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Presenilin-Based Genetic Screens in *Drosophila melanogaster* Identify Novel Notch Pathway Modifiers

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ABSTRACT

Presenilin is the enzymatic component of γ-secretase, a multisubunit intramembrane protease that processes several transmembrane receptors, such as the amyloid precursor protein (APP). Mutations in human Presenilins lead to altered APP cleavage and early-onset Alzheimer's disease. Presenilins also play an essential role in Notch receptor cleavage and signaling. The Notch pathway is a highly conserved signaling pathway that functions during the development of multicellular organisms, including vertebrates, Drosophila, and *C. elegans*. Recent studies have shown that Notch signaling is sensitive to perturbations in subcellular trafficking, although the specific mechanisms are largely unknown. To identify genes that regulate Notch pathway function, we have performed two genetic screens in Drosophila for modifiers of Presenilin-dependent Notch phenotypes. We describe here the cloning and identification of 19 modifiers, including *nicastrin* and several genes with previously undescribed involvement in Notch biology. The predicted functions of these newly identified genes are consistent with extracellular matrix and vesicular trafficking mechanisms in Presenilin and Notch pathway regulation and suggest a novel role for γ-tubulin in the pathway.

THE Presenilin genes encode eight-pass transmembrane proteins found in most metazoans, including mammals, Drosophila, and *Caenorhabditis elegans* (reviewed in Selkoe 2000; Wolfe and Kopan 2004). In humans, mutations in the two Presenilin genes, PS1 and PS2, account for the majority of familial early-onset Alzheimer's disease (reviewed in Tanzi and Bertram 2001). Presenilin is the catalytic component of the γ-secretase complex that is responsible for the cleavage of the transmembrane protein, amyloid precursor protein (APP) (reviewed in De Strooper 2003). APP cleavage, first by β-secretase and subsequently by γ-secretase, results primarily in the release of the 40-amino-acid amyloid β-peptide (Aβ40). Alzheimer's disease-associated mutations in PS1 or PS2 subtly alter this cleavage pattern, causing increased production of a longer, more cytotoxic form of the amyloid β-peptide (Aβ42). Aβ-peptides are the major component of amyloid plaques in the brains of Alzheimer's disease patients. Higher Aβ42 levels are thought to accelerate the aggregation of Aβ into toxic oligomers and the deposition of extracellular plaque material (reviewed in Wolfe and Haass 2001; Selkoe 2004).

The γ-secretase complex is composed of at least three proteins in addition to Presenilin: nicastrin, aph-1, and pen-2 (Yu *et al.* 2000; Francis *et al.* 2002; Goutte *et al.* 2002). These four transmembrane proteins constitute the γ-secretase core complex, yet little is known about its regulation and activity. γ-Secretase recognizes and cleaves a growing list of transmembrane proteins with very short extracellular domains generated by prior processing (Struhl and Adachi 2000; reviewed in De Strooper 2003; Wolfe and Kopan 2004). A

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functional role for γ-secretase cleavage has not been demonstrated for most substrates. In such cases, γ-secretase may serve simply to eliminate transmembrane stubs of proteins after extracellular domain shedding (Struhl and Adachi 2000). However, in the case of the Notch family of receptors, γ-secretase plays an essential role in signaling. Genetic studies in *C. elegans* initially established that Presenilin is required for Notch pathway signaling (Levitan and Greenwald 1995; Li and Greenwald 1997), and this has now been confirmed in Drosophila, mouse, and human systems (reviewed in Wolfe and Kopan 2004). Following ligand binding and subsequent cleavage of Notch by ADAM/TACE proteins, γ-secretase cleavage of Notch results in the release of the Notch intracellular domain (NICD). NICD translocates to the nucleus where it activates transcription of target genes in conjunction with the Suppressor of Hairless [Su(H)] and mastermind proteins (reviewed in Baron 2003; Kadesch 2004; Weng and Aster 2004). Notch signaling is involved in a wide variety of cell signaling events in development and in the regeneration and homeostasis of adult tissues. Defects in Notch signaling have been linked to a number of human developmental syndromes and cancers, including Alagille syndrome, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), and T cell acute lymphoblastic leukemia/lymphoma (reviewed in Gridley 2003; Weng and Aster 2004).

In Drosophila, Notch signaling is required during most stages of development and functions in many cell fate specification events in the wing, bristle, and eye (reviewed in Muskavitch 1994; Artavanis-Tsakonas *et al.* 1999; Portin 2002). *Presenilin* (*Psn*) and *nicastrin* (*nct*) loss-of-function mutations in Drosophila have been shown to cause similar developmental defects (Guo *et al.* 1999; Struhl and Greenwald 1999; Ye *et al.* 1999; Hu *et al.* 2002; Lopez-Schier and St. Johnston 2002).

Genetic screens in Drosophila and *C. elegans* have identified many proteins in the Notch pathway. These include Delta/Serrate/Lag-2 type ligands, cytoplasmic/nuclear proteins such as Su(H) and mastermind, and Notch-regulated target genes such as the *Enhancer of split complex* genes (Kimble and Simpson 1997; Greenwald 1998; reviewed in Baron 2003). Recently, proteins involved in modification, trafficking, and degradation of Notch pathway components have begun to be elucidated, including proteases (furin, kuzbanian, TACE), enzymes involved in glycosylation and/or in chaperone function (fringe, O-fut), members of the ubiquitin machinery (neuralized, mindbomb, deltex, fat facets), and clathrin-coated pit components (dynamin, clathrin, epsin, α-adaptin) (reviewed in Haltiwanger and Stanley 2002; Baron 2003; Schweisguth 2004; Le Borgne *et al.* 2005; see also Cadavid *et al.* 2000; Okajima *et al.* 2005). Notch signaling appears to be particularly sensitive to alterations in subcellular trafficking. Genes involved in vesicular trafficking have been implicated in the activation of Delta, in Notch dissociation and *trans*-endocytosis, and in Notch degradation (reviewed in Le Borgne *et al.* 2005). The molecular mechanisms that underlie the requirements for these genes in Notch signaling remain largely unknown.

We have performed two screens in Drosophila to identify genes that interact with Presenilin and the Notch signaling pathway. By screening for modifiers of *Psn* hypomorphic alleles, we hoped to isolate genes that might directly regulate Presenilin activity. The first screen employed a small deletion within *Psn* (Psn^{143}) to identify genes that result in dominant Notch pathway mutant phenotypes in the presence of the Psn^{143} heterozygote. In this screen we recovered a *Psn* hypomorphic allele, Psn^{9}, as well as several other second-site modifiers. The second screen utilized the viable *Psn* hypomorphic genotype, Psn^{9}/Psn^{143}, to screen for second-site enhancers and suppressors of the Psn^{9}/Psn^{143} small, rough eye. We recovered a total of 23 complementation groups and successfully identified 19 genes. These genes include *nct*, other known Notch interactors, and several genes with previously undescribed involvement in Notch or Presenilin biology, including genes with roles in the extracellular matrix (ECM), Notch transcriptional activity, and vesicular trafficking.

MATERIALS AND METHODS

Drosophila handling and isolation of the Psn^{143} allele: All fly stocks and crosses were handled using standard procedures at 25°, unless otherwise noted. Psn^{143} was generated from a lethal screen performed against deficiency Df(3L)rdgC-co2 [77A1;77D1; Bloomington Stock Center (BSC) stock 2052], which uncovers the region containing *Psn* (77C3) (data not shown). The Psn^{143} allele contains a 268-bp deletion that removes amino acids 136–224. There are no associated phenotypes in Psn^{143} heterozygotes; homozygotes exhibit pupal lethality, which can be rescued by a wild-type *Psn* transgene (data not shown).

Screen A: Isogenic w^{1118} males were mutagenized by overnight feeding of 25 mm EMS in a 10% sucrose solution after a 2-hr starvation period. Mutagenized males were mated to w^{1118}; Psn^{143} *FRT*(w^{+})*(2G)/TM6B Hu Tb* virgin females (Figure 1A). F_1 $Psn^{143}/+$ progeny were scored for dominant Notch pathway phenotypes. This screen generated a *Psn* hypomorph allele, Psn^{9}. The Psn^{9} chromosome carries an extraneous lethal mutation uncovered by deficiency Df(3R)Antp17 (84A6–D14; BSC stock 1842).

