DOI: 10.1534/genetics.104.039933

# Drosophila Signal Peptide Peptidase Is an Essential Protease for Larval Development

**David J. Casso,* Soichi Tanda,† Brian Biehs,* Bruno Martoglio‡ and Thomas B. Kornberg*,[1]**

**Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, †Department of Biological Sciences, Ohio University, Athens, Ohio 45701 and ‡Institute of Biochemistry, Swiss Federal Institute of Technology, ETH-Hönggerberg, 8093 Zürich, Switzerland*

Manuscript received December 15, 2004
Accepted for publication January 26, 2005

ABSTRACT

We identified the *Drosophila melanogaster Signal peptide peptidase* gene (*Spp*) that encodes a multipass transmembrane aspartyl protease. Drosophila SPP is homologous to the human signal peptide peptidase (SPP) and is distantly related to the presenilins. We show that, like human SPP, Drosophila SPP can proteolyze a model signal peptide and is sensitive to an SPP protease inhibitor and that it localizes to the endoplasmic reticulum. Expression of Drosophila SPP was first apparent at germ band extension, and in late embryos it was robust in the salivary glands, proventriculus, and tracheae. Flies bearing mutations in conserved residues or carrying deficiencies for the *Spp* gene had defective tracheae and died as larvae.

THE distribution of proteins in cell membranes can shift rapidly in response to changes in metabolic or developmental conditions. Among the enzymes that help to produce these rapid changes are proteases that bring about the maturation, activation, or relocalization of membrane proteins. Some of these proteases are themselves membrane proteins that cleave within the membrane anchoring transmembrane domain (TMD) of their substrates (Rudner *et al.* 1999; Weihofen and Martoglio 2003). These proteases, called intramembrane-cleaving proteases (I-CLiP's), are thought to have a catalytic domain within the plane of the lipid bilayer. Here we report the identification of a Drosophila signal peptide peptidase (SPP), an I-CLiP with activity against the TMD of signal peptides.

Intramembrane proteases have been implicated in developmental and disease processes, including cholesterol biosynthesis (Brown and Goldstein 1999), Notch signaling (Levitan and Greenwald 1995; De Strooper *et al.* 1999; Struhl and Greenwald 1999; Ye *et al.* 1999; Saxena *et al.* 2001), EGF signaling (Lee *et al.* 2001; Urban *et al.* 2001, 2002), signal peptide processing (Weihofen *et al.* 2002), and Alzheimer's disease (Scheuner *et al.* 1996; De Strooper *et al.* 1998; Kimberly *et al.* 2001). Presenilin is a member of this class of protease. It is an I-CLiP that is thought to constitute the catalytic core of the γ-secretase complex (Wolfe *et al.* 1999), a multisubunit enzyme first identified genetically by association with and thereby implicated in familial early onset Alzheimer's disease (Levy-Lahad *et al.* 1995; Sherrington *et al.* 1995). Predictions based on hydrophobicity calculations suggest that presenilin has eight TMDs, two of which contain aspartyl residues that lie within the plane of the bilayer and that form a catalytic site. Among the known substrates of presenilin are amyloid precursor protein (APP) (Kimberly *et al.* 2001), Notch (Levitan and Greenwald 1995; De Strooper *et al.* 1999; Struhl and Greenwald 1999; Ye *et al.* 1999; Saxena *et al.* 2001), and the Notch ligands, Delta and Jagged (Bland *et al.* 2003; Ikeuchi and Sisodia 2003; LaVoie and Selkoe 2003).

SPP is an intramembrane aspartyl protease with structural and catalytic similarities to presenilins (Weihofen *et al.* 2002). The human enzyme has been identified and characterized and has been shown to cleave signal peptide fragments that are released during maturation of precursor proteins. Its kinship with presenilin is revealed by its sensitivity to inhibitors directed against the active site of presenilin/γ-secretase (Weihofen *et al.* 2003; Nyborg *et al.* 2004). Like presenilin, SPP has multiple transmembrane regions that may assemble its catalytic site in the plane of the lipid bilayer. The active site motifs YD and LGLGD are in the center of adjacent TMDs (Weihofen *et al.* 2002; Urny *et al.* 2003). However, the orientation of the SPP active site is apparently inverted relative to the active site of presenilin, and it is predicted to cleave type II transmembrane domains that have their amino termini on the cytoplasmic side of the membrane, as opposed to presenilin substrates that have a type I orientation. Although the role of SPP is not fully understood, it seems likely that its function is not solely to cleanse ER membranes of released signal peptides. Peptides produced by SPP cleavage have been shown to bind and regulate calmodulin and class I major histocompatibility molecules (Martoglio *et al.* 1997;

[1]*Corresponding author:* Department of Biochemistry and Biophysics, 1550 4th St., University of California, San Francisco, CA 94143. E-mail: tkornberg@biochem.ucsf.edu

Braud *et al.* 1998; Lemberg *et al.* 2001), so it is possible that SPP functions to produce and release functional peptides or proteins from membranes and could be regulated to target specific substrates.

Our goal in this study was to determine whether SPP plays a role in animal development. We identified the Drosophila SPP ortholog and found that, like the human SPP, this enzyme has proteolytic activity *in vitro*. We also identified a number of mutants defective in Drosophila SPP and found that SPP has an essential role during larval development.

## MATERIALS AND METHODS

**Cloning of *Spp* and rescue construct:** The proximal 5-kb *Eco*RI fragment of the λ-phage clone S2-6 (Schneitz *et al.* 1993) was used to isolate CG11840 cDNA clones from a library of embryo cDNA's (Brown and Kafatos 1988); this gene was initially named *shanti*, but with the demonstration that it encodes a signal peptide peptidase, is henceforth called *Spp*. Two *Spp* cDNA's (LD08101 and CK00414) were also identified in the EST database of Berkeley *Drosophila* Genome Project; sequence analysis revealed that LD08101 is full length. Comparison of LD08101 and the 5-kb *Eco*RI genomic fragment revealed the presence of two small introns (65 and 66 bp), which have consensus 5′ and 3′ splice sites.

The pP(W8, $Spp^{+}$) rescue construct was made by inserting a 5.3-kbp genomic DNA fragment containing the *Spp* gene into the *P*-element vector P(W8) (Klemenz *et al.* 1987). This genomic region includes the complete SPP coding sequence, beginning 3677 bases upstream of the initiating methionine and extending 381 nucleotides (5360–4979) beyond the stop codon. This region does not include the protein coding sequence from *lwr*. The pP(W8, $Spp^{M}$) rescue construct is identical except that the two catalytic site aspartates were mutated to alanines. For pP(H-Pelican, $lwr^{+}$), a 2.4-kb *lwr* genomic sequence was amplified from genomic DNA using oligo lwrF1 (CCATCTACCGCAGTCCATAGCTC) and oligo lwr1214-220 (CGTTGGTAGCCTACTAGAAG). The amplified fragments were cloned into the pCR 2.1 vector (TOPO-TA cloning kit, Invitrogen, Carlsbad, CA) and then into the *P*-element vector pP(H-Pelican) (Barolo *et al.* 2000). pP(H-Pelican, $lwr^{+}$) contains the entire *lwr* coding region, but not the *Spp* coding region. Germline transformation was performed as described by Spradling (1986).

