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# The serpin Spn5 is essential for wing expansion in *Drosophila melanogaster*

YVES CHARRON[1], RIME MADANI[1], CHANTAL COMBEPINE[1], VINCENT GAJDOSIK[2], YEUKUANG HWU[3], GIORGIO MARGARITONDO[2] and JEAN-DOMINIQUE VASSALLI[1,*]

*[1]Department of Genetic Medicine and Development, Faculty of Medicine, University of Geneva, Switzerland, [2]School of Basic Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland and [3]Institute of Physics, Academica Sinica, Nankang, Taiwan, ROC.*

**ABSTRACT Serpins, a superfamily of protease inhibitors, control proteolytic cascades in many physiological processes. Genomic studies have revealed the presence of a high number of serpin-encoding genes in *Drosophila melanogaster*, but their functions remain largely unknown. In a biochemical screen designed to detect protease inhibitors that may be implicated in early *Drosophila* development, we identified in embryos a ligand that forms a 67 kDa SDS-stable complex with the broad spectrum protease trypsin. Characterization of this ligand revealed it to be the recently described serpin, Spn5. Expression analysis by *in situ* and Northern blot hybridization indicated maternal transmission of the transcript as well as zygotic expression in many larval, pupal and adult tissues. Targeted repression by RNA interference did not alter early embryogenesis but resulted in a complete defect in the unfolding and expansion of the wings of freshly eclosed mutant flies, without other detectable effects on development.**

KEY WORDS: *Spn5, serpin, Drosophila, embryo, wing expansion*

## Introduction

Serpins constitute the largest family of peptidase inhibitors with over 800 serpin-encoding genes identified throughout the animal, plant, and microbial kingdoms (Silverman *et al.*, 2001; Gettins, 2002; van Gent *et al.*, 2003; Rawlings *et al.*, 2004; Silverman and Lomas, 2004). Serpins are, for the most part, secreted glycoproteins of ~350-400 amino acid residues. They inhibit their target serine proteases by an irreversible suicide substrate mechanism (Gettins, 2002) leading to the formation of a kinetically stabilised (or covalent) complex between the serpin and its target enzyme, which is resistant to denaturants such as SDS (Cohen *et al.*, 1977, Lawrence *et al.*, 1990), and subsequently to the trapping, deformation and hence inhibition of the target protease (Huntington *et al.*, 2000). As antiproteases, serpins play key roles in regulating biological phenomena such as blood coagulation, fibrinolysis, complement activation, fertilization, inflammation, malignancy, tissue remodeling and apoptosis. In this regard they are of medical interest because mutations in serpin genes can cause diseases such as pulmonary emphysema, cirrhosis, arthritis, blood clotting disorders and dementia (Boswell and Carrell, 1987; Carrell and Lomas, 1997).

The *Drosophila melanogaster* genome reveals a total of 29 annotated genes encoding proteins with a putative serpin domain (Rubin *et al.*, 2000; Adams *et al.*, 2000; Reichhart, 2005). *Drosophila* serpins have received relatively little attention until now, and it is only recently that functions of three members of this family have been identified (for review see Reichhart, 2005). Spn43Ac has been implicated in the regulation of an unknown serine protease cascade that plays a central role in the Toll signalling pathway, a key defence mechanism against pathogens in the innate immune systems of both insects and mammals (Levashina

*Abbreviations used in this paper:* βME, β-mercaptoethanol; DEAE, diethylaminoethyl; DPCC, diphenyl carbamyl chloride; ECM, extracellular matrix; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetate; EST, expressed sequence tag; HIS, histidine; IgGs, immunoglobulins; IR, inverted repeat; L1-L3, first, second and third instar larva; Ni-NTA, nickel-nitriloacetic acid; O, ovaries; P, pupae; PBS, phosphate-buffered saline; PC2, Prohormone Convertase 2; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RCL, reactive center loop; rp49, ribosomal protein 49; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; serpin, serine protease inhibitor; SPC, subtilisin-like proprotein convertase; Spn1-6, serpins 1-6; Spn27A, serpin 27A; Spn43Ac, serpin 43Ac; TEI, Tris EDTA iodoacetamide; TPCK, L-1-Tosylamide-2-pheny-lethyl chloromethyl ketone; UAS, Upstream Activating Sequence; UTP, uridine triphosphate.