Screen B: *F_1 screen (Figure 1B):* w^{1118}; Psn^{143}/*TM6B* males were mutagenized with EMS as above or by exposure to X-ray irradiation (4000 rad) using the Faxitron X-ray cabinet system. Mutagenized males were mated with *winscy* (y^{c4} sc^{8} sc^{S1} w^{1}); sp^{2}; Psn^{9} e^{11}/*TM6B* virgin females. Reverse crosses were also conducted [mutagenized w^{1118}; Psn^{9}/*TM6B* males were crossed to *winscy*; sp^{2}; Psn^{143} *FRT* (w^{+}) e^{11}/*TM6B* virgin females]. Psn^{9}/Psn^{143} F_1 progeny were scored for modification of the Psn^{9}/Psn^{143} small, rough eye and for other *Psn*-dependent Notch pathway phenotypes.

F_2 screen (Figure 1B): Balanced F_1 male siblings (*winscy*; +/sp^{2}; Psn^{143} or Psn^{9}/*TM6B*) carrying mutagenized chromosomes were collected and mated as above to the reciprocal *Psn* allele. A small number of F_1 female siblings were also collected and

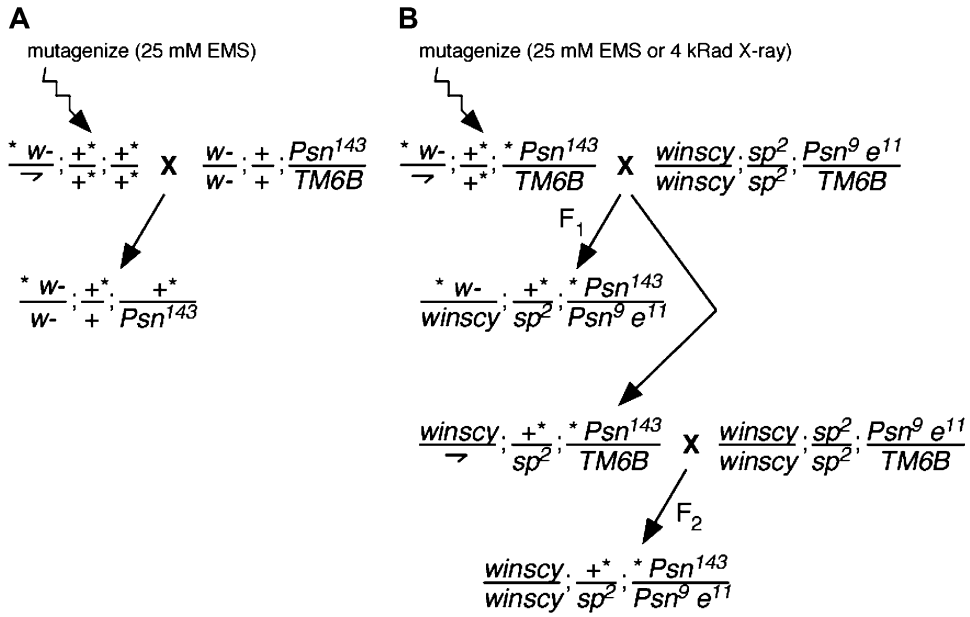

FIGURE 1.—(A) Screen A. F_1 progeny were screened for Notch pathway phenotypes in the presence of $+/Psn^{143}$. (B) Screen B. F_1 or F_2 progeny were screened for enhanced small eye or other Notch pathway phenotypes in the presence of Psn^{9}/Psn^{143}.

mated to recover modifiers on the X chromosome. F_2 progeny were scored for *Psn* modifier phenotypes. These crosses enjoyed a much higher rate of fertility than did the F_1 crosses and resulted in the retention of increased numbers of modifiers, including lethal interactors in the Psn^{9}/Psn^{143} background.

Complementation analysis and mapping procedures: Mutations on chromosomes 2 and 3 that were homozygous lethal or homozygous viable with a visible phenotype were analyzed in standard complementation matrices. Complementation for modifiers on the third chromosome (which also carries a copy of either Psn^{9} or Psn^{143}) could be assessed only as *Psn trans*-heterozygotes, because both Psn^{9} and Psn^{143} chromosomes are homozygous lethal.

Two representatives from each complementation group were mapped via recombination with *P*-element-containing chromosomes to identify a candidate region of 8–10 Mb. This was followed by sequence analysis of single-nucleotide polymorphisms (SNPs) to narrow the region to 1–2 Mb (HOSKINS *et al.* 2001). High-resolution mapping using SNP analysis on recombinants generated between two flanking *P* elements (each marked with a miniwhite gene) usually narrowed the candidate region to 25–200 kb (HOSKINS *et al.* 2001). Recombinant chromosomes were scored for lethality with other complementation group members and for the original modification phenotype in the Psn^{9}/Psn^{143} or $+/Psn^{143}$ background. The length of the SNP-defined intervals containing each modifier gene is indicated in Table 2. We sequenced most or all genes within these regions. For complementation groups, gene identification was considered valid if mutations were identified in at least two members and were not present in the parental strain. For the two genes represented by single alleles, identification was considered valid if mutations were not present in the parental strain and were confirmed by noncomplementation with known alleles. Additional evidence for candidate regions came from deficiency mapping and, whenever possible, candidate genes were confirmed by lack of complementation with known alleles.

Some X-ray-induced modifiers were identified cytologically by utilizing *in situ* hybridization on polytene chromosomes to molecularly define rearrangements and generate narrow regions of interest (PARDUE 1986). *dp* alleles were meiotically mapped and identified by their notal pit phenotypes and via lack of complementation with known alleles. *H* and *S* were identified by their dominant phenotypes and by lack of complementation with known alleles (see Table 2 for details).

Scanning electron microscopy: Adult flies stored in 70% ethanol were dehydrated through an ethanol series, dried using hexamethyldisilazane (BRAET *et al.* 1997), mounted on stubs, and sputter coated with a 20-nm coat of gold/palladium in an E5400 Sputter Coater. Prepared tissue was viewed and photographed on either an Electroscan E3 ESEM or an ISI DS-130.

Immunohistochemistry: mAb22C10, which recognizes futsch, a cytoplasmic protein primarily expressed in neuronal cells, was the kind gift of Seymour Benzer (California Institute of Technology, Pasadena, CA). For antibody staining of pupal wings, pupae were removed from their cases at ~30 hr after puparium formation and fixed in 4% paraformaldehyde for 30 min. Pupal wings were then removed from the cuticle and fixed for an additional 30 min before washing and staining with mAb22C10 diluted 1:100 (as in PARKS *et al.* 1995 without silver enhancement).

RESULTS

***Psn* modifier screens identify 19 genes:** We conducted two screens to identify enhancers and suppressors of Presenilin-dependent Notch-like phenotypes. Flies heterozygous for the null allele Psn^{143} display no visible phenotypes. In screen A (Figure 1A), we screened for mutations causing dominant Notch mutant phenotypes in the $+/Psn^{143}$ background. These phenotypes include eye reduction and roughness, wing margin loss, ectopic wing veins and vein thickening, and gain or loss of sensory bristles. Twenty-four modifiers of Psn^{143} were recovered from 18,332 F_1 progeny screened (Table 1). We sequenced the *Psn* genomic region from viable third chromosome mutants and identified a missense mutation, L499Q, in one allele, which we named Psn^{9}. Psn^{9}/Psn^{143} flies have a reduced and roughened eye (Figure 2D) and distal wing vein thickening (Figures 3E and 4C),

TABLE 1

Summary of screen hits

Screen	No. screened[a]	No. primary hits[b]	No. final hits[c]
Screen A	18,332	295	24
Screen B (F_1)	67,079	1,266	73
Screen B (F_2)	8,821	366	171
Totals	94,232	1,927	268

[a] Number of critical class (screen A, Psn^{143}/+; screen B, Psn^{9}/Psn^{143}) flies screened.
[b] Number of initial modifiers recovered during primary screening.
[c] Number of viable, fertile modifiers that retested.

consistent with reduced Presenilin function and Notch signaling in eye and wing development. The Psn^{9} mutation resides in the eighth transmembrane domain of Presenilin, which forms part of the C-terminal PAL domain that is critical for γ-secretase activity. This domain is highly conserved in the Presenilins and in homologous transmembrane proteases (reviewed in Brunkan and Goate 2005). Missense mutations in the residues directly flanking L425 (equivalent to Drosophila L499) have been observed in human PS1 in familial Alzheimer's disease patients. In subsequent experiments, the eye phenotype associated with Psn^{9} was mapped via meiotic recombination to the interval 73A–83A that contains the *Psn* gene. Together, these observations implicate the Psn^{9} allele as a hypomorphic allele responsible for the reduced eye phenotype observed in Psn^{9}/Psn^{143}. Additional modifiers from screen A that did not carry *Psn* mutations were characterized along with the modifiers from screen B (see below).