A second chromosome integrant of P(H-Pelican, $lwr^{+}$) was recombined with the $Df(2L)lwr^{14}$ deficiency. This chromosome was viable in *trans* with both $lwr^{05486}$ and $lwr^{5}$, indicating that P(H-Pelican, $lwr^{+}$) can rescue mutations in *lwr*; however, $Df(2L)lwr^{14}$ is not rescued by one or two copies of P(H-Pelican, $lwr^{+}$), indicating that this deficiency removes at least one other essential gene. A second chromosome integrant of pP(W8, $Spp^{+}$) was recombined with $Df(2L)lwr^{14}$. The resulting $Df(2L)lwr^{14}$, pP(W8, $Spp^{+}$) chromosome was viable with $Df(2L)lwr^{14}$, P(H-Pelican, $lwr^{+}$), indicating that the $lwr^{14}$ deficiency carries essential mutations in both the *lwr* and the *Spp* genes. Germline clones were made from $Df(2L)lwr^{14}$, P(H-Pelican, $lwr^{+}$), P($ry^{+r7.2}$=*neoFRT*)$^{40A}$ females.

**Mutagenesis of *Spp* and sequence analysis:** Flies were cultured on standard molasses/cornmeal medium at 25°. Mutagenesis was carried out by feeding EMS to 150 isogenized $cn^{1}$ $bw^{1}$ males as by the method of Lewis and Bacher (Lewis 1968). These males were mated *en masse* for 3 days with *Sco/Cyo* females and individual $F_1$ progeny were tested for complementation with $Df(2L)lwr^{14}$, P(H-Pelican, $lwr^{+}$). $Df(2L)lwr^{14}$ removes part of both the *lwr* and the *Spp* genes. Five second chromosomes were recovered that failed to complement $Df(2L)lwr^{14}$, P(H-Pelican, $lwr^{+}$), and the recovered alleles defined a single complementation group; three of these were characterized further.

To analyze the lesions in the $Spp^{5}$ and $Spp^{7}$ mutants, genomic DNA was isolated from homozygous mutant larvae, which were identified as the nonfluorescent progeny in cultures of $Spp^{5}$/CKG19 and $Spp^{7}$/CKG19 flies (*CKG19*: *CyO, Kr-GAL4, UAS-GFPS65T*; Casso *et al.* 2000). Genomic DNA was isolated from extracts of 10 homozygous mutant $Spp^{5}$ and $Spp^{7}$ larvae using a protocol for DNA extraction from single flies (Ashburner protocol 48). The $Spp^{6}$ mutation was characterized using DNA extracted from $Spp^{6}$/SM6a adult flies. The entire *Spp* coding region was amplified in three independent PCR reactions using oligos F (ggcgattttcaggaacggattggattgg) and R (aaagtc gagggaacattttctacaattg), and sequences were obtained from cloned PCR products.

**Cell culture and histology:** c-MYC epitope tags (EQKLI SEEDL) were added to the N terminus (using oligos aaaATG gaacaaaaacttatttctgaagaagacctgGCGGAGGAAGTCATCGGA ACCG and taactcgagCTACTTGCCCTTTTTCGACTCC) and to the C terminus (using oligos ttagaattcgtATGGCGGAGG AAGTCATCG and acaagcttctaCAGGTCTTCTTCAGAGATG AGTTTTTGTTCCTTGCCCTTTTTCGACTCC) of SPP by PCR. After the sequences were confirmed by DNA sequence analysis, the fusion constructs were cloned into vector pUAST (Brand and Perrimon 1993) in which expression is controlled by the *Saccharomyces cerevisiae* enhancer binding protein GAL4 to create both pUAS-*SppCmyc*, which has a carboxy-terminal cMyc tag, and pUAS-*SppNmyc*, which has an amino-terminal tag.

S2 cells were grown in Shields and Sang M3 Media (Sigma-Aldrich) supplemented with 12.5% heat-inactivated fetal bovine serum, 2.5 g/liter Bacto-peptone, and 1 g/liter yeastolate. Cells were cotransfected with pA5c-GAL4, a plasmid in which the expression of GAL4 is controlled by the Drosophila actin *Act5C* promoter (Ishikawa *et al.* 1999), and either pUAS-*SppNmyc* or pUAS-*SppCmyc* using Effectene (QIAGEN, Valencia, CA). Cotransfected with these was marker plasmid pA5c GG105, expressing a fusion of calreticulin (*Crc*, CG9429), GFP, and KDEL, which marks ER only, or marker plasmid pA5c GG112, expressing a fusion of KDEL receptor (KdelR, CG5183) with GFP, which localizes to the Golgi and ER. In our experiments, KdelR-GFP localization in punctate cytoplasmic structures characteristic of the Golgi was the most prominent. Two days after transfection, cells were plated on Permanox chamber slides coated with concanavalin-A to facilitate adherence to the substrate (Rogers *et al.* 2002), fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 20 min, washed in PBS, permeabilized in PBS containing 0.1% Tween-20 and 3% BSA for 30 min (PBSTw), and washed. Slides were then incubated with mouse anti-Myc antibody at 1:1000 (sc-789, Santa Cruz Biotechnology) followed by Cy3-conjugated donkey anti-mouse secondary antibody at 1:1000 (Jackson Laboratory), both in PBSTw.

*In situ* hybridizations were carried out as described (O'Neill and Bier 1994; Biehs *et al.* 1998) using a digoxygenin-labeled *Spp* anti-sense riboprobe to the entire 1.1-kb coding region. Embryos were from an overnight collection from *y w* or $Scr^{2}$/*TM3-ftz-lacZ* flies, and salivary glands were from wandering stage third instar *y w* larvae. Control hybridizations with a sense *Spp* digoxygenin-labeled riboprobe showed no specific staining.

The state of air filling in the dorsal trunks of the trachea of homozygous mutant first instar larvae was assessed just after hatching using standard optics on a Leica DMR compound microscope. Upon air filling, the trachea become more refrac-

tive and appear dark. Mutant phenotypes ranged from breaks in normal air filling to larvae without any discernible air filling (Figure 4, K and L). The percentage of larvae with normal air filling was determined (Table 1). The non-air-filled portions of the tracheal trunks were visualized using Nomarski optics (data not shown).

**Proteolysis assays:** The *Spp* gene coding region was cloned into p426gal1 (Mumberg *et al.* 1995) in which its expression is controlled by a GAL4-dependent promoter. This plasmid (p426gal-*Spp*), p426gal1, and pDAW300 [p426gal carrying human SPP (Weihofen *et al.* 2002)] were transformed into *S. cerevisiae* (*MAT*α*; his3*Δ*, leu2*Δ*0; lys2*Δ*0; ura3*Δ*0*) and expression was induced in SC media containing 2% galactose overnight at 20° to an OD of 0.6. SPP assays were done as described (Weihofen *et al.* 2002), with extracts prepared from crude microsomes by solubilizing in buffer containing CHAPS. The model human HLA-E signal sequence substrate (HLA-A/24) was resolved by SDS-PAGE.

**Manipulation of SPP levels by ectopic expression and RNA interference:** Wild-type *Spp* cDNA and $Spp^{AS}$, an *Spp* cDNA in which the putative catalytic aspartates were replaced with alanine (228) and serine (274), were subcloned into pUAST (Brand and Perrimon 1993). An 1167-bp fragment comprising the entire *Spp* coding region minus the initiating methionine codon was subcloned in a tail-to-tail orientation in pWIZ, a *P*-element vector for the GAL4-dependent expression of double-stranded RNA hairpins (Lee and Carthew 2003). The sequence of these constructs was confirmed by sequence analysis. The two insertions with strongest expression were both mapped to the second chromosome and then recombined onto a single second chromosome.