***Address correspondence to:** Jean-Dominique Vassalli. Department of Genetic Medicine and Development. Faculty of Medicine, University of Geneva, 1, Rue Michel-Servet, CH-1211 Geneva 4, Switzerland. Fax: +41-22-379-5978. e-mail: Jean-Dominique.Vassalli@medecine.unige.ch

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*et al.*, 1999). Spn27A is a second serpin involved in another of the multiple host defence mechanisms in *Drosophila*, the melanization cascade; this process is controlled by a serine protease which is inhibited and restricted to the site of injury or infection by Spn27A, thereby preventing the insect from excessive melanization (De Gregorio *et al.*, 2002; Ligoxygakis *et al.*, 2002). In addition to their implication in immunity, Toll and Spn27A are essential for dorsoventral patterning in early *Drosophila* embryogenesis; the Toll pathway is initiated by a signal restricted to the ventral side of the extracellular compartment between the embryo and the eggshell and involving four trypsin-like extracellular serine proteases (LeMosy *et al.*, 1998, Morisato and Anderson, 1995) (for review see Moussian and Roth, 2005). Since embryos lacking maternal Spn27A function are strongly ventralized, it has been proposed that Spn27A is also essential to ventrally restrict this proteolytic cascade, and to establish a correct embryonic dorsoventral axis of polarity (Hashimoto *et al.*, 2003; Ligoxygakis *et al.*, 2003). It is interesting to note that, in addition to the melanisation and developmental phenotypes, the few Spn27A deficient flies that eclosed also showed a defect in wing expansion (De Gregorio *et al.*, 2002). The third *Drosophila* serpin that has been studied in detail is Spn4; it is transiently expressed in the developing nervous system and appears to be the closest *Drosophila* homolog to neuroserpin, a vertebrate neuronal serpin (Osterwalder *et al.*, 2004). Recombinant Spn4 binds and inhibits *in vitro* human furin and its *Drosophila* homolog Prohormone Convertase 2 (PC2) (Oley *et al.*, 2004; Osterwalder *et al.*, 2004; Richer *et al.*, 2004), which belong to the subtilisin-like proprotein convertase (SPC) family known to regulate the maturation of many secreted proteins (Steiner, 1998).

Taken together, these data suggest that *Drosophila* could be a model to understand the role of serpins in multiple processes, both during development and in the adult. However, most serpins identified in the *Drosophila* genome are only annotated sequences in the data bank and neither their expression profiles nor functions are known. At the time we began this study, only one serpin had been identified in *Drosophila melanogaster* (Coleman *et al.*, 1995; Wolfner *et al.*, 1997). In order to define a role for new serpins in the fruit fly, we investigated the presence of such molecules using an enzyme-binding assay based on the formation of stable, detergent-resistant, complexes between serpins and radio-iodinated proteases. We purified, from *Drosophila melanogaster* embryo extracts, a trypsin ligand and identified it as an active serpin that had recently been annotated as Spn5 in the data bank. Expression analysis by *in situ* and Northern blot hybridization suggested a potential role in early *Drosophila* development but also at later stages. Targeted repression of Spn5 by RNA interference did not have obvious effects on early development, but resulted in a complete defect in the unfolding of the wings.

## Results

### *Identification and characterization of a trypsin-binding protein in* Drosophila *embryos*

To investigate the presence of inhibitors of proteases of tryptic specificity in *Drosophila* embryo protein extracts, we performed a binding assay using radio-labeled trypsin. With this assay, the complexes formed between proteases and inhibitors are detected by SDS-PAGE and autoradiography. Extracts incubated with $^{125}$I-trypsin contained a single, SDS resistant, $^{125}$I-trypsin-ligand complex which migrated according to a molecular mass of 67 kDa under native and reducing conditions (Fig. 1A). Assuming a 1:1 stoichiometry of the complex, and given a molecular mass for trypsin of 23 kDa, the ligand appeared to have a molecular mass of approximately 44 kDa. Further characterization after partial purification of the ligand by DEAE Sepharose ion exchange chromatography suggested that the ligand is a serpin class antiproteases (data not shown, see below).