In the F_1 generation of screen B (Figure 1B), we screened for enhancement or suppression of the small, rough eye phenotype present in Psn^{9}/Psn^{143} as well as for the presence of any other Notch-like phenotypes. In addition, an F_2 screen was performed by crossing F_1 generation $Psn^{-}/TM6B$ males heterozygous for mutagenized chromosomes (Figure 1B) to females carrying the reciprocal *Psn* allele. Their progeny were scored for Notch-like phenotypes as well as lethality. We recovered 244 modifiers from 75,900 total *trans*-heterozygous progeny from the F_1 and F_2 portions of screen B (Table 1).

From these modifiers, we identified 21 lethal complementation groups and two complementation groups displaying phenotypic interactions. In total, 19 modifier genes were successfully mapped and identified, including 17 defined by complementation groups and two genes represented by single alleles (Table 2). Additionally we recovered secondary mutations in the *Psn* gene in *cis* to the Psn^{9} mutation. These mutations, like *Psn* deficiencies, are lethal in *trans* to Psn^{143}, suggesting that they are severe hypomorphic or null alleles.

Nicastrin, a γ-secretase core complex member: We recovered seven alleles of the γ-secretase core complex gene, *nct*, from screen B. These *nct* alleles enhance the Psn^{9}/Psn^{143} reduced eye (Figure 2E) and wing vein thickening and also exhibit wing notching (data not shown). They show no phenotype as heterozygotes in *cis* with either *Psn* allele alone. Two alleles, $nct^{SGE\text{-}9}$ and $nct^{SIE\text{-}22}$, have missense mutations that result in substitutions in two adjacent amino acids (Table 2). This region is located two amino acids upstream of an aspartate conserved in the bacterial zinc aminopeptidase and glutaminyl cyclase G-protein families. These two amino acids may be critical for nicastrin function as part of a putative catalytic or structural domain involved in either assembly of the active Presenilin complex or interactions with γ-secretase substrates such as Notch, Delta, or APP.

Although this screen isolated *nct* alleles, we note that the background is not sensitive enough to recover all γ-secretase complex members. Heterozygosity for a recessive lethal *aph-1* allele, $aph\text{-}1^{D35}$, does not modify Psn^{9}/Psn^{143}. However, $aph\text{-}1^{D35}$ did show interactions with $nct^{SGE\text{-}9}$ Psn^{9}/Psn^{143}, including slightly smaller, rougher eyes and severe loss of abdominal bristles (data not shown).

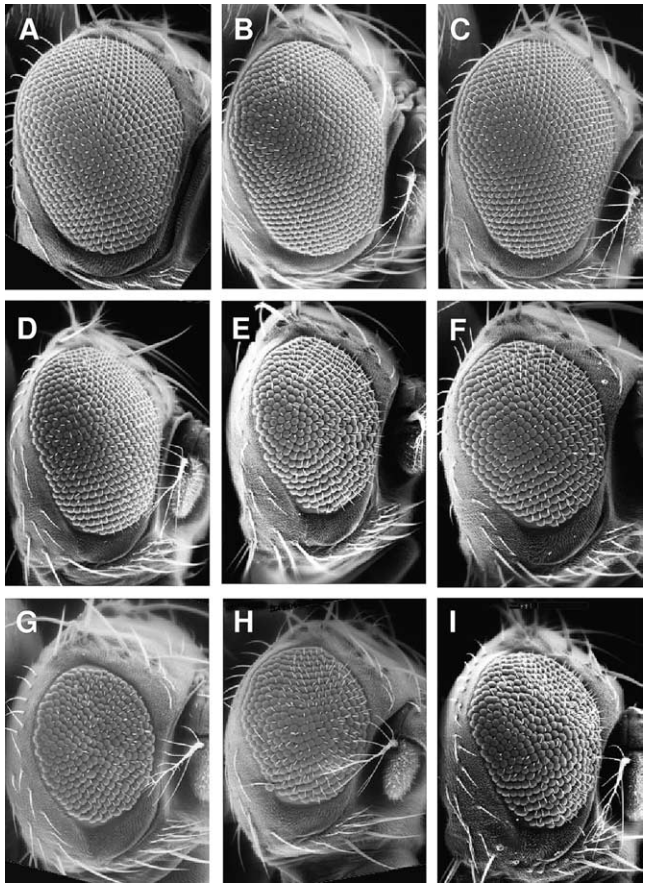

Figure 2.—Enhancers of Psn^{9}/Psn^{143} have smaller and/or rougher eyes. Scanning electron micrographs of adult eyes from control, Psn^{9}/Psn^{143}, and a selection of modifiers are shown. (A) Wild type. (B) Psn^{143}/+. (C) Psn^{9}/+. (D) Psn^{9}/Psn^{143}. (E) Psn^{143} $nct^{SGE\text{-}9}/Psn^{9}$. (F) Psn^{143} $AP\text{-}47^{SHE\text{-}11}/Psn^{9}$. (G) γ-$Tub^{bmps4}$/+; Psn^{9}/Psn^{143}. (H) $Spt5^{SIE\text{-}27}$/+; Psn^{9}/Psn^{143}. (I) $opa^{SIE\text{-}19}$/+; Psn^{9}/Psn^{143}.

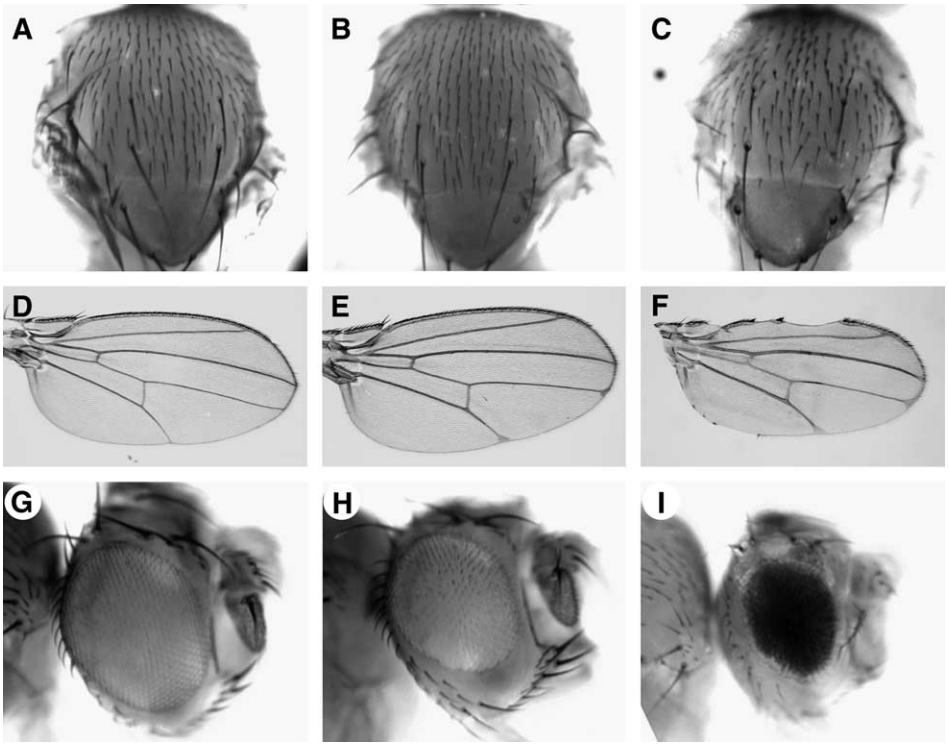

FIGURE 3.—Reductions in AP-47 function enhance *Psn* loss-of-function phenotypes in multiple tissues. (A, D, and G) Wild type. (B, E, and H) Psn^{9}/Psn^{143}. (C, F, and I) Psn^{9} $AP\text{-}47^{SAE\text{-}10}/Psn^{143}$ $AP\text{-}47^{SHE\text{-}11}$. In addition to a smaller, slightly rougher eye (H), Psn^{9}/Psn^{143} flies display slight wing vein thickening (E); the notum is relatively normal (B). In contrast, Psn^{9} $AP\text{-}47^{SAE\text{-}10}/Psn^{143}$ $AP\text{-}47^{SHE\text{-}11}$ flies have much smaller and rougher eyes (I) (the w+ eye is derived from a recombinant chromosome carrying a w+ P element generated during mapping). They also display dorsal and ventral wing notching (F), enhanced wing vein thickening (F), and notal bristle (microchaetae) loss (C).

Future screens using modifiers of this genotype or a clonal screen to recover recessive modifier mutations may yield additional regulators of Presenilin and the Notch pathway.