## RESULTS

**Identification of Drosophila *Spp*:** This investigation began as a search for the gene altered in the *oroshigane* mutant strain, which has a phenotype that resembles *hedgehog* loss of function (Epps *et al.* 1997). Although we did not identify a gene in the *oroshigane* region that could be correlated with the mutant phenotype, an unrelated gene in the region (CG11840) that encodes a candidate I-CLiP was found in the course of these studies. This article describes the general properties and preliminary genetic characterization of this gene, which we call *signal peptide peptidase* (*Spp*).

DNA sequence was obtained from wild-type flies for the *Spp* genomic region and for two *Spp* cDNA's (Figure 1). The *Spp* transcription unit spans 1757 bases and has two small introns (65 and 66 nucleotides). The encoded protein has 389 residues that topology predictions estimate will span the membrane nine times. TMDs 6 and 7 have centrally located aspartates, which may comprise active site residues in the catalytic domain of the enzyme in a manner similar to intramembrane cleaving aspartyl proteases such as presenilin. A BLASTP score of 439 for SPP (Altschul *et al.* 1990) suggests that SPP shares functional homology with human aspartyl protease SPP (Weihofen *et al.* 2002) and kinship with a family of I-CliP's that has known members in plants, vertebrates, and invertebrates (Ponting *et al.* 2002). Figure 1A illustrates the apparent consensus sequence for this family and shows that the regions with greatest conservation reside in the portions that are predicted to form the active site, helices 6 and 7. The consensus sequences in helices 6 and 7, YD and LGLGD, respectively, are also found in the active site of presenilin. The C terminus of SPP has a likely ER retention sequence, KKXX. Assuming that KKXX is directed to the cytoplasmic compartment, the orientation of helices 6 and 7 and of the active site is predicted to be opposite to that of presenilin and to be the same as that of human SPP.

When a MYC-SPP fusion was expressed in cultured Drosophila S2 cells, it distributed both in a perinuclear ring and in a lacy reticular pattern outside the nucleus. This distribution is consistent with ER localization (Figure 2). In cells that were cotransfected with both MYC-SPP and a calreticulin-GFP-KDEL fusion protein (a marker for the ER), significant colocalization was observed (Figure 2, bottom). However, MYC-SPP only weakly colocalized with a KDEL-Receptor-GFP fusion, a marker for Golgi (see materials and methods; Figure 2, top). Similar patterns of subcellular localization were observed with SPP that had an epitope tag on either the amino or the carboxy terminus. Human SPP also terminates with KKXX and localizes to the ER as a C-terminally tagged protein (Nyborg *et al.* 2004). These data are consistent with the ER localization of SPP and with similar studies showing that murine (Urny *et al.* 2003) and human (Friedmann *et al.* 2004) SPP also localize to the ER. We suggest that SPPs may contain ER localization signals in addition to KKXX or that ectopically expressed SPP may associate with endogenous SPP to form a dimer that is retained in the ER/pre-Golgi.

To establish whether the protein encoded by *Spp* has enzymatic activity as a signal peptide peptidase, we expressed both the human and the Drosophila SPP in *S. cerevisiae* and assayed extracts from these yeast strains for proteolytic activity on a 24-amino-acid signal peptide derived from the human HLA-A signal sequence. Previous studies showed that this peptide is a substrate for human SPP and that human SPP proteolytic activity is sensitive to 1,3-di-(*N*-carboxybenzoyl-L-leucyl-L-leucyl) amino acetone [$(Z\text{-LL})_2$-ketone] (Weihofen *et al.* 2002). This protease inhibitor was shown to block signal peptide processing, but not to affect signal peptidase or other proteases such as cathepsin or the proteasome (Weihofen *et al.* 2000). We found that the HLA-A derived signal peptide, a previously described substrate of human SPP (Lemberg *et al.* 2001; Weihofen *et al.* 2002), was quantitatively cleaved by extracts that contained either human or Drosophila SPP (Figure 3). The peptide was also cleaved by an extract of canine pancreatic microsomes, which has been shown previously to be a source of SPP activity (Weihofen *et al.* 2002). In contrast, the peptide was stable after incubation with buffer or control yeast extracts or after incubation in the presence of the SPP inhibitor $(Z\text{-LL})_2$-ketone. We conclude that the Drosophila *Spp* gene encodes a functional SPP enzyme.