### *Purification of the ligand and identification as Spn5*

DEAE-Sepharose-purified material from embryonic extracts was incubated with biotinylated trypsin and applied to an avidin-agarose column. Elution was achieved with SDS/βME or with $NH_4OH$ and eluted material was analyzed by SDS-PAGE, silver staining and autoradiography (Fig. 1B). The SDS/βME eluate contained a 23 kDa band corresponding to free trypsin and the 67 kDa complex, which could be revealed by silver staining (Fig. 1B, lane 3), as well as by autoradiography (Fig. 1B, lane 1). Additional treatment of the SDS/βME eluate with $NH_4OH$, which dissociates the trypsin-ligand complex (Fig. 1B, lane 2), yielded an additional 43 kDa silver-stained band corresponding to the dissociated ligand (Fig. 1B, lane 4). Direct elution of the avidin-agarose column with $NH_4OH$ yielded a single silver-stained band corresponding to the purified ligand (Fig. 1B, lane 5). This band was electroblotted onto a polyvinylidene fluoride (PVDF) membrane and subjected to amino-terminal sequence analysis. The N-terminal sequence of the purified ligand was determined and sequence comparison analysis in the Swiss-Prot data bank revealed that the peptide was identical to a portion of the *Drosophila* serpin, Spn5, previously identified as a serpin gene with maternal and zygotic expression (Han *et al.*, 2000). The C-terminal region of Spn5 contains highly conserved residues forming the reactive center loop (RCL) with a putative cleavage site for tryptic enzymes as its target proteases. This is compatible with our purification strategy based on formation of a stable complex with trypsin. To confirm that Spn5 is indeed an inhibitor of trypsin, we prepared a recombinant construct encoding the protein with a polyhistidine tag at its C-terminus. Using a bacterial expression system, purified recombinant 6XHIS-Spn5 was obtained after elution from a Ni-NTA agarose column. To assay for inhibition, we incubated recombinant polyhistidine-tagged Spn5 with trypsin and analyzed the samples in parallel by enzyme binding and caseinolytic assays. By SDS-PAGE analysis, we observed increasing amounts of the 67 kDa Spn5/trypsin complex with increasing amounts of added recombinant Spn5 (Fig. 1C). The caseinolytic assay performed on the same samples revealed a good correlation between increasing amounts of complexed trypsin and inhibition of trypsin proteolytic activity (Fig. 1D). We did not determine the kinetic parameters of the reaction, however, because we did not obtain enough high purity Spn5 to achieve saturation under our experimental conditions.

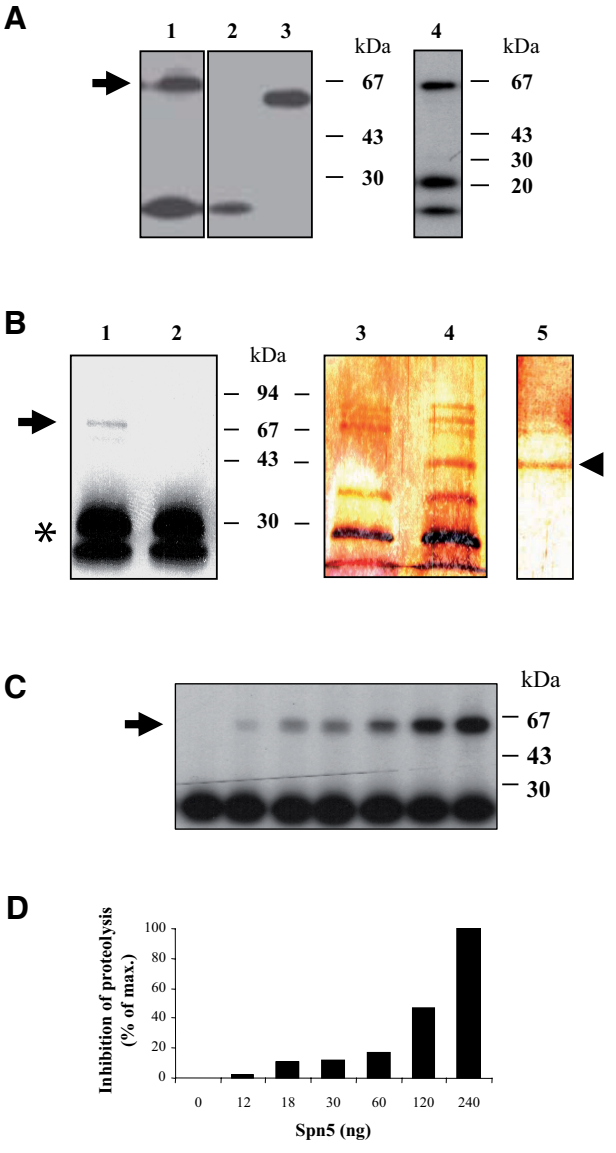
### *Expression of Spn5 during* Drosophila *development*