Modifiers with established roles in Notch signaling: In addition to *nct*, we identified a number of genes with well-characterized roles in Notch signaling. Nine alleles of *Delta* (*Dl*), one of the two Drosophila Notch ligands, were isolated either as enhancers of the Psn^{9}/Psn^{143} eye or wing phenotype or as lethal interactors. All display dominant vein thickening at the wing margin, a common *Dl* mutant phenotype, in a $Psn^{9}/+$ or a $Psn^{143}/+$ background. Interestingly, mutations in a number of alleles affect cysteine residues in epidermal growth factor (EGF)-like repeats (ELRs) 4, 6, 7, 8, and 9. Previous results indicate that mutations in many of the *Dl* ELRs are correlated with loss-of-function phenotypes and abnormal subcellular Delta distribution (PARKS *et al.* 2000; J. R. STOUT, A. DOS SANTOS and M. A. T. MUSKAVITCH, personal communication).

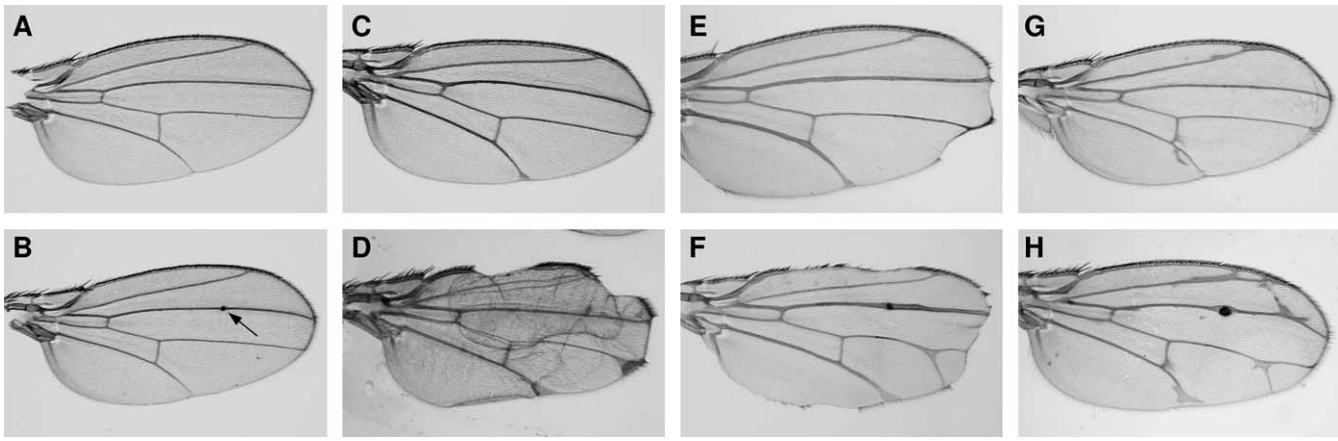

FIGURE 4.—Wing phenotypes caused by loss of Presenilin, Notch, or Delta function are enhanced by mutations in γ-*Tub23C*. (A) Wild type. (B) $\gamma\text{-}Tub23C^{bmps4}/+$ wings display small- to moderate-sized bumps (arrow points to small bump), usually along wing veins, and occasional nicking (not shown). (C) Psn^{9}/Psn^{143} wings show slight vein thickening at the wing margin. (D) $\gamma\text{-}Tub23C^{bmps4}/+$; Psn^{9}/Psn^{143} wings display notching and enhanced vein thickening compared to C. (E) $N^{81k1}/+$ wings have distal notching and wing vein thickening. (F) $N^{81k1}/+$; $\gamma\text{-}Tub23C^{bmps4}/+$ wings have enhanced notching (dorsal, ventral, and distal) compared to E and both thickened and ectopic veins. (G) $Dl^{eA7}/+$ wings show some vein thickening as well as small amounts of ectopic vein. (H) $\gamma\text{-}Tub23C^{bmps4}/+$; $Dl^{eA7}/+$ wings develop more ectopic vein and have mildly enhanced wing vein thickening compared to G.

TABLE 2

Mapped and identified modifiers

Gene	Cytological site	Accession no.	Allele[a]	Mutations[b]	Phenotypes in *Psn* mutant background[c,d]	Gene identification methods (maximal interval defined by SNPs, phenotype mapped)[e]
dac	36A1	NM_165161	MEE-2*	367–794 DEL and FS	Enhanced eye	Meiotic (40 kb, enhanced eye) and noncomplementation with *dac[3]* and *dac[4]*
			MHE-1	Y259N	Enhanced eye	
			SFE-8*	C272Y; 887–1074 DEL and FS	Enhanced eye	
dp	24F4–25A1	NM_175960.2	MFE-1*	Not determined	Notal pits	Meiotic (7 Mb, notal pits) and noncomplementation with *CyO dp[lvI]*, *Df(2L)dp-h25*, *dp[olvR]*, *Tp(2;3)dp[h27]*, and *dp[olvDG10]*
			PGE-8	Not determined	Enhanced eye	
			PHE-5	Not determined	Lethal	
			PIE-10*	Not determined	Notal pits	
eya	26E1–E2	NM_078768	SJE-1	Translocation	Enhanced eye	Cytology, *in situ*
			SLE-2	Not determined	Enhanced eye	
γ-*Tub23C*	23C3–C4	NM_057456	bmps1[d,*]	M382I	Enhanced eye, wing bumps and notching	Meiotic (26.1 kb, enhanced eye)
			bmps2*	M382I	Enhanced eye, wing bumps and notching	
			bmps3	P358L	Enhanced eye, wing bumps and notching, vein thickening	
			bmps4	P358L	Enhanced eye, wing bumps and notching, vein thickening	
			bmps5	P358L	Enhanced eye, wing bumps and notching, vein thickening	
so	43C1	NM_057385	SHE-7*	R274Q	Enhanced eye	Meiotic (48 kb, enhanced eye)
			SKE-1[d,*]	281–416 DEL	Enhanced eye	
Spt5	56D5–D7	NM_144353	MGE-3*	K236*	Enhanced eye	Meiotic (36.2 kb, enhanced eye)
			SIE-27*	Q632*	Enhanced eye	
S	21E2–E3	NM_078726	16 alleles	Not determined	Enhanced eye	Noncomplementation with *S[BTE]*
vg	49E1	NM_078999	PHE-4	Not determined	Wing notching	Noncomplementation with *Df(2R)vg133* and *vg[1]*
			SDE-1	Splice @435	Enhanced eye, wing notching	
			SGE-4	Q349*	Wing notching	
			SGE-11	Not determined	Enhanced eye, wing notching	
AP-47	85D24	NM_141649	SAE-10*	S366N	Enhanced eye, vein thickening	Meiotic (85 kb, enhanced eye)
			SHE-11*	146–158 DEL and FS	Enhanced eye, vein thickening, low penetrance pupal lethal	
Dl	92A1–2	NM_057916	MIE-19	W252*	Lethal	Meiotic (86 kb, enhanced eye) and noncomplementation with known alleles
			MIE-22*	C433S; C506W	Enhanced eye, vein thickening	
			PFE-5	C488Y	Lethal	
			PFE-6	1–20 DEL	Enhanced eye, vein thickening	
			PGE-2	1st exon deleted	Enhanced eye, vein thickening	
			SDE-4*	C538R	Enhanced eye, vein thickening	
			SHE-5	C362Y	Wings held out, wing notching	
			SHE-11	Splice @124	Enhanced eye, vein thickening, low penetrant pupal lethal	
			SIE-36	Translocation	Vein thickening, low penetrant pupal lethal	

(*continued*)

TABLE 2

(Continued)