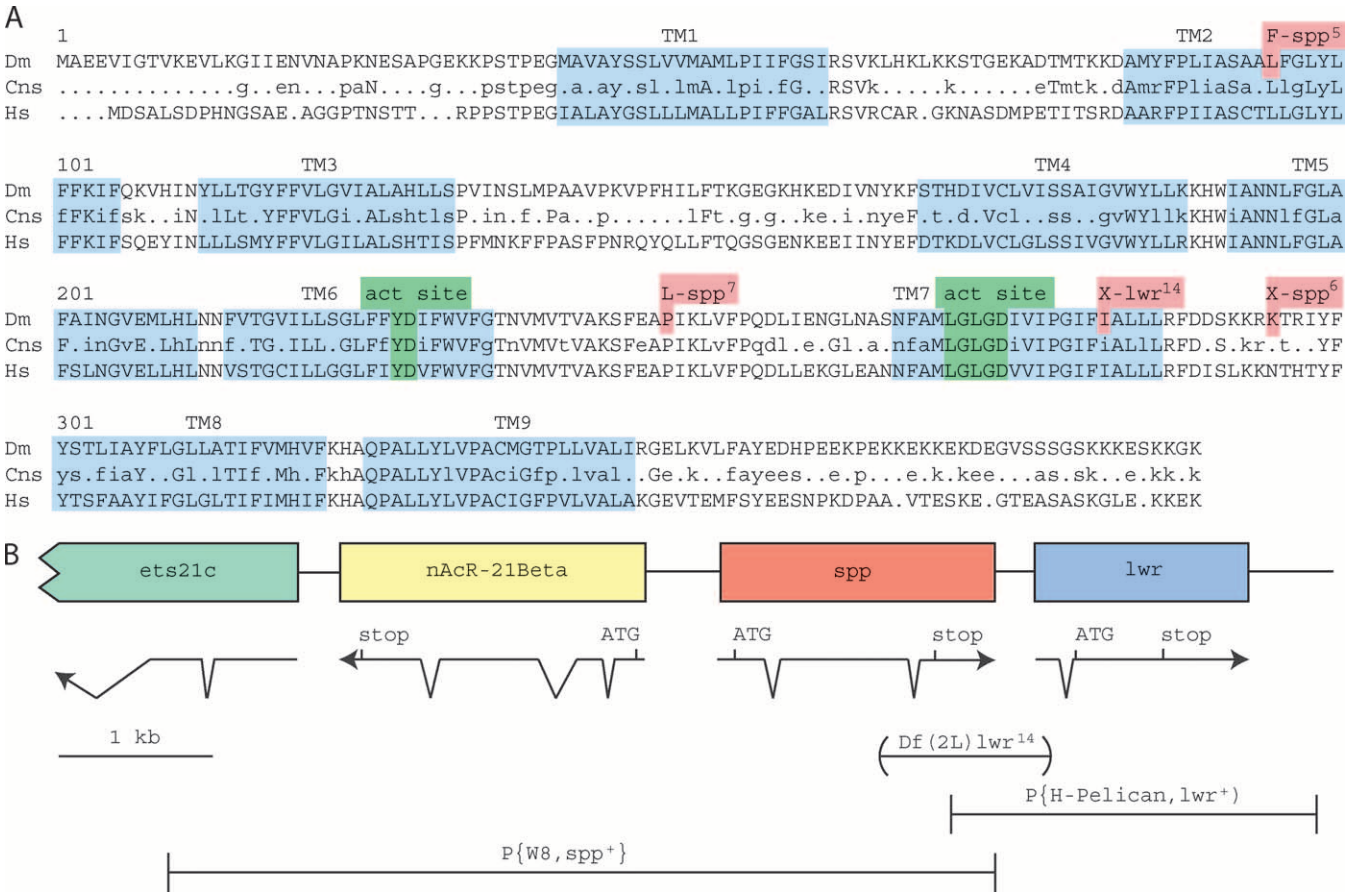


FIGURE 1.—The Drosophila *Spp* gene. (A) The protein sequence of Drosophila SPP (Dm) aligned with human SPP (Hs) and an SPP family consensus sequence derived from four species [*D. melanogaster* (CG11840), human (NP_110416), *Mus musculus* (BAC25752), and *Arabidopsis thaliana* (NP_565294)] using the program MultAlin (CORPET 1988). Capitalized amino acids indicate absolute conservation among the four species, while lowercase characters represent amino acids conserved in three of the four. Residues mutated in alleles 5, 6, and 7 (red) and those that make up the catalytic core (green) are highlighted. Transmembrane regions (TM1–9) shown in blue are based on predictions by the algorithm TopPred II (CLAROS and VON HEIJNE 1994). TM5 was predicted for Drosophila and Arabidopsis SPP, but not for the human or the mouse sequences. (B) The *Spp* locus and neighboring genes are shown. Predicted translational start and stop codons are shown above a diagram of the intron/exon structures. The regions used in two genomic rescue constructs P(W8, $Spp^+$) and P(H-Pelican, $lwr^+$) as well as the sequence deleted in $Df(2L)lwr^{14}$ are shown.

**Expression and role of *Spp* during Drosophila development:** We monitored expression of *Spp* mRNA in Drosophila embryos and larvae by *in situ* hybridization (Figure 4). No expression was observed during the cellularization or gastrulation stages. Hybridization to *Spp* probes was first apparent during germ band extension in a region caudal to the stomodeal invagination in the approximate location of invaginating salivary placodes, and it was enhanced very slightly in the epidermal cells adjacent to the segmental folds. Expression increased during germ band retraction and, in later stages, was most prominent in the salivary glands, proventriculus, and trachea. Expression of *Spp* in the salivary placodes and the embryo salivary glands was dependent on *Sex combs reduced* (*Scr*), a homeotic gene whose function is necessary for salivary gland fate (Figure 4, G–J). Expression of *Spp* was observed in the embryo proventriculus but not the salivary placodes and salivary glands of *scr* mutant embryos (Figure 4, G–J). In third instar larvae, prominent expression was observed in stalk cells of salivary glands (Figure 4F) and in a nonuniform pattern in wing and leg imaginal discs (not shown). Although murine *Spp* transcripts have been reported to be abundant in both CNS and PNS tissues (URNY *et al.* 2003), we did not detect *Spp* mRNA at significant levels in either the Drosophila CNS or PNS.

To investigate the role of SPP, we generated and analyzed mutants that delete or otherwise alter the *Spp* gene. A synthetic deletion of the C-terminal 106 residues was constructed using $Df(2L)lwr^{14}$, which deletes 1136 bp from the 5′ proximal region of *Spp* and extends into the neighboring *lesswright* (*lwr*) gene (Figure 1B) (APIONISHEV *et al.* 2001). *lwr* encodes an essential SUMO-transferase, and its loss results in embryonic lethality and cuticle defects (EPPS and TANDA 1998). We restored lwr function with P(H-Pelican, $lwr^+$), which contains a

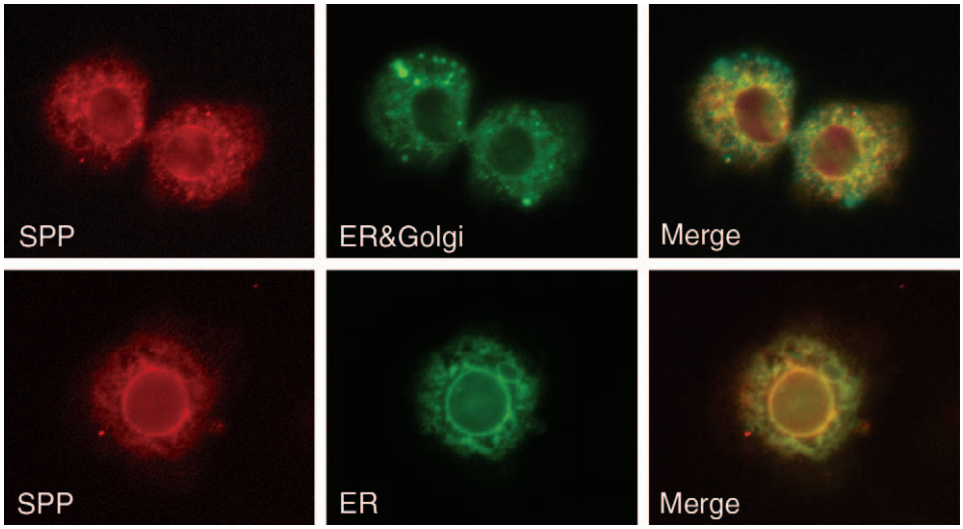


FIGURE 2.—Immunofluorescence of Schneider-2 cells expressing MYC-tagged Drosophila SPP. Drosophila SPP that was tagged on either the N terminus (top) or the C terminus (bottom) revealed perinuclear and reticular localization. Left, SPP (red); middle, KDEL-receptor-GFP fusion protein (top) and calreticulin-GFP-KDEL fusion protein (bottom), labeled ER + Golgi and ER, respectively (green); right, merged images.

2-kb genomic fragment that rescues the lethality of *lwr* mutants carrying *Df(2L)lwr*$^{14}$, *lwr*$^{05486}$, and *lwr*$^{5}$. *Df(2L)lwr*$^{14}$, P(H-Pelican, *lwr*$^{+}$) homozygous animals, which should lack Spp function only, failed to develop beyond the larval period. These animals arrested growth soon after embryo hatching, surviving for as much as several weeks as small second instar larvae. This phenotype was unchanged in animals that developed from *Df(2L)lwr*$^{14}$, P(H-Pelican, *lwr*$^{+}$), P(*ry*$^{+r7.2}$=*neoFRT*)$^{40A}$ germline clones, indicating that the survival of the mutants to the larval period is not due to partial rescue by maternal product.

To verify that the lethality of the *Spp* deficiency was due to loss of SPP, we introduced a 5360-bp genomic fragment containing the *Spp* gene. Flies homozygous for *Df(2L)lwr*$^{14}$ with either the P(H-Pelican, *lwr*$^{+}$) or the pP(W8, *Spp*$^{+}$) were not viable. However, homozygotes harboring both rescue constructs developed to adults. This result demonstrates that the lwr and Spp functions are the only ones defective in *Df(2L)lwr*$^{14}$ and indicates that *Spp* encodes an essential function for normal development. To determine whether the predicted aspartyl protease activity of SPP was responsible for this rescue, the catalytic site aspartyl diad was mutated to alanines to create pP(W8, *Spp*$^{M}$). Unlike its wild-type counterpart, pP(W8, *Spp*$^{M}$) was unable to rescue *Df(2L)lwr*$^{14}$ in combination with P(H-Pelican, *lwr*$^{+}$), implying a critical role for the protease activity of SPP.