To determine the temporal and spatial pattern of Spn5 expression during *Drosophila* development, we first performed Northern blot hybridizations on total RNA prepared from all developmental stages. Using a ~0.5 kb cRNA specific for Spn5 as a probe, we

detected Spn5 mRNA at the expected size of ~1.4 kb (Fig. 2A). Spn5 transcripts were abundant in ovaries and at all embryonic stages except between five and fifteen hours of development. Spn5 was highly expressed throughout larval and pupal developments as well as in adults (Fig. 2A).

The early expression of Spn5 in ovaries and in early embryos before the initiation of zygotic transcription indicated that Spn5 is a maternal transcript that accumulates during oogenesis. To confirm this, we analyzed the presence of Spn5 transcripts in ovaries by whole mount *in situ* hybridization. We detected Spn5 mRNA in the cytoplasm of nurse cells and in oocytes, but not in the follicle cells, confirming that Spn5 is a maternally-expressed gene (Fig. 2 B-C).

By RNA *in situ* hybridization of whole mount embryos, we determined the distribution of Spn5 mRNA throughout embryogenesis (Fig. 2 D-J). Blastoderm and gastrulating embryos (stages 5-6; 0-5 hours) showed a uniform distribution of Spn5 mRNA (Fig. 2D). At later stages, between five and fifteen hours of development (stages 10-16), Spn5 transcripts were not detected, in accord with the results from Northern hybridizations (Fig. 2F). Toward the end of embryogenesis (stages 16-17; 15-24 hours), Spn5 expression resumed and became more apparent in specific tissues such as ventral and dorsal epidermis, embryonic/larval posterior spiracles, embryonic foregut and hindgut, sensory nervous system primordium, pharynx and respiratory embryonic/larval tracheal system (Fig. 2H-J).




### *Functional analysis of Spn5 by RNA interference*

We used the RNA interference (RNAi) approach to investigate the role of Spn5 *in vivo,* taking advantage of the Gal4-UAS system (Brand and Perrimon, 1993) to express an inducible inverted repeat homologous to the Spn5 target mRNA, so as to repress its function. A UAS-Spn5-IR (Inverted-Repeat) construct, containing two copies of a ~0.9 kb Spn5 fragment in an inverted repeat arrangement and separated by an intron, was prepared and different transgenic lines carrying the UAS-Spn5-IR construct were generated by P-element insertion. Established homozygous lines were crossed separately to homozygous (*GMR-Gal4*, *nos-Gal4:VP16*, *nos-Gal4-NGT$^{40}$*, *da-Gal4*) or balanced (*Act5C-Gal4/TM6b*, *Tb*, *Hu*) driver transgenic lines (see Materials and Methods). Flies heterozygous for the different drivers and transformant lines displayed no detectable phenotype, and analysis of Spn5 mRNA levels by Northern blot revealed a normal expression of the endogenous wild type Spn5 gene at larval stages (data not shown). In order to produce homozygous strains carrying four copies of the UAS-driven transgene, we recombined two different UAS-Spn5-IR P-element insertions on the same chromosome and established eight stocks, each homozygous for a different combination of two UAS-Spn5-IR insertions (UAS-Spn5-IR$^{R}$).