Gene	Cytological site	Accession no.	Allele[a]	Mutations[b]	Phenotypes in *Psn* mutant background[c,d]	Gene identification methods (maximal interval defined by SNPs, phenotype mapped)[e]
gl	91A3	NM_057506	SAE-9[d]	R466*	Enhanced eye	Noncomplementation with *gl[2], gl[3]*
H	92F3	NM_079694	PFE-1	Not determined	Bristle shaft to socket transformations, vein loss	Noncomplementation with known alleles
			PGE-1	Not determined	Bristle shaft to socket transformations, vein loss	
			PGE-4	Not determined	Bristle shaft to socket transformations, vein loss	
			PGE-6	Not determined	Bristle shaft to socket transformations, vein loss	
			SDS-1	Not determined	Suppressed eye, bristle shaft to socket transformations, vein loss	
			SLS-1	Not determined	Suppressed eye, bristle shaft to socket transformations, vein loss	
hh	94E1	NM_079735.3	PIE-7*	G257D	Enhanced eye, extra vein material between veins 2 and 3	Meiotic (161 kb, enhanced eye) and noncomplementation with known alleles
			SCE-2	Translocation	Enhanced eye, wing notching	
			SFE-4	284–462 DEL and FS	Enhanced eye	
			SGE-2	N141I	Enhanced eye	
			SHE-2*	S286N	Enhanced eye	
			SLE-1*	Uncharacterized deletion	Enhanced eye	
kkv	83A1	NM_079509	A1	G824D	Enhanced eye	Cytology, *in situ* on a transposition that fails to complement this group[f]
			BM1	P764N	Enhanced eye	
			SIE-29	I1043N	Enhanced eye	
Nsf2	87F15	NM_176499	A6[d,*]	A597V	Enhanced eye	Meiotic (24 kb, enhanced eye)
			A15*	555–641 DEL and FS	Enhanced eye	
nct	96B1	NM_143040	PIE-6	Point mutation in 3′ UTR	Enhanced eye	Meiotic (1.46 Mb, enhanced eye)
			SGE-3	K476*	Enhanced eye	
			SGE-8	Q177*	Enhanced eye, wing vein thickening and notching	
			SGE-9	R273C	Enhanced eye, wing vein thickening and notching	
			SGE-10*	Uncharacterized deletion	Enhanced eye	
			SIE-28*	Not determined	Enhanced eye, wing vein thickening and notching	
			SIE-22	C272Y	Enhanced eye, wing notching, missing microchaetae	
Opa	82E1	NM_079504	A3	Uncharacterized deletion	Enhanced eye, tufted vibrissae	Cytology, *in situ*
			B28	Y275N	Enhanced eye, tufted vibrissae	
			SIE-19	Y242D	Enhanced eye, tufted vibrissae	
			SIE-24	Q104*	Enhanced eye, tufted vibrissae	
			SJE-2	Translocation	Enhanced eye, tufted vibrissae, wing notching	
			SKE-5	Uncharacterized deletion	Enhanced eye, tufted vibrissae	

(*continued*)

TABLE 2

(Continued)

Gene	Cytological site	Accession no.	Allele[a]	Mutations[b]	Phenotypes in *Psn* mutant background[c,d]	Gene identification methods (maximal interval defined by SNPs, phenotype mapped)[e]
Psn	77C3	NM_079460	9	L499Q	Enhanced eye	Sequencing lethal and viable noncomplementers
			DIE-2[g]	Q244*	Lethal	
			MIE-5[g]	Not determined	Lethal	
			MIE-12[g]	Q523*	Lethal	
			MIE-15[g]	Not determined	Lethal	
			PIE-11[g]	G446S	Lethal	
			SIE-5[g]	D110L	Lethal	
R	62B7	NM_057509	PIE-6[d]	L120F	Enhanced eye	Noncomplementation with *R[1]*
Ras85D	85D21	NM_057351	MAE-2*	M67I	Enhanced eye	Meiotic (106 kb, enhanced eye) and noncomplementation with *Ras85[D06677]* and *Ras85[D05703]*
			MDE-7*	G60S	Enhanced eye	

[a] Meiotically mapped alleles are indicated by an *.

[b] The mutation for each allele is indicated. Amino acid numbers are derived from the reference sequence listed in the accession number column. "Splice @" is defined as a nucleotide change in the donor/acceptor "GU/AG" sequence at the referenced amino acid position. "DEL" refers to a deletion of the referenced amino acids and "FS" refers to a predicted frameshift.

[c] *Psn* background: screen A, Psn^{143}/+ (alleles: 9, bmps1, bmps2, A1, BM1. A6, A15, A3, B28); screen B, Psn^{9}/Psn^{143} (all other alleles).

[d] Phenotypes in a *Psn* wild-type background were not assessed, with the following exceptions: $NSF2^{A6}$ (see RESULTS), γ-*Tub23C* (see RESULTS), $gf^{SAE\text{-}9}$ (homozygous viable and displays a very small eye), $so^{SKE\text{-}1}$ (homozygous viable and displays a very small eye and loss of ocelli), and $R^{PIE\text{-}6}$ (homozygous viable and displays slightly reduced and severely rough eyes).

[e] Method(s) of gene identification are indicated for each group. Final meiotic mapping interval and the phenotype mapped are indicated in parentheses. The alleles meiotically mapped for each group are indicated by an * in the "Allele" column.

[f] Three additional alleles of *kkv* were found by testing kkv^{A1} and kkv^{BM1} for noncomplementation with a set of lethal mutations within the 82F region recovered by CARPENTER (1999). One of these alleles consisted of an X-ray-induced transposition [T(2;3)82Fh^2], which was used to cytologically locate the gene by *in situ* hybridization analysis on polytene chromosomes.

[g] These alleles also carry the Psn^{9} L499Q mutation in *cis* to the reported mutation.

Six alleles of *Hairless* (*H*) were identified. *H* encodes a negative regulator of Notch signaling (Bang *et al.* 1995; Lyman and Yedvobnick 1995; Schweisguth and Lecourtois 1998) and, consistent with this, two of our alleles suppressed the Psn^{9}/Psn^{143} reduced eye (Table 2). All display dominant phenotypes associated with reduced Hairless activity (*e.g.*, transformation of the bristle shaft to a socket cell and shortening of the fourth wing vein) in a $Psn^{9}/+$ or a $Psn^{143}/+$ background.

Alleles of *vestigial* (*vg*) were recovered as wing modifiers, exhibiting wing notching in the Psn^{9}/Psn^{143} background; $vg^{PHE\text{-}4}$ also exhibits mild wing nicking in the presence of Psn^{143} alone. All *trans*-heterozygous combinations of these alleles result in severe reduction or loss of wings. Two alleles, $vg^{SGE\text{-}11}$ and $vg^{SDE\text{-}1}$, also display a slightly smaller eye with Psn^{9}/Psn^{143}. Notch signaling, in addition to wingless signaling, is required for *vg* expression (Couso *et al.* 1995; Kim *et al.* 1996; Neumann and Cohen 1996). The genetic interactions in our screen suggest that *vg* is an important Notch pathway downstream effector in the eye as well as in the wing.

Modifiers involved in eye development: We recovered alleles of eight genes with known roles in eye development: *dachshund* (*dac*), *sine oculis* (*so*), *eyes absent* (*eya*), *Star* (*S*), *Ras85D, Roughened* (*R*), *glass* (*gl*), and *hedgehog* (*hh*). All alleles, with the exception of $hh^{PIE\text{-}7}$ (see Table 2), exhibited a small rough eye in the presence of Psn^{143} or were enhancers of the Psn^{9}/Psn^{143} reduced eye phenotype. Eyes absent, sine oculis, and dachshund function downstream of eyeless during early eye development and positively regulate specification of the eye (reviewed in Silver and Rebay 2005). The small GTPase Ras85D functions downstream of multiple receptors during eye development, including the EGF receptor and Sevenless, and plays roles during many different stages of eye development (Simon *et al.* 1991; Halfar *et al.* 2001; Kumar and Moses 2001b; Yang and Baker 2001, 2003; Strutt and Strutt 2003). Star is required during eye development (Heberlein and Rubin 1991; Heberlein *et al.* 1993) for the correct trafficking of the EGF receptor ligand, spitz, to the cell surface (Bang and Kintner 2000; Lee *et al.* 2001; Tsruya *et al.* 2002). The Notch and EGF receptor signaling pathways have been shown to act together and/or in opposition during the specification of most retinal cell fates (Flores *et al.* 2000; Kumar and Moses 2001a,b; Tsuda *et al.* 2002; reviewed in Voas and Rebay 2004). However, we note that Star may also be required directly by the Notch pathway for proper transport of Notch, its ligands, or components of the Presenilin complex, in a manner analogous to that of spitz. *R* encodes a Ras-related Rap GTPase that has been implicated in the regulation of the development of cell morphology during eye imaginal development (Hariharan *et al.* 1991; Asha *et al.* 1999). The transcription factor, glass, is required for photoreceptor development (Moses *et al.* 1989; Dickson and Hafen 1993; O'Neill *et al.* 1995), while hedgehog is involved in both specification of the early eye primordium (Royet and Finkelstein 1997) and the progression of the morphogenetic furrow (reviewed in Heberlein and Moses 1995). The eye phenotypes associated with these eight genes in the *Psn* mutant background are likely due to additive effects on eye development or to reduced Notch induction resulting from alterations in these pathways.