New alleles of *Spp* were generated by chemical mutagenesis. Five mutants were isolated that failed to complement the *Df(2L)lwr*$^{14}$, P(H-Pelican, *lwr*$^{+}$) synthetic deficiency or the large deficiency *Df(2L)BSC16* that deletes the *Spp* gene. All were larval lethal and no complementation was observed between any two mutants. We analyzed the three with the most severe larval growth phenotypes: *Spp*$^{5}$, *Spp*$^{6}$, and *Spp*$^{7}$ (Table 1; Figure 1A). *Spp*$^{6}$ is the most severe; it truncates SPP at residue 322. *Spp*$^{6}$ mutants died as first instar larvae, showing a greater degree of growth retardation than *Df(2L)lwr*$^{14}$. *Spp*$^{5}$ and *Spp*$^{7}$ have point mutations in conserved residues. The leucine residue mutated in *Spp*$^{5}$ is conserved in the Psn/SPP superfamily (PONTING *et al.* 2002); the proline residue mutated in *Spp*$^{7}$ is conserved in the SPP family and is in a putative membrane-spanning domain.

Mutant *Spp* larvae had tracheal air-filling defects. During stage 16 of normal embryogenesis, the luminal cavities of the tracheal tubes evacuate liquid and fill with air. We monitored the extent of air filling in the dorsal trunks of the trachea of first instar larvae. Compared to either wild-type larvae or heterozygous siblings, the trachea of *Spp* homozygous mutants often failed to fill with air either partially or entirely (Figure 4, K and L; Table 1). This defect in tracheal physiology was rescued in *Spp* mutants carrying the *Spp* genomic rescue construct pP(W8, *Spp*$^{+}$). Partial rescue was achieved by heat-shock-dependent expression of the *Spp* cDNA (data not shown). This phenotype is consistent with both the ex-

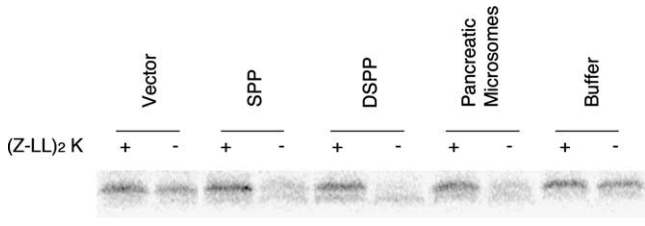


FIGURE 3.—Signal peptide peptidase activity of Drosophila SPP. In this assay, SDS-PAGE resolves the test peptide but cleavage products could not be identified. In the left six lanes, extracts of solubilized crude microsomes from *S. cerevisiae* expressing either human SPP or Drosophila SPP or carrying the empty expression vector (vector) were added to radiolabeled human HLA signal peptide (top band) in either the presence or the absence of the SPP inhibitor $(Z\text{-}LL)_2$-ketone. In the right four lanes, a solubilized extract of canine pancreatic microsomes (pancreatic microsomes) or solubilization buffer (buffer) as a control were assayed with the same substrate and inhibitor. SPP activity is indicated by the $(Z\text{-}LL)_2$-ketone-sensitive loss of the HLA signal peptide band.

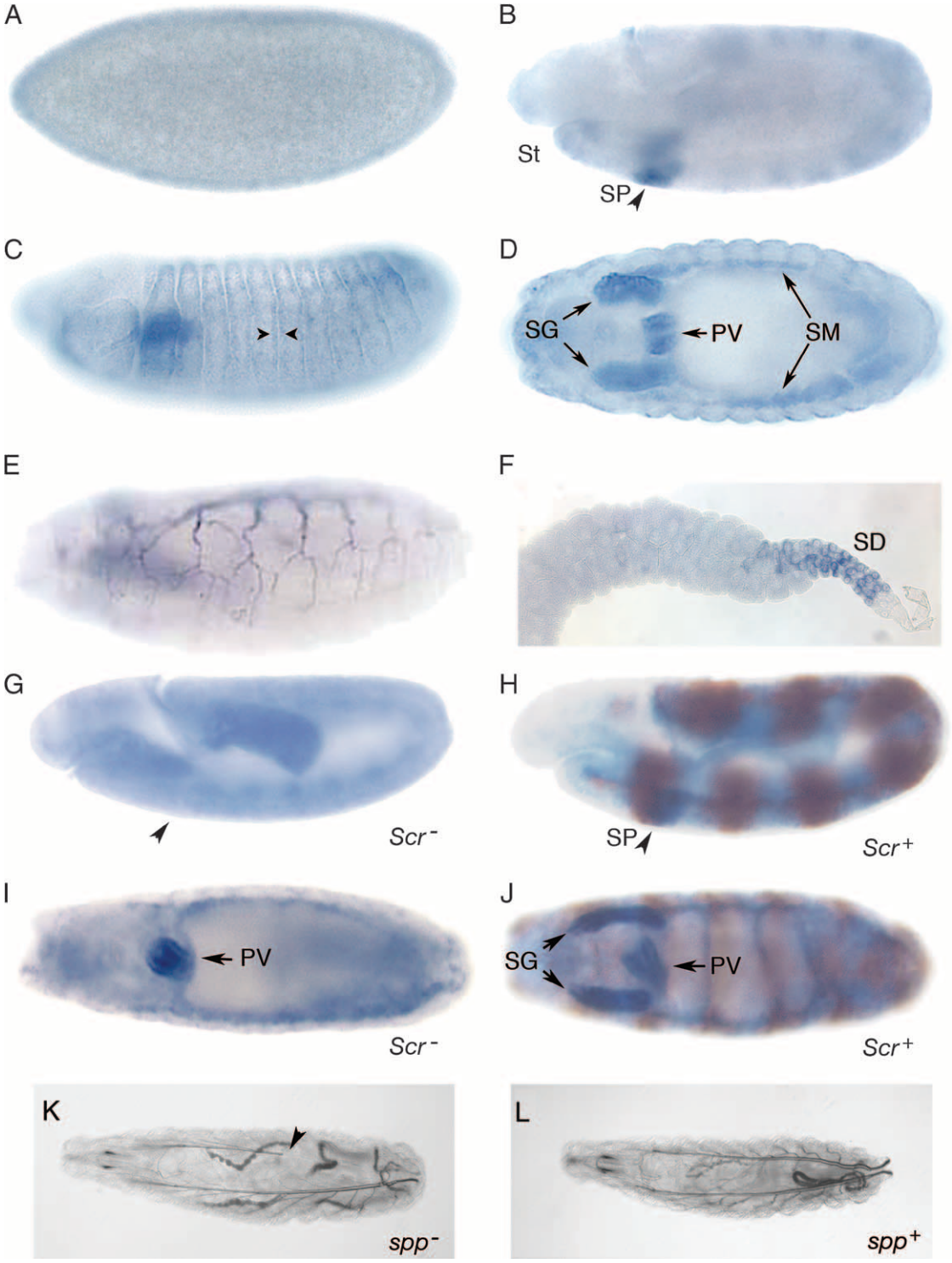


FIGURE 4.—*Spp* expression in embryos and larvae. (A–D) Expression in embryos was not detectable at cellularization (A), but at germ band extension was present (B, arrowhead) in the salivary primordium (SP) posterior to the stomodeal invagination (St). Enhanced levels of expression are seen in older embryos adjacent to the segmental involutions (C, arrowheads) and in the salivary glands (SG), proventriculus (PV), and somatic musculature (SM). (E) Expression in the tracheal branches of a late stage embryo. (F) Expression in the salivary gland duct cells (SD) of a third instar larva. Expression in $Scr^2$ mutants (G and I) and their $Scr^2/+$ siblings (H and J). Brown stripes indicate anti-β-galactosidase staining from a balancer chromosome. Light micrograph showing the longitudinal dorsal tracheal trunks of an *Spp* mutant (K) and wild-type (L) first instar larva. Incomplete tracheal air filling in the tracheal dorsal trunks in the *Spp* mutant is indicated with an arrow. Anterior is left except in F.