**Fig. 1. Detection, purification and characterization of a trypsin ligand in *Drosophila* embryos. (A)** *50 and 10 μg of total* Drosophila *embryo protein extract (lanes 1 and 4 respectively) were incubated at 37°C for 30 minutes with 6 ng of $^{125}$I-trypsin. Lane 2, $^{125}$I-trypsin alone; lane 3, $^{125}$I-trypsin incubated with a known murine serpin, Spi3/Serpinb6. The samples were subjected to SDS-PAGE under non-reducing (lanes 1-3) or reducing (lane 4) conditions and the gel analyzed by autoradiography. The arrow points to a 67 kDa SDS-stable complex comprising $^{125}$I-trypsin. Under reducing conditions free $^{125}$I-trypsin yields two bands corresponding to the β-single chain- and α-two chain- forms of the enzyme (lower bands in lane 4).* **(B)** *Purification of the ligand by affinity chromatography. DEAE-purified material was incubated with biotinylated-$^{125}$I-trypsin, the complex was adsorbed on an avidin-derivatized resin and eluted with SDS/βME, (lane 1, autoradiography; lane 3, silver-stained gel). A subsequent treatment of the SDS/βME eluate with $NH_4OH$ led to dissociation of the 67 kDa complex (lane 2: autoradiography, lane 4: silver-stained gel) and to the appearance of a new band at 45 kDa corresponding to the dissociated and purified ligand (lane 4: silver-stained gel). Direct elution from the resin with $NH_4OH$ yielded the pure ligand (lane 5: silver-stained gel). The arrow points to the 67 kDa complex, the arrowhead points to the purified ligand and the asterix points to free $^{125}$I-trypsin at 30kDa; under reducing conditions free trypsin yields two bands corresponding to the β-single chain- and α-two chain- forms of the enzyme.* **(C,D)** *Increasing amounts of recombinant Spn5 (0, 12, 18, 30, 60, 120 and 240 ng) were incubated at 37°C with 100 ng of trypsin and 6 ng of $^{125}$I-trypsin in the presence of 1% casein.* **(C)** *Spn5-trypsin complex formation measured by enzyme binding assay. After 30 minutes an aliquot of each sample was analyzed as in (A). The arrow points to the 67 kDa complex.* **(D)** *Inhibition of trypsin in a caseinolytic assay. Absorbance at 410 nm, which decreases proportionally to trypsin-catalyzed proteolysis, was measured after 240 minutes.*