Odd-paired: Six alleles of *odd-paired* (*opa*) were recovered as mild enhancers of the Psn^{9}/Psn^{143} eye phenotype (Figure 2I) and all display tufted vibrissae (data not shown). Opa is homologous to the Zic family of transcription factors, which function prominently in vertebrate neuronal development (reviewed in Aruga 2004). During embryonic development in Drosophila, opa is required for the correct level and temporal pattern of *wingless* (*wg*) and *engrailed* expression (Benedyk *et al.* 1994 and references therein) as well as for the expression of the proneural gene, *achaete* (*ac*) (Skeath *et al.* 1992). In vertebrates, Zic1 has been shown to affect the expression levels of several members of the Notch pathway (Aruga *et al.* 2002). Our data demonstrate a novel function for opa in the development of adult eyes and head bristles. We propose that opa plays a role in determining the positioning and number of vibrissae via regulation of *wg*, *ac*, and *N*. This phenotype and subtle changes in eye size are likely the result of the additive effects of altering Wingless, Achaete, and Notch signaling.

Spt5: We recovered two alleles of *Spt5* as enhancers of the Psn^{9}/Psn^{143} reduced eye phenotype (Figure 2H). Spt5 is one of a group of transcriptional regulatory factors named after their initial isolation in yeast genetic screens as suppressors of Ty insertions. Spt5 appears to play both positive and negative roles during transcription, possibly by forming a complex with Spt4 and by interacting with both a positive transcription elongation factor (P-TEFb) and RNA polymerase II (Pol II) (Hartzog *et al.* 1998; Wada *et al.* 1998a,b).

In yeast, Spt5 forms a physical complex with another elongation factor, Spt6, and in humans, Spt6 can stimulate transcription in conjunction with the Spt5/Spt4 complex (Lindstrom *et al.* 2003; Endoh *et al.* 2004). In Drosophila, Spt5 and Spt6 may play both positive and negative roles in transcription elongation. They colocalize to actively transcribed regions of the chromosome and are recruited to the heat-shock genes following heat shock. Spt5 mutant embryos display reduced levels of heat-shock proteins following heat shock, suggesting that Spt5 plays a positive role in the transcription of these genes (Andrulis *et al.* 2000, 2002; Kaplan *et al.* 2000; Jennings *et al.* 2004). In contrast, *even-skipped* transcription increases in Spt5 mutant embryos, suggesting that Spt5 acts to negatively regulate expression of this gene (Jennings *et al.* 2004).

Genetic and biochemical studies suggest that Spt6 may interact with histones H3 and H4 and may help regulate chromatin structure (Bortvin and Winston

1996). Interestingly, the *C. elegans* homolog of Spt6, EMB-5, has mutant phenotypes and genetic interactions consistent with a role in Notch signaling (Hubbard *et al.* 1996). It has also been shown by yeast two-hybrid analysis to associate with the intracellular domains of the *C. elegans* Notch homologs, LIN-12 and GLP-1, and to biochemically contribute to NICD transcriptional activity (Hubbard *et al.* 1996). Fryer *et al.* (2004) observed that human SPT6 is present at Notch-regulated promoters and increases upon Notch stimulation, although a physical interaction of NICD and SPT6 was not detected. The genetic interactions of Spt5 and Spt6 with Notch signaling implicate regulated transcriptional elongation by the Pol II transcriptional machinery in the function of the NICD transcription complex

Novel Presenilin-dependent Notch interactions in the ECM: We recovered four alleles of *dumpy* (*dp*) from screen B. $dp^{PHE\text{-}5}$ was recovered as a modifier causing Psn^{9}/Psn^{143} lethality. $dp^{PGE\text{-}8}$ enhances the Psn^{9}/Psn^{143} eye phenotype and two alleles, $dp^{MFE\text{-}1}$ and $dp^{PIE\text{-}10}$, exhibit Psn^{9}/Psn^{143}-dependent *dp*-like pits in the anterior of the notum. $dp^{PIE\text{-}10}$ also displays mild *dp*-like pits in a $Psn^{9}/+$ background.

dp encodes a very large protein predicted to contain 308 EGF-like repeats, a zona pellucida (ZP) domain, and a membrane anchor sequence and likely functions as part of the ECM (Wilkin *et al.* 2000). Dumpy appears to play roles in the organization of the cuticle, tracheal development, attachment of epithelial cells to overlying cuticle, and in cell growth and differentiation (Wilkin *et al.* 2000 and references therein; Denholm and Skaer 2003; Jazwinska *et al.* 2003). Recent studies have also suggested that ZP-containing proteins, including dumpy, may be involved in cell adhesion to the apical extracellular matrix (Bokel *et al.* 2005). Dumpy may be involved in mediating cell–cell interactions in the ECM between Notch and its ligands or possibly in localizing the Presenilin complex to specific regions of the membrane. Alternatively, loss of dumpy activity may cause cell adhesion abnormalities that, in addition to reductions in Notch signaling, result in the observed modifications.

A total of three alleles of *krotzkopf verkehrt (kkv)* were isolated from the two screens. Two alleles cause smaller eyes in the presence of Psn^{143} and one enhances the Psn^{9}/Psn^{143} eye phenotype. *kkv* encodes one of two chitin synthases found in Drosophila. It is a multipass transmembrane protein that converts UDP-N-acetyl-D-glucosamine into UDP and chitin, an insoluble polymer consisting of 1,4-linked N-acetylglucosamine residues. It has two conserved aspartates and a QXXRW sequence motif necessary for substrate binding and catalysis (Saxena *et al.* 1995). In mammals, these same motifs are conserved in hyaluronan synthase (HAS), and recent studies have demonstrated that insects can produce hyaluronan when the murine HAS2 gene is introduced, suggesting that the chitin and hyaluronan synthetic pathways are highly related (Takeo *et al.* 2004). All three mutations in the *kkv* gene lie within conserved stretches of amino acids in this region of HAS homology. We propose that alterations of chitin synthesis adversely affect cell–cell adhesion in the ECM, thereby disrupting the interactions of Notch with its ligands, although alternative models in which altered ECM integrity disrupts general cell–cell interactions independently of Notch cannot be ruled out.

Nsf2 and AP-47 implicate vesicular trafficking in Notch signaling: We recovered two *Psn* modifiers with known functions in subcellular protein and vesicular trafficking. The first of these modifiers, *NEM-sensitive fusion protein 2* (*Nsf2*), encodes an AAA ATPase family member, which functions as a chaperone-type protein that utilizes ATP hydrolysis to drive conformational changes in target proteins (reviewed in Whiteheart and Matveeva 2004). We identified two alleles of *Nsf2* that cause subtle eye roughness in the Psn^{143} background, one of which, $Nsf2^{A6}$, is homozygous viable with small rough eyes.

In Drosophila, phenotypes resulting from expression of dominant-negative forms of Nsf2 suggest that Nsf2 plays roles in Notch and Wingless signaling (Stewart *et al.* 2001), and our mutant alleles confirm this observation. In humans, NSF likely functions in synaptic vesicle fusion by altering the conformation of SNAP–SNARE complexes. A similar chaperone activity contributes to the regulation of the β-2-adrenergic receptor by altering the conformation of the adrenergic receptor-binding protein, β-arrestin, which affects β-arrestin's interactions with the cytoskeleton or with proteins such as clathrin (McDonald *et al.* 1999; McDonald and Lefkowitz 2001; Miller *et al.* 2001). In addition, NSF may be involved in disassembly and recycling of the glutamate receptor complex (Nishimune *et al.* 1998; Song *et al.* 1998; Noel *et al.* 1999). Several models are possible for the role of Nsf2 in Notch signaling. Nsf2 may be required for the endocytosis of Notch receptors and ligands that has been shown to be essential for Notch signaling (see discussion). Alternatively, Nsf2 might be essential for the assembly of mature γ-secretase complexes or for trafficking and recycling of γ-secretase during Notch signaling.

We recovered two alleles of *AP-47*, the μ-subunit of the AP-1 clathrin adaptor complex, as mild enhancers of the Psn^{9}/Psn^{143} reduced eye (Figure 2F). $AP\text{-}47^{SAE\text{-}10}$ displayed mild vein thickening in the Psn^{9}/Psn^{143} background. As *trans*-heterozygotes, Psn^{9} $AP\text{-}47^{SAE\text{-}10}/Psn^{143}$ $AP\text{-}47^{SHE\text{-}11}$ are viable and display striking Notch loss-of-function phenotypes in the wing, the notum, and the eye (Figure 3). In the course of genetic mapping, we generated a recombinant $AP\text{-}47^{SHE\text{-}11}$ chromosome lacking the Psn^{143} mutation. Psn^{9} $AP\text{-}47^{SAE\text{-}10}/AP\text{-}47^{SHE\text{-}11}$ flies are essentially wild type (data not shown), suggesting that the *AP-47* Notch-like interaction phenotypes are dependent on reduced Notch signaling. We suggest that AP-47 functions in Notch signaling via its role as a clathrin adaptor complex member (see discussion).