**TABLE 1**

**SPP mutants: lesions and tracheal phenotypes**

| *Spp* allele | Protein mutation | DNA mutation | Tracheal air filling (%) |
|---|---|---|---|
| *Df(2L)lwr*$^{14}$ | Fusion of aa 1–282 to 21 novel residues | 1137-base deletion | 15 |
| *Spp*$^{5}$ | L → F 95 | C → T 283 | 70 |
| *Spp*$^{6}$ | K → stop 295 | 29-base deletion | 0 |
| *Spp*$^{7}$ | P → L 248 | C → T 743 | 38 |

Four recessive lethal alleles of the Drosophila *Spp* gene are shown, along with a description of their genetic lesions and predicted effects on the resulting SPP proteins. The percentage of larvae with normal air filling is shown. A total of 95% of both wild-type and heterozygous *Spp* mutant animals had normal tracheal air filling.

pression of *Spp* in the trachea and the observation that *Spp* larvae are markedly lethargic compared to their heterozygous siblings. Using the extent of air filling in mutant larvae to rank the relative severity of the *Spp*$^{6}$, *Spp*$^{7}$, and *Spp*$^{5}$ mutants, *Spp*$^{5}$ was the least severe and *Spp*$^{6}$ was the most severe.

Growth arrest of strong mutant combinations occurred predominantly in the first and second instars as determined by mouth hook morphology. Growth arrest in *Spp* mutants was not apparently caused by molting defects, since none of the larvae had two pairs of mouth hooks (a characteristic of mutants defective in the ecdysone pathway). The mutant larvae frequently lived for several weeks and were able to ingest food, but did not grow notably in size and did not progress beyond the larval stages. This "failure to thrive" phenotype suggests a defect in feeding or digestion, but we have no direct evidence for this. To determine if growth arrest in the mutant animals was caused by generalized cell death, we stained both wild-type and mutant *Spp*$^{6}$ larvae with propidium iodide, which is excluded by live cells. No differences in number or type of cells stained with propidium iodide were detected between normal and mutant larvae (not shown).

**Ectopic expression of SPP in the wing:** To determine if the level of SPP is important to the normal development of the adult animal, we ectopically expressed SPP in the wing disc using the Gal4 UAS system (Brand and Perrimon 1993). When SPP was ectopically expressed at high levels in the posterior compartment, significant loss of tissue resulted and the size of this region was dramatically reduced (Figure 5, A and B). Notching of these wings was similar to the phenotype caused by ectopic apoptosis that has been observed after dREF overexpression (Yoshida *et al.* 2001), but we did not find evidence indicating that wing notching caused by ectopic expression of SPP had a similar etiology. It was not suppressed by coexpression of the antiapoptotic protein p35 or by reduction to one copy of the genes *reaper*, *grim*, or *head involution defective*, which are required for induction of apoptosis. The wing notching phenotype was enhanced at higher temperature, suggesting that it is sensitive to levels of *Spp* expression, and it was suppressed by coexpression of a double-stranded RNAi that targets *Spp* (Figure 5C). Induction of this *Spp* RNAi transgene alone had no phenotypic consequence (not shown).

To obtain additional evidence that the wing notching phenotype was caused by the activity of SPP, we engineered a gene encoding mutant protein in which the putative catalytic aspartyl residues at positions 228 and 272 were replaced with alanine and serine, respectively. Expression of this mutated gene in the posterior compartment did not cause loss of tissue (Figure 5D). This result supports the conclusion that the wing notching phenotype was caused by elevated levels of SPP activity.

## DISCUSSION

Our goal in this work was to determine if signal peptide peptidase is necessary for normal animal development. We present data showing that Drosophila *Spp* encodes the fly ortholog of human signal peptide peptidase and show that Drosophila *Spp* provides an essential function required during the larval stages. We also show that SPP is strongly expressed in only a limited set of cells and that the mutant phenotype is consistent with a need for its function in these tissues. Further work will be needed to establish whether the role of Drosophila SPP is a general one that cleanses membranes of signal peptides or if it has specific targets and generates essential products through its action.

Human SPP is an intramembrane aspartyl protease whose active site is predicted to be buried within the lipid bilayer. It belongs to a family of enzymes conserved among animals, plants, and fungi (Ponting *et al.* 2002; Weihofen *et al.* 2002). The Drosophila and human SPPs have strong sequence similarity, with the strongest conservation near the catalytic aspartyl residues. The similarity between the human and fly enzymes also includes the transmembrane topology of these proteins, as both are predicted to span the lipid bilayer nine times (Friedmann *et al.* 2004). In addition, the SPPs contain a conserved carboxy-terminal ER retention signal KKXX. Human SSP localizes to the ER (Urny *et al.* 2003) and we found that the Drosophila SPP does as well. The strongest evidence that Drosophila SPP is a signal peptide peptidase is its enzymatic activity. We show that the

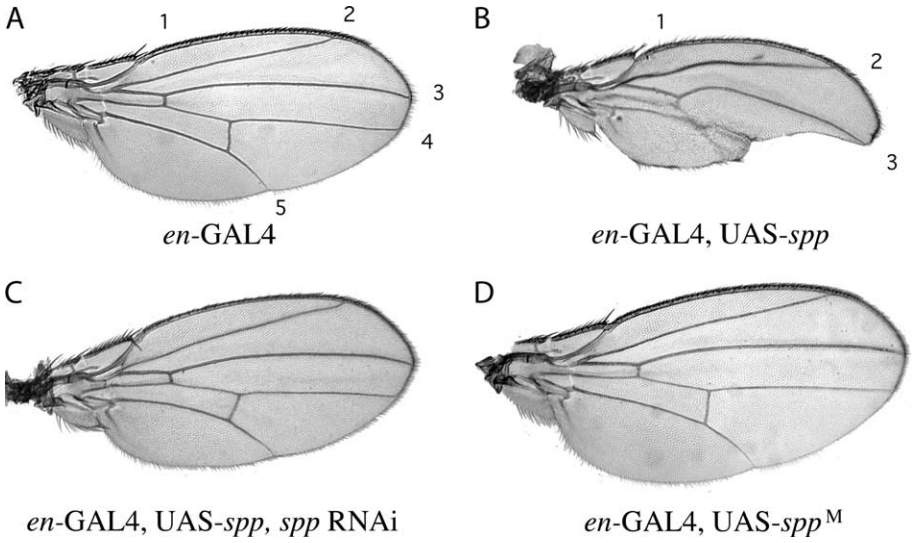


FIGURE 5.—Ectopic expression of *Spp* in the posterior compartment of the wing. Genotypes were as follows: *en-GAL4* alone (A); *en-Gal4, UAS-Spp* (B); *en-Gal4, UAS-Spp, Spp RNAi* (C); and *en-Gal4, UAS-Spp*$^{M}$ (D). Numbers refer to the five wing veins.

Drosophila SPP can proteolyze a model signal peptide and that this activity is sensitive to the SPP protease inhibitor, $(Z\text{-}LL)_2$-ketone.