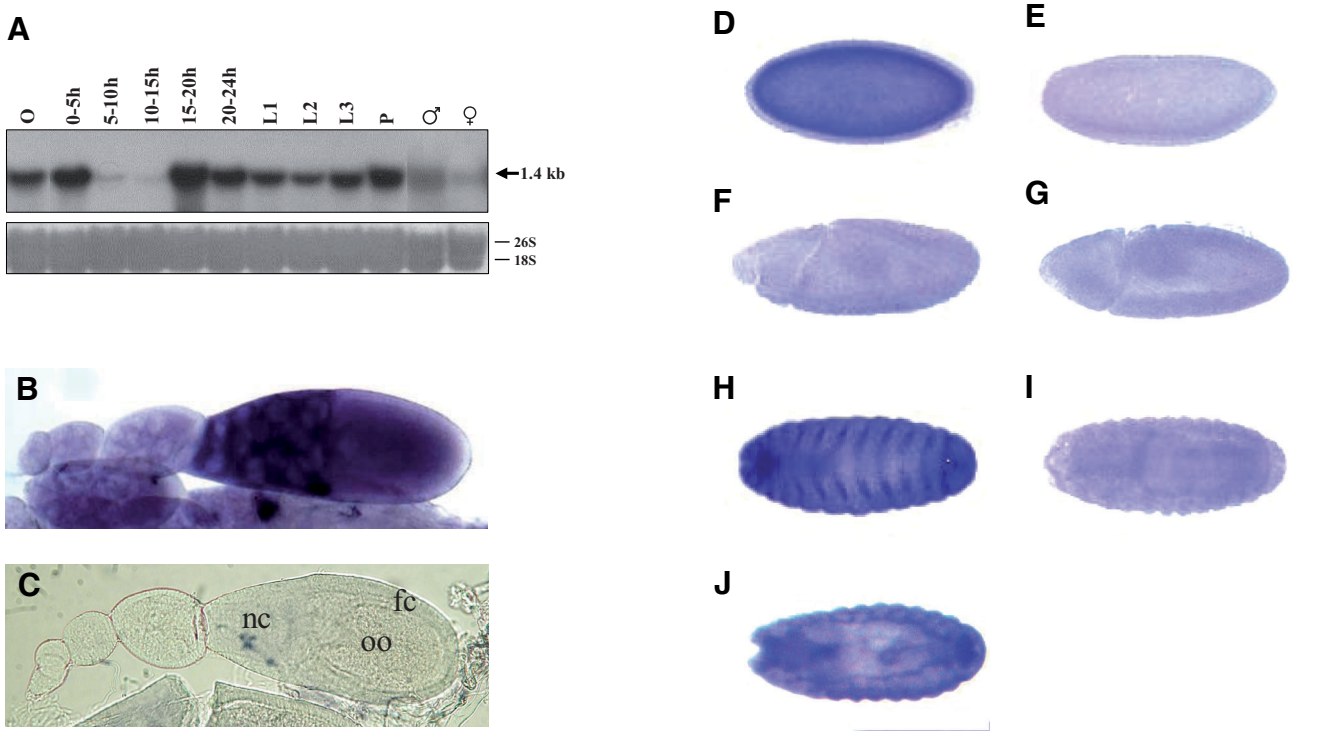



**Fig. 2. Developmental expression analysis of *Spn5* by Northern blot and *in situ* hybridization. (A)** *Northern blot analysis of total RNA from ovaries (O), 0 to 24 hour embryos, first, second and third instar larvae (L1-L3), pupae (P) and adult male (♂) and female (♀)* Drosophila. *The size expected for full-length* Spn5 *mRNA is indicated by an arrow. Staining of the membrane (lower panel) shows 26S and 18S rRNAs as loading controls.* **(B,C)** *RNA* in situ *hybridization analysis of whole mount ovaries with antisense (B) and sense (C) Dig-labelled riboprobes complementary to a 457 bp fragment of* Spn5 *cDNA. The different cell types of ovaries are indicated (nc, nurse cells; fc, follicle cells; oo, oocyte).* **(D-J)** *RNA* in situ *hybridization analysis of whole mount embryos at stages 5-6* **(D-E)**, *stages 10-16* **(F-G)** *and stages 16-17* **(H-J)** *of development using the antisense (D, F, H and J) and sense (E, G and I) Dig-labelled riboprobes. The anterior part of embryos is on the left. At the later stages,* Spn5 *expression is observed in ventral and dorsal epidermis (strips in H), embryonic/larval posterior spiracles (posterior labeling in J), embryonic foregut and hindgut (anterior labeling in H,J), sensory nervous system primordium, pharynx and respiratory embryonic/larval tracheal system (two canals in J).*

We crossed each recombinant line with the ubiquitous driver *Act5C-Gal4*, and analysed by Northern blot total RNA from heterozygous larvae. A strong decrease in Spn5 mRNA levels was detected in larvae carrying the *Act5C-Gal4* driver and the UAS-Spn5-IR$^R$ transgenes (data not shown). RNAi silencing was not observed with all transformant lines; this can be explained by chromosomal position effects influencing the expression levels of the various UAS-Spn5-IR transgenic insertions. Despite decreased Spn5 expression in larvae, no visible phenotypic effect was observed: embryos and larvae developed normally into viable and fertile flies. No decrease in Spn5 mRNA was observed in either pupae or adults carrying *Act5C-Gal4* in combination with any of the UAS-Spn5-IR$^R$ recombinant chromosomes (data not shown). However, these results demonstrated that we could target Spn5 expression by RNAi during *Drosophila* development. The 0.9 kbp Spn5 fragment used in the RNAi inverted construct showed a 14.7% homology with CG6686, another serpin-encoding gene. By RT-PCR analysis, expression of CG6687 was similar in samples from mutant and control larvae, thus rendering an off-target-effect of the RNAi construct unlikely (data not shown).

We obtained the same results by combining the UAS-Spn5-IR$^R$ construct with the maternal germline-expressed *nos-Gal4:VP16* or *nos-Gal4-NGT$^{40}$* drivers (data not shown). A clear reduction of Spn5 mRNA abundance was measured in ovaries of heterozygous females carrying the driver and the recombinant RNAi construct and also after fertilization during the first hours of embryogenesis, but development was not detectably affected (data not shown).

### *Spn5 is involved in wing unfolding*

To repress Spn5 expression throughout the entire *Drosophila* life cycle, we crossed the ubiquitous driver strain *daughterless-Gal4* (*da-Gal4*) with recombinant lines and analysed the presence of Spn5 mRNA by Northern blot at different stages. In three heterozygous lines carrying both the *da-Gal4* driver and the UAS-Spn5-IR$^R$ transgene, we detected a selective reduction in Spn5 mRNA abundance at larval (Fig. 3A) and pupal stages (data not shown), but not in embryos or in adults. Flies resulting from this cross eclosed normally, but a clear and striking phenotype was observed; indeed, while the wings unfold after wild type or control flies hatch from pupae, all mutant flies carrying both the *da-Gal4* driver and the UAS-Spn5-IR$^R$ transgene, except the R7 mutant, were affected in the unfolding and expansion of their wings (Fig. 4A). These remained folded and hypotrophic (Fig. 4A) throughout the adult life of the mutant flies, whose life span was also severely compromised since all had died by 5 days after eclosion.