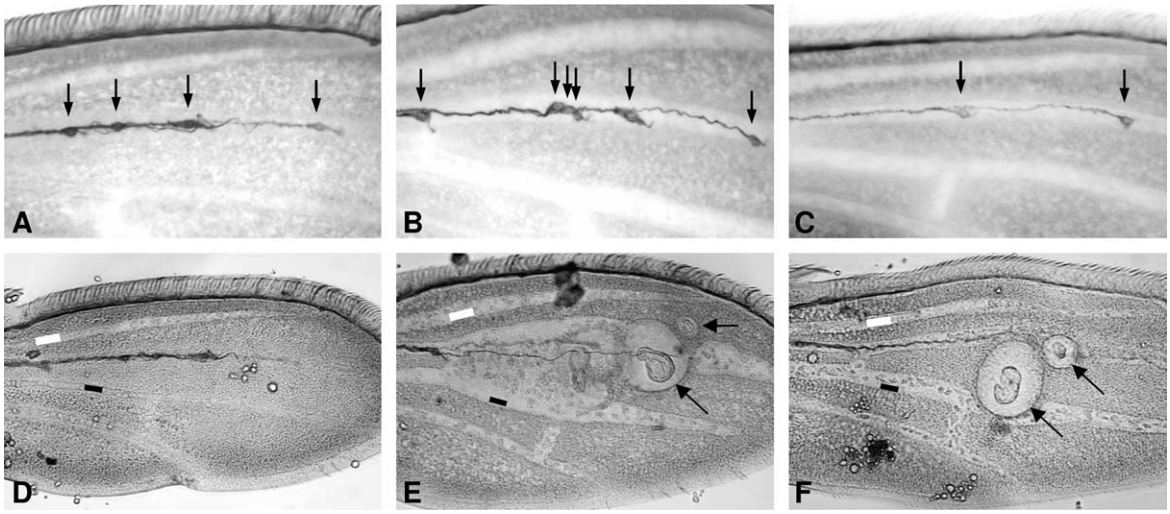

FIGURE 5.—Mutations in γ-*Tub23C* result in abnormal numbers of neurons and thickened veins in pupal wings. (A–C) Thirty hours after puparium formation (APF) pupal wings were immunostained with mAb22C10 to identify neurons. (D–F) Pupal wings were treated as in A–C except that photos were taken under high contrast to more easily visualize veins. (A–C) Wild-type pupal wings (A) have four neurons (arrows) spaced along the third wing vein. In contrast, γ-*Tub23C*^{bmps1}/+ pupal wings have either too many neurons (arrows in B) or too few neurons (arrows in C). (D–F) In comparison to wild type (D), γ-*Tub23C*^{bmps4}/+; *Psn*^{9}/+ pupal wing veins are abnormally wide (E and F). The open and solid rectangles in D–F indicate the widths of the wild-type veins in D to facilitate comparison. Arrows in E and F indicate bumps (see text).

γ-Tubulin: We isolated five alleles of γ-*Tubulin23C* (γ-*Tub23C*) that exhibit small, rough eyes, enhanced wing vein thickening, and wing nicking in combination with *Psn*^{9}/*Psn*^{143} (Figures 2G and 4, C and D). γ-*Tub23C* mutants exhibit two additional phenotypes as pupae that may result from Notch signaling defects. Wild-type pupal wings contain four neuronal cell bodies spaced along the third wing vein (Figure 5A, arrows), three of which are likely associated with the campaniform sensillae. In contrast, γ-*Tub23C* mutant wings often show a reduced number of neuronal cell bodies, neurons spaced incorrectly, or, less frequently, extra neurons at a single site (Figure 5, B and C). Sensory organ loss and the inappropriate adoption of the neuronal fate by sibling cells resulting in clusters of neurons are typical phenotypes caused by alterations in Notch signaling. In addition, 30 hr after puparium formation (APF), γ-*Tub23C* mutant pupal wings appear to have grossly thickened veins (compare Figure 5, E and F, with 5D), a typical Notch pathway loss-of-function phenotype. These thickened veins appear to recover during subsequent development, resulting in essentially wild-type veins in the adult. Transient vein thickening associated with loss of Notch signaling has also been observed in conditional dynamin mutants (PARKS *et al.* 2000).

γ-*Tub23C* mutants are homozygous lethal. Heterozygotes display several dominant, Presenilin-independent phenotypes in the adult. Approximately 8% of γ-*Tub23C* adults have nicked wings and γ-*Tub23C*^{bmps1}, γ-*Tub23C*^{bmps2}, and γ-*Tub23C*^{bmps4} adults display a very mild rough eye phenotype (data not shown) that is not highly penetrant and is not strong enough to account for the enhanced small, rough eye observed in the *Psn*^{9}/*Psn*^{143} background (Figure 2G). Strikingly, a large fraction of γ-*Tub23C* adults have wings with "bumps" (Figure 4B, arrow). This phenotype appears to be temperature sensitive. At 18°, 10% ($n = 19$) and at 23°, 6% ($n = 31$) of γ-*Tub23C* ^{bmps1}/*CyO* display bumps, whereas, at 27°, ~92% display bumps ($n = 40$). Similarly, 0–5% of γ-*Tub23C* ^{bmps4}/*CyO* adults display bumps at 18° or 23° ($n = 149$ and 37, respectively), whereas 47% display bumps at 27° ($n = 113$). The majority (90%) of these bumps occur along the third wing vein (L3) and most of these (91%) occur in the mid-distal portion of the vein (data not shown). Bumps have also been observed on the fourth wing vein, on crossveins, and in intervein regions. The location of the majority of bumps on L3 coincides with the region in which campaniform sensillae are found. However, examination of 30-hr APF wings suggests that there is no correlation between the neurons associated with the campaniform sensillae and the location of these masses. Bumps appear sometime between 0 and 30 hr APF and seem to consist of a mass of extracellular material deposited between the dorsal and ventral epithelial sheets that form the wing (Figure 5, E and F, arrows). There are no cells associated with these masses as judged by the absence of DAPI-positive nuclei (data not shown). In addition, there is no clear correlation between the severity of the bumps phenotype and reductions in Presenilin, Notch, or Delta function (data not shown). These results indicate that this phenotype is not directly related to Notch signaling.

In addition to strong genetic interactions with *Psn* mutations, γ-*Tub23C* alleles show significant genetic interactions with *N* and *Dl* alleles. γ-*Tub23C* mutants

strongly enhance the wing-notching phenotype associated with *N* hypomorphs. For example, nicking associated with N^{81k1} occurs primarily in the distal portion of the wing with little or no anterior nicking (Figure 4E). In contrast, notching in N^{81k1}/+ ; γ-*Tub23C*^{bmps4}/+ occurs throughout the entirety of the wing margin and is accompanied by a mild increase in extra vein material, especially at vein termini (Figure 4F). γ-*Tub23C* mutations also enhance the vein thickening and ectopic vein phenotypes associated with *Dl* mutations (Figure 4, G and H). No interactions were seen with alleles of *Su*(*H*), *aph-1*, *mastermind*, *deltex*, *bigbrain*, *EGFR*, or *rhomboid* (data not shown).

The mutational changes present in our γ-*Tub23C* alleles suggest that they are not null alleles. The five alleles arose in two separate rounds of mutagenesis in each of the two screens and thus originated from at least four independent mutational events. Nonetheless, γ-*Tub23C*^{bmps1} and γ-*Tub23C*^{bmps2} both share a change from Met382 to Ile, while γ-*Tub23C*^{bmps3}, γ-*Tub23C*^{bmps4}, and γ-*Tub23C*^{bmps5} share a change from Pro358 to Leu. Met382 is conserved in both humans and yeast γ-tubulin, but is changed to Leu in *C. elegans*. Pro358 is conserved in humans, yeast, and *C. elegans* γ-tubulin. Genetic data are consistent with the suggestion that these γ-*Tub23C* alleles are not null alleles as γ-*Tub23C*^{bmps4} is semiviable in *trans* with a deficiency that deletes γ-*Tub23C* [*Df(2L)JS17 dpp*$^{d\text{-}ho}$; BSC stock 1567]. The surviving adults are all male and display small, crumpled, blistered wings; small, rough eyes; missing macrochaetae; and microchaeta polarity defects (data not shown). The recurrence of these two amino acid changes in our alleles suggests that they cause aberrant γ-tubulin function that can reduce Notch pathway signaling.