We examined the expression of *Spp* during Drosophila embryogenesis. Earliest expression was during germ band extension. Expression in the germ band was nonuniform, with areas of weak enhancement that had a segmental periodicity; the most pronounced expression was in the salivary placodes. Later in embryogenesis, prominent expression was in three tubular organ systems: the salivary glands, the proventriculus (an anterior segment of the gut), and the trachea. In preliminary experiments with an *Spp* promoter-GFP fusion reporter, *Spp*-dependent GFP fluorescence was observed in similar patterns in the salivary glands, proventriculus, and trachea of both embryos and larvae (data not shown). Although other tissues such as wing and leg imaginal discs also expressed low levels of *Spp*, increasing levels of SPP by ectopic expression caused malformations in adult wings, legs, eyes, and sensory structures (Figure 5; data not shown). We conclude that expression of *Spp* is predominantly in salivary glands, proventriculus, and trachea and that normal development in other tissues requires that expression not rise above low levels.

We showed that *Spp* expression is necessary for Drosophila development by identifying recessive lethal mutations in *Spp*. These *Spp* alleles define a single complementation group. We identified two chromosomes with small deletions in the *Spp* gene. These deletions remove the two final transmembrane helices containing the QPALLY region highly conserved throughout evolution (Figure 1). In addition, the two missense mutations that were characterized ($Spp^5$ and $Spp^7$) changed conserved residues in the second transmembrane domain and third intracellular loop, respectively. Our molecular analysis therefore confirms the importance of these residues to SPP function that is implied by their conservation. We also introduced mutations in the conserved catalytic aspartyl diad. The aspartates in this domain are essential for the protease activity of human SPP as well as for the related polytopic intramembrane aspartyl protease, presenilin (WOLFE *et al.* 1999; WEIHOFEN *et al.* 2002). We found that a wild-type *Spp* construct could rescue *Spp* mutants, but that a similar construct encoding a protein with mutated catalytic domain aspartates could not. This result implies that the proteolytic activity of SPP is critical to its function. Our analysis of *Spp* mutants suggests that this activity is required specifically in the tracheae. We found that the dorsal trunks of mutant embryos were abnormal, with mutant phenotypes ranging from entire tracheal systems devoid of air to dorsal trunks that did not completely fill with air. Defects in this essential oxygen delivery system are consistent with the lethargic nature of *Spp* mutant larvae.

Having established that SPP has an essential role, we now hope to identify and characterize the critical processes and substrates it controls. SPP was originally identified as an enzyme that cleaves signal peptides, potentially cleansing the ER membrane of the signal sequence remnants of secreted proteins. It also appears to regulate vital systems by producing bioactive peptides, as is evident in the metabolism of human MHC (LEMBERG *et al.* 2001). It is also possible that SPP could function to release proteins from the membrane by cleaving their transmembrane domain tether. Intramembrane proteolysis by presenilin has been implicated in the processing and reverse signaling of a number of type I transmembrane proteins such as Notch, Delta, and Jagged (LEVITAN and GREENWALD 1995; DE STROOPER *et al.* 1999; STRUHL and GREENWALD 1999; YE *et al.* 1999; SAXENA *et al.* 2001; BLAND *et al.* 2003; LAVOIE and SELKOE 2003; IKEUCHI and SISODIA 2003). Presenilin cleaves the transmembrane domains of these proteins, releasing their respective cytoplasmic domains to migrate to

the nucleus where they function as transcriptional regulators. Since the active site of SPP is predicted to be inverted within the bilayer by 180° compared to the presenilin active site, the substrates of SPP would be type II transmembrane proteins, inverted in the membrane compared to the type I presenilin targets. In support of this type of activity, human SPP can activate an artificial ATF6 transcription factor construct that is tethered to the membrane in a type II orientation (Nyborg *et al.* 2004).

The essential nature of the SPP function is not limited to Drosophila. A recent publication describes the *Caenorhabditis elegans spp* homolog *imp-2*, which is required for normal molting in nematode development (Grigorenko *et al.* 2004). It appears that cholesterol and lipid metabolism are involved in this SPP-dependent process. It will be interesting to investigate whether there is also a connection between these processes and spp function in Drosophila, especially since intramembrane proteolysis plays a critical role in the activation of the SREBP pathway (Rawson 2003). More generally, we are most interested in identifying the targets of Drosophila SPP whose cleavage is necessary for normal development. To this end, we have begun to analyze *Spp* mutant animals using DNA microarray expression profiling. Preliminary comparisons of mutant and wild-type larvae revealed significant differences in transcription profiles (data not shown).

Finally, we note that SPP has been defined in part by its membership in a conserved family of intramembrane proteases (Ponting *et al.* 2002; Friedmann *et al.* 2004) and by its activity directed against signal peptides (Weihofen *et al.* 2000). Animal genomes are known to encode several different proteins with high sequence identity to SPP, and the Drosophila genome encodes two: CG11840 (*Spp*) and CG17030. While we have clearly established that SPP has an essential role in Drosophila development, characterization of CG17030 mutants and of *Spp* CG17030 double mutants will be important to further define the roles they play and to establish whether these genes have redundant functions. The results presented in this article suggest that this system holds great promise to help define the roles and mechanisms of action of this recently identified and interesting family of intramembrane proteases.

We are especially grateful to Helen Salz for her willingness to allow us to name the Drosophila *Spp* gene as we have. We thank the following for reagents and help with experiments: Chen-ming Fan, Elena Friedman, Songmei Liu, Andreas Weihofen, Marius Lemberg, Joy Gu, Heying Lin, Susan Younger, Janet Epps, Steven Beckendorf, Vidya Chandrasekaran, Deepak Malhotra, Robert Threlkeld, Gohta Goshima, and Ron Vale. We also thank the following for helpful discussions: Kevin Hill, Brenda Ng, Gretchen Ehrenkaufer, Felipe-Andrés Ramírez-Weber, David Iwaki, Frank Hsiung, Xingwu Lu, Francoise Chanut, and Arjun Guha. We acknowledge Hee Jae and the University of California-San Francisco Genomics Core Facility for DNA sequencing. D.J.C. received support from National Institute on Aging Training grant AG00278; this work was supported by grants from the National Institutes of Health to S.T. and T.B.K.

## LITERATURE CITED

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Apionishev, S., D. Malhotra, S. Raghavachari, S. Tanda and R. S. Rasooly, 2001 The Drosophila UBC9 homologue lesswright mediates the disjunction of homologues in meiosis I. Genes Cells **6:** 215–224.

Barolo, S., L. A. Carver and J. W. Posakony, 2000 GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. Biotechniques **29:** 726, 728, 730, 732.

Biehs, B., M. A. Sturtevant and E. Bier, 1998 Boundaries in the Drosophila wing imaginal disc organize vein-specific genetic programs. Development **125:** 4245–4257.

Bland, C. E., P. Kimberly and M. D. Rand, 2003 Notch-induced proteolysis and nuclear localization of the Delta ligand. J. Biol. Chem. **278:** 13607–13610.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea *et al.*, 1998 HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature **391:** 795–799.

Brown, M. S., and J. L. Goldstein, 1999 A proteolytic pathway that controls the cholesterol content of membranes, cells and blood. Proc. Natl. Acad. Sci. USA **96:** 11041–11048.

Brown, N. H., and F. C. Kafatos, 1988 Functional cDNA libraries from Drosophila embryos. J. Mol. Biol. **203:** 425–437.

Casso, D., F. Ramirez-Weber and T. B. Kornberg, 2000 GFP-tagged balancer chromosomes for Drosophila melanogaster. Mech. Dev. **91:** 451–454.