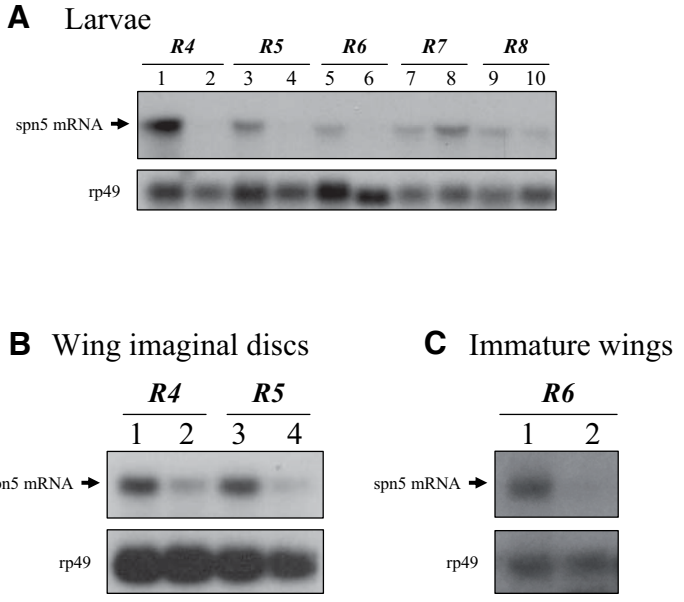
Shortly after eclosion the size and shape of the unopened mutant wings were identical to that of their wild type unopened

counterparts. Histological analysis revealed no differences at this resolution (Fig. 4B-C), neither could any obvious anatomical variation be observed by X-ray microradiology (Fig. 4D-E); in particular, all pattern elements of the wings (veins, bristles and hairs) appeared to be present and normal. The phenotype therefore did not involve gross developmental defects. It was fully penetrant in all different transgenic lines analyzed, except the R7 line which never showed an RNAi silencing effect and displayed a wild type phenotype. The wing phenotype was dependent on the ubiquitous *da-Gal4* driver, since tissue-specific repression of Spn5 in wing imaginal discs or wing structures using other different specific Gal4 driver lines (*engrailed-Gal4*, *apterous-Gal4*, *hemese-Gal4* and *spalt-Gal4*) did not interfere with wing expansion (data not shown).

To further explore how Spn5 might be involved in normal wing maturation, we investigated its expression in wing imaginal discs (Fig. 3B) and immature folded wings (Fig. 3C). Spn5 mRNA was detected in samples from control flies, and a selective repression of Spn5 expression was observed in samples from the RNAi mutants. These results were confirmed by enzyme binding assay (data not shown). Thus, Spn5 is expressed in wing imaginal discs at the pupal stage and also in folded wings of the newly eclosed fly, where it could play a local role in wing development and maturation. In any event, specific silencing of Spn5 expression in larvae and pupae results in a dramatic defect in wing unfolding, and thus demonstrates a critical role for Spn5 in this process.

## Discussion

In a study aimed at revealing antiproteases that could be involved in early *Drosophila* development, we identified a ligand, present in embryos, which formed a stable 67 kDa complex with $^{125}$I-trypsin. Preliminary evidence showed that this trypsin-binding protein was a member of the serpin family of antiproteases; purification and sequencing allowed us to identify it as Spn5, a *Drosophila* serpin first described by Han *et al.* (Han *et al.*, 2000). Spn5 is known to be expressed in the ovary and in embryos and to encode a protein of 427 amino acids, with a predicted signal



