps + Filamin wing discs detected by immunostaining. Note that overexpression of Dps alone does not change the distribution or levels of filamin in wing discs. In contrast, F_1 progeny that contain both the Cut-Dps and EP line insertion do overexpress filamin at the margin. The phenotype of wild-type, *cut-Dps* and *cut-Dps + filamin* adult wings is also shown. Note that the notched wing phenotype produced by overexpression of Cut-Dps is suppressed when filamin is overexpressed in the same tissue. (C) The ability of filamin to interact with Dps was also determined by crossing flies that contained a pnr-GAL4 driver and UAS-Dps transgene to the EP insertion in the filamin gene. The F_1 progeny from this cross would overexpress both Dps and filamin in the dorsal region of the presumptive notum in third larval instar wing imaginal discs (see arrowheads). Shown is the expression of Dps and filamin in wild-type (ORE-R), Pnr-Dps and Pnr-Dps + Filamin wing discs, detected by immunostaining. Note that overexpression of Dps alone does not change the distribution or levels of filamin in the presumptive notum of wing discs. In contrast, F_1 progeny that contain both the Pnr-Dps and EP line insertion do overexpress filamin at the margin. The phenotype of wild-type, Pnr-Dps and Pnr-Dps + filamin adult notums is also shown. Note that the increase in scutellar macrochaetes produced by overexpression of Pnr-Dps (arrowheads) is suppressed when filamin is overexpressed in the same tissue.

overexpress *filamin*, we first crossed the EP line to a *daughterless*-GAL4 driver that is ubiquitously expressed during development and showed an increase in the levels of both Filamin 240 and Filamin 90 by western blotting that could not be detected in the control EP line alone (Fig. 6A). We then crossed the EP line to a recombinant line containing both a *cut*-GAL4 and UAS-*Dps* transgene. This line has a dominant notched wing phenotype due to the overexpression of *Dps* along the presumptive wing margin by the cut-GAL4 driver, during third instar larval imaginal disc development (Fig. 6B). In the progeny of this cross, the *cut*-GAL4 line would drive expression of both the UAS-*Dps* transgene and filamin via the EP line. We found that overexpression of filamin suppressed the dominant notched wing phenotype produced by overexpression of *Dps*. In contrast, overexpression of two independent EP lines or a UAS-lacZ transgene had no effect (data not shown), demonstrating that the suppression observed by overexpression of filamin is not due to dilution of the GAL4 driver. Similar effects were also observed using a *pnr*-GAL4 driver that causes a dominant adult bristle phenotype when crossed to UAS-*Dps*, suggesting that the ability of overexpression of filamin to suppress the *Dps* phenotype is not tissue specific (Fig. 6C). Taken together, these experiments demonstrate that filamin can functionally interact with Dps during development.

DISCUSSION

Presenilins are a novel class of transmembrane domain proteins that were first identified because mutations in them are causative factors in FAD; more recently, they have also been implicated in Notch signaling events during normal development. To gain insight into the mechanisms underlying presenilin function, we and others have used yeast two-hybrid techniques to identify presenilin-binding proteins. Many of these studies have used the hydrophilic loop domain between TM6 and TM7 of presenilins as bait since it is oriented to the cytoplasm and therefore accessible to interacting proteins; it is also the site of endoproteolytic cleavage and, more importantly, it is the site of numerous FAD mutations. However, although presenilins are proteolytically cleaved in most species, both the length and amino acid composition of the loop are poorly conserved, raising the possibility that this loop is not a functionally important domain of the protein. Here we have shown that the loop domain from two Dps isoforms that differ by only 14 amino acids within this region, bind to the C-terminal domain of the actin-binding protein filamin. More importantly, we show that *Drosophila* filamin can also bind to the loop domain from human PS1 and PS2, indicating that the interaction domains on both proteins must be conserved. During the course of these studies, another report demonstrated that human PS1 and PS2 proteins interact with human filamins (Zhang et al., 1998), emphasizing the universality of this interaction. Interestingly, there is very poor sequence conservation between the cytoplasmic loop domain of *Drosophila* and human presenilins, suggesting that a structural motif is responsible for the interaction between presenilins and filamin.

The membrane of cells is lined by a network of actin filaments crosslinked by a variety of proteins. Filamin, also known as ABP-280, is one of these crosslinking proteins that consists of a dimer composed of two identical subunits. At the N terminus of the protein is an actin-binding domain whose amino acid sequence is similar to that of other actin binding proteins including α-actinin, β-spectrin and dystrophin. This is followed by a backbone composed of 24 repeats, each of approximately 96 residues, separated by two hinge regions between repeats 15-16 and 23-24. The C-terminal domain of filamin is critical for dimerization and attachment to integral membrane proteins, thereby providing a direct link between the membrane and actin filaments. Interestingly, *Drosophila* has two distinct forms of filamin that are differentially expressed and arise from alternative splicing of a single gene. The longest form, Filamin 240, shares 41% overall amino acid identity to vertebrate ABP-280. In contrast, Filamin 90 is much shorter and does not contain an actin binding domain. Both forms contain the C-terminal domain that binds to presenilin. Our current studies demonstrate that Dps and filamin are expressed in overlapping patterns during development and that both filamin and Dps are enriched in the central nervous system. It will be interesting to determine if binding of Dps to different filamin forms mediates different functions during development or in different tissues.

Filamin has been implicated in numerous cellular functions. Although a major function is predicted to be linking membrane receptors and the actin cytoskeleton, filamin has also been shown to regulate the formation of actin stress fibers and adhesive contacts and to bind to other regulators of the cytoskeleton. Here, we show that filamin can also bind to presenilins. Previous studies have also shown that presenilins can bind to Notch (Ray et al., 1999), suggesting that these three proteins may form a larger complex. Interestingly, we find that overexpression of Dps gives rise to a Notch loss-of-function phenotype at the wing margin and on the notum. Since Dps would be a central component of this larger complex, increasing the levels of Dps could be predicted to reduce the formation of this complex in a dominant-negative manner by saturating individual binding sites on filamin and Notch. In this study we show that overexpression of filamin can suppress dominant phenotypes produced by Dps overexpression. This may indicate that overexpression of filamin does so by restoring the balance between the available Dps molecules that can bind to Notch and filamin.

How such a complex would then regulate Notch signaling is still unclear. Filamin binding to Dps could provide a link between the Notch receptor and the actin cytoskeleton that may be required for intracellular trafficking events. Consistent with this model, we have previously shown that both mutations in *Dps* and overexpression of *Dps* can affect the subcellular distribution of Notch (Guo et al., 1999). Filamin binding to Dps could also function as a link between membrane-bound substrates such as Notch or APP and proteases known to affect their proteolytic processing. As such, it is interesting to note that filamin has been shown to bind to the cytosolic domain of furin through its internal repeats (Liu et al., 1997). Furin is a protease that catalyzes the proteolytic maturation of many proproteins within the trans-Golgi network and endosomal system. Specifically, filamin has been shown to directly tether furin molecules to the cell surface and modulate the rate of furin internalization and the processing of furin substrates within the endocytic

pathway, but does not affect its activation in the biosynthetic pathways (Liu et al., 1997). Whether filamin can also recruit other proteases to their substrates remains unclear. Recent studies have demonstrated that presenilin is found in a high molecular weight complex that contains γ-secretase activity (Esler et al., 2000; Li et al., 2000a,b). Whether presenilin encodes the γ-secretase itself or acts as a cofactor in its activity remains unclear, as does the identity of the other proteins within the complex. It will be interesting to determine if filamin is a component of this complex and acts as a link between the γ-secretase and its substrates.

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