Claros, M. G., and G. von Heijne, 1994 TopPred II: an improved software for membrane protein structure predictions. Comput. Appl. Biosci. **10:** 685–686.

Corpet, F., 1988 Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16:** 10881–10890.

De Strooper, B., P. Saftig, K. Craessaerts, H. Vanderstichele, G. Guhde *et al.*, 1998 Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature **391:** 387–390.

De Strooper, B., W. Annaert, P. Cupers, P. Saftig, K. Craessaerts *et al.*, 1999 A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature **398:** 518–522.

Epps, J. L., and S. Tanda, 1998 The Drosophila semushi mutation blocks nuclear import of bicoid during embryogenesis. Curr. Biol. **8:** 1277–1280.

Epps, J. L., J. B. Jones and S. Tanda, 1997 oroshigane, a new segment polarity gene of Drosophila melanogaster, functions in hedgehog signal transduction. Genetics **145:** 1041–1052.

Friedmann, E., M. K. Lemberg, A. Weihofen, K. K. Dev, U. Dengler *et al.*, 2004 Consensus analysis of signal peptide peptidase and homologous human aspartic proteases reveals opposite topology of catalytic domains compared with presenilins. J. Biol. Chem. **279:** 50790–50798.

Grigorenko, A. P., Y. K. Moliaka, M. C. Soto, C. C. Mello and E. I. Rogaev, 2004 The Caenorhabditis elegans IMPAS gene, imp-2, is essential for development and is functionally distinct from related presenilins. Proc. Natl. Acad. Sci. USA **101:** 14955–14960.

Ikeuchi, T., and S. S. Sisodia, 2003 The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent "gamma-secretase" cleavage. J. Biol. Chem. **278:** 7751–7754.

Ishikawa, T., A. Matsumoto, T. Kato, Jr., S. Togashi, H. Ryo *et al.*, 1999 DCRY is a Drosophila photoreceptor protein implicated in light entrainment of circadian rhythm. Genes Cells **4:** 57–65.

Kimberly, W. T., J. B. Zheng, S. Y. Guenette and D. J. Selkoe, 2001 The intracellular domain of the beta-amyloid precursor protein

is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. J Biol. Chem. **276:** 40288–40292.

Klemenz, R., U. Weber and W. J. Gehring, 1987 The white gene as a marker in a new P-element vector for gene transfer in Drosophila. Nucleic Acids Res. **15:** 3947–3959.

LaVoie, M. J., and D. J. Selkoe, 2003 The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J. Biol. Chem. **278:** 34427–34437.

Lee, J. R., S. Urban, C. F. Garvey and M. Freeman, 2001 Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell **107:** 161–171.

Lee, Y. S., and R. W. Carthew, 2003 Making a better RNAi vector for Drosophila: use of intron spacers. Methods **30:** 322–329.

Lemberg, M. K., F. A. Bland, A. Weihofen, V. M. Braud and B. Martoglio, 2001 Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. J. Immunol. **167:** 6441–6446.

Levitan, D., and I. Greenwald, 1995 Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. Nature **377:** 351–354.

Levy-Lahad, E., W. Wasco, P. Poorkaj, D. M. Romano, J. Oshima *et al.*, 1995 Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science **269:** 973–977.

Lewis, E., 1968 Method of feeding ethyl methane sulfonat (EMS) to Drosophila males. Dros. Inf. Serv. **43:** 193.

Martoglio, B., R. Graf and B. Dobberstein, 1997 Signal peptide fragments of preprolactin and HIV-1 p-gp160 interact with calmodulin. EMBO J. **16:** 6636–6645.

Mumberg, D., R. Muller and M. Funk, 1995 Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156:** 119–122.

Nyborg, A. C., A. Y. Kornilova, K. Jansen, T. B. Ladd, M. S. Wolfe *et al.*, 2004 Signal peptide peptidase forms a homodimer that is labeled by an active site-directed gamma-secretase inhibitor. J. Biol. Chem. **279:** 15153–15160.

O'Neill, J. W., and E. Bier, 1994 Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. Biotechniques **17:** 870, 874–875.

Ponting, C. P., M. Hutton, A. Nyborg, M. Baker, K. Jansen *et al.*, 2002 Identification of a novel family of presenilin homologues. Hum. Mol. Genet. **11:** 1037–1044.

Rawson, R. B., 2003 The SREBP pathway—insights from Insigs and insects. Nat. Rev. Mol. Cell. Biol. **4:** 631–640.

Rogers, S. L., G. C. Rogers, D. J. Sharp and R. D. Vale, 2002 Drosophila EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. J. Cell Biol. **158:** 873–884.

Rudner, D. Z., P. Fawcett and R. Losick, 1999 A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. Proc. Natl. Acad. Sci. USA **96:** 14765–14770.

Saxena, M. T., E. H. Schroeter, J. S. Mumm and R. Kopan, 2001 Murine notch homologs (N1–4) undergo presenilin-dependent proteolysis. J. Biol. Chem. **276:** 40268–40273.

Scheuner, D., C. Eckman, M. Jensen, X. Song, M. Citron *et al.*, 1996 Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat. Med. **2:** 864–870.

Schneitz, K., P. Spielmann and M. Noll, 1993 Molecular genetics of aristaless, a prd-type homeo box gene involved in the morphogenesis of proximal and distal pattern elements in a subset of appendages in Drosophila. Genes Dev. **7:** 114–129.

Sherrington, R., E. I. Rogaev, Y. Liang, E. A. Rogaeva, G. Levesque *et al.*, 1995 Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature **375:** 754–760.

Spradling, A. C., 1986 P-element mediated transformation, pp. 175–197 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Struhl, G., and I. Greenwald, 1999 Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature **398:** 522–525.

Urban, S., J. R. Lee and M. Freeman, 2001 Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell **107:** 173–182.

Urban, S., J. R. Lee and M. Freeman, 2002 A family of Rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. EMBO J. **21:** 4277–4286.

Urny, J., I. Hermans-Borgmeyer, G. Gercken and H. C. Schaller, 2003 Expression of the presenilin-like signal peptide peptidase (SPP) in mouse adult brain and during development. Gene Expr. Patterns **3:** 685–691.

Weihofen, A., and B. Martoglio, 2003 Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. Trends Cell Biol. **13:** 71–78.

Weihofen, A., M. K. Lemberg, H. L. Ploegh, M. Bogyo and B. Martoglio, 2000 Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. J. Biol. Chem. **275:** 30951–30956.

Weihofen, A., K. Binns, M. K. Lemberg, K. Ashman and B. Martoglio, 2002 Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science **296:** 2215–2218.

Weihofen, A., M. K. Lemberg, E. Friedmann, H. Rueeger, A. Schmitz *et al.*, 2003 Targeting presenilin-type aspartic protease signal peptide peptidase with gamma-secretase inhibitors. J. Biol. Chem. **278:** 16528–16533.

Wolfe, M. S., W. Xia, B. L. Ostaszewski, T. S. Diehl, W. T. Kimberly *et al.*, 1999 Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature **398:** 513–517.

Ye, Y., N. Lukinova and M. E. Fortini, 1999 Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. Nature **398:** 525–529.

Yoshida, H., Y. H. Inoue, F. Hirose, K. Sakaguchi, A. Matsukage *et al.*, 2001 Over-expression of DREF in the Drosophila wing imaginal disc induces apoptosis and a notching wing phenotype. Genes Cells **6:** 877–886.

Communicating editor: K. V. Anderson