DISCUSSION

We performed two genetic screens in Drosophila and identified 19 modifiers of Presenilin-dependent Notch phenotypes caused by *Psn* hypomorphic mutations. We identified genes required for general eye development as well as known members of the Notch pathway. The screen isolated several *nct* mutations, indicating that the Psn^{9}/Psn^{143} genotype provides a sensitized background for recovering Notch pathway interactors, including those directly involved in γ-secretase function.

We identified *Nsf2*, *AP-47*, and γ-*Tubulin23C* as regulators of the Notch pathway. Nsf2 has well-defined functions in protein trafficking and has been previously tied to Notch signaling using overexpression of a dominant-negative Nsf2 protein (Stewart *et al.* 2001). Our screens now confirm Nsf2 involvement in Notch signaling with the recovery of loss-of-function alleles. In contrast, AP-47 and γ-tubulin have not been linked to Notch signaling in the past. AP-47 has well-defined functions in vesicular trafficking and likely functions in Notch signaling in this capacity, while the mechanism of γ-tubulin function in the pathway is less clear.

Recent work has implicated several proteins involved in vesicular trafficking in both positive and negative regulation of the Notch pathway (reviewed in Le Borgne *et al.* 2005). The best studied of these is dynamin, the GTPase responsible for formation and pinching off of vesicles. Loss of dynamin function results in loss of Delta endocytosis, loss of dissociation of the Notch extracellular and intracellular domains, and strong Notch loss-of-function phenotypes (Poodry 1990; Seugnet *et al.* 1997; Parks *et al.* 2000). Dynamin appears to be required in both Delta- and Notch-expressing cells for Notch signaling to occur, but its precise role has yet to be determined (Seugnet *et al.* 1997; Parks *et al.* 2000). Other proteins that positively regulate Notch signaling include the clathrin coat components, clathrin heavy chain, α-adaptin, and epsin (Cadavid *et al.* 2000; Tian *et al.* 2004; Wang and Struhl 2004, 2005) and the regulator, Nsf2 (see results; Stewart *et al.* 2001). Finally, three ubiquitin ligases, neuralized, mindbomb, and deltex, act to positively regulate trafficking and signaling of Notch pathway members. Neuralized and mindbomb are thought to ubiquitinate Delta and/or Serrate to promote ligand endocytosis and activation of signal (reviewed in Le Borgne *et al.* 2005). Deltex likely ubiquitinates Notch to promote sorting into an undefined intracellular compartment where ligand- and Su(H)-independent signaling may occur (Hori *et al.* 2004 and references therein).

It is apparent that the endocytic machinery can be regulated at numerous steps to positively affect Notch signaling, yet the role that these proteins play remains unclear. Endocytosis of Delta bound to Notch could result in conformational changes in Notch necessary for its cleavage by ADAM/TACE proteins and γ-secretases and subsequent release of the intracellular domain (Parks *et al.* 2000). Endocytic proteins may also recruit cofactors necessary for Delta–Notch signaling or may contribute to colocalization of Notch receptors and secretases. In addition, endocytosis through a recycling endosome has been proposed as a mechanism for converting a Delta "pro-ligand" into an active form (Wang and Struhl 2004). It is not known if similar mechanisms directly regulate the activity and recycling of γ-secretase complexes.

Mutations in *AP-47,* the Drosophila μ1 protein of the clathrin adaptor complex AP-1, result in typical Notch loss-of-function phenotypes in the Psn^{9}/Psn^{143} background. There are at least four distinct adaptor protein (AP) complexes that link clathrin to membranes, coordinate clathrin coat assembly, and recruit cargo proteins. AP-1 functions in multiple steps in vesicle trafficking and cargo sorting from the Golgi to endosomes and the plasma membrane and is critical for the sorting and recycling of receptors to correct plasma membrane domains (Futter *et al.* 1998; Nakagawa

et al. 2000; Orzech *et al.* 2001; Gan *et al.* 2002; Pagano *et al.* 2004). The μ-chain of AP-1 appears to be responsible for sorting cargo proteins into developing vesicles. In kidney epithelial cells, μ1A mediates sorting to endosomes, while μ1B mediates the targeting of proteins to the basolateral plasma membrane (Sugimoto *et al.* 2002; Folsch *et al.* 2003 and references therein). In *C. elegans*, *unc-101* encodes a μ-subunit closely related to mammalian AP-47 (Lee *et al.* 1994). In chemosensory neurons, loss of *unc-101* function results in abnormal membrane trafficking of a certain set of proteins (Dwyer *et al.* 2001). These data suggest that AP-1 μ-chains can recognize and target specific proteins to specific cellular destinations. Preliminary data suggest that *unc-101* enhances a *Presenilin* loss-of-function egg-laying phenotype in *C. elegans* (R. Francis and G. McGrath, personal communication). This, in combination with our genetic data, implies that AP-47 plays a key regulatory role in Notch pathway function through the sorting, trafficking, and/or recycling of the Notch receptors, ligands, and secretases to their correct cellular destinations. Alternatively, AP-47 could function as part of the recycling endosomal pathway suggested to be required for Delta activation (Wang and Struhl 2004, 2005).

We recovered five alleles of γ-*Tub23C*. These alleles display loss-of-function Notch-like phenotypes in pupae and adults in the absence of any sensitizing mutations and have strong genetic interactions not only with *Psn* mutations, but also with *Dl* and *N* alleles. These alleles do not appear to behave as nulls (see results), but rather may impair or impart a specific interaction between γ-tubulin and Presenilin, Notch, or other members of the pathway.

There are currently two primary functions attributed to γ-tubulin: nucleation of microtubules as part of the centrosomal complex (Oakley 2000; reviewed in Moritz and Agard 2001) and capping of microtubule "minus" ends (Wiese and Zheng 2000), which may regulate microtubule growth. It has also been hypothesized that the centrosomal complex may serve as a site to concentrate proteins involved in the cell cycle and that some of these proteins may bind to γ-tubulin (Prigozhina *et al.* 2004). Interestingly, PS1 is functionally associated with the cytoskeleton (Pigino *et al.* 2001 and references therein), perhaps through interactions with microtubule-binding proteins such as CLIP-170 (Tezapsidis *et al.* 2003). PS1 and PS2 have also been detected at centrosomes (Li *et al.* 1997), suggesting the possibility of a functional interaction with γ-tubulin. This notion is supported by the observation that mutations in a *C. elegans* Presenilin gene, *spe-4*, display defective spermatogenesis accompanied by aberrant tubulin accumulation (Arduengo *et al.* 1998). Finally, recent research has indicated that in the two-cell stage in developing Drosophila bristle organs, Delta accumulates in Rab11-positive recycling endosomes in one cell but not in the other (Emery *et al.* 2005). These endosomes are pericentrosomal and their asymmetric accumulation appears to require asymmetric accumulation of the protein Nuclear Fallout, the Drosophila homolog of Arfophilin/Rab11-FIB3, which is also known to concentrate at centrosomes (Emery *et al.* 2005).

Centrosomal and/or cytoplasmic γ-tubulin may play a role in regulating cellular architecture via the nucleation of microtubules from the centrosome, capping of minus ends, and mediating microtubule growth in the cytoplasm and/or recruitment and localization of proteins. These functions may regulate vesicle trafficking through the secretory and endocytic pathways, which could influence the subcellular localization of Presenilin or other Notch pathway components. Additional experiments will be required to determine if the γ-tubulin missense mutations described here are gain or loss of function, whether they interact directly with Presenilin or Notch pathway components, or whether they modulate the pathway indirectly through effects on other processes, such as vesicle trafficking.

In conclusion, we have performed two genetic screens and identified 19 modifiers of Presenilin-dependent Notch pathway phenotypes. We recovered a number of proteins not previously implicated in Notch signaling, including Spt5, a transcription elongation factor that may interact with the Notch intracellular domain through Spt6, and two proteins involved in ECM function, kkv and dumpy. In addition, we have discovered a novel role for AP-47 that reinforces current research suggesting that the subcellular trafficking machinery is an important regulator of Notch signaling, and we implicate γ-tubulin as a Notch pathway interactor. These findings provide new insights into the mechanisms by which Notch signaling is regulated in development and suggest novel candidate approaches for targeting human disorders, including cancer and Alzheimer's disease.

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