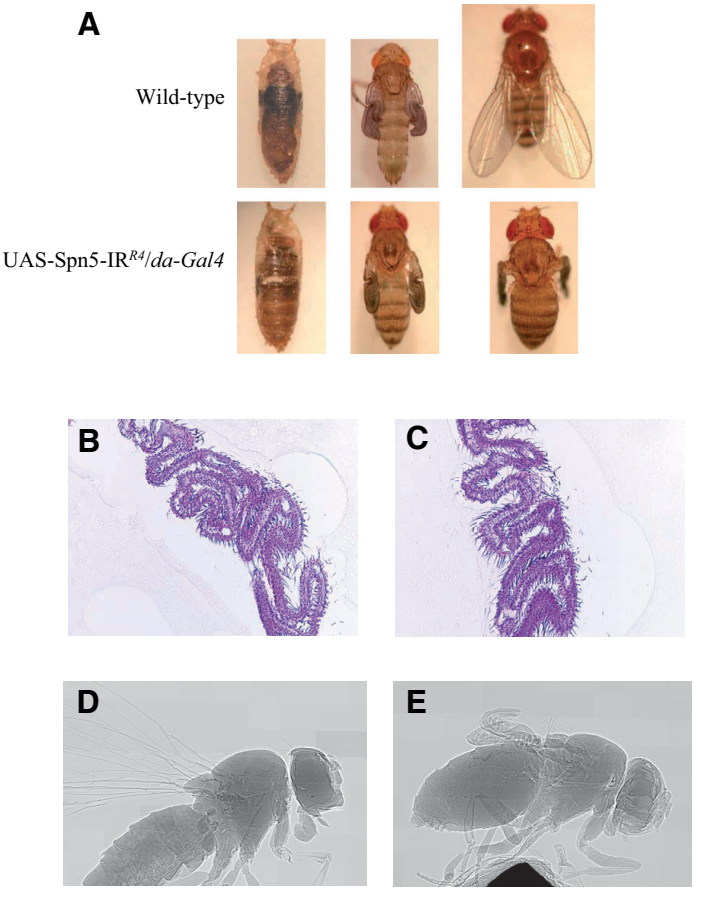




**Fig. 3 (Left). *Spn5* RNAi silencing in larvae, wing imaginal discs and unopened immature wings using the *da-Gal4* driver. (A)** *Northern blot analysis of total RNA isolated from control UAS-Spn5-IR$^{R}$ larvae (odd-numbered lanes), and from* Spn5 *RNAi expressing larvae carrying the* da-Gal4 *driver and the UAS-Spn5-IR$^{R}$ construct (even-numbered lanes). Five recombinant lines (R4-R8) were analyzed and a marked RNAi-induced decrease in* Spn5 *mRNA (arrow) was detected in three lines. Hybridization with a rp49 probe was used as a loading control.* **(B)** *Northern blot analysis of total RNA isolated from wing imaginal discs of control UAS-Spn5-IR$^{R}$ (lanes 1 and 3) and of Spn5 RNAi-expressing* da-Gal4 */ UAS-Spn5-IR$^{R}$ (lanes 2 and 4) larvae. Recombinant lines R4 and R5 were analyzed and an RNAi induced decrease in* Spn5 *mRNA (arrow) was detected. Hybridization with a rp49 probe was used as a loading control.* **(C)** *Northern blot analysis of total RNA isolated from immature wings of controls UAS-Spn5-IR$^{R6}$ (lane 1) and* Spn5 *RNAi-expressing* da-Gal4 */ UAS-Spn5-IR$^{R6}$ (lane 2) flies. Hybridization with a rp49 probe was used as a loading control.*

**Fig. 4 (Right). Phenotype of *Spn5* RNAi mutants obtained using the *da-Gal4* driver. (A)** *Wild-type (top) and* da-Gal4 */ UAS-Spn5-IR$^{R4}$ (bottom) pupae (left), newly eclosed (middle) and two days-old flies (right).* **(B,C)** *Cross section of agar-embedded control* **(B)** *and* Spn5 *RNAi expressing* da-Gal4 */ UAS-Spn5-IR$^{R4}$ unopened wings* **(C)**. *Tissue was prepared as described in Materials and Methods and stained with hematoxylin/eosin (x20).* **(D,E)** *X-ray microradiology analysis of control UAS-Spn5-IR$^{R4}$* **(D)** *and* Spn5 *RNAi expressing* da-Gal4 */ UAS-Spn5-IR$^{R4}$* **(E)** *flies two days after eclosion.*

sequence at its N-terminus suggesting its secretion into the extracellular medium (Han *et al.*, 2000). The amino acid sequence shows clear homology with other members of the serpin family and the reactive centre residues, i.e. Arg-Ser, predict that Spn5 should be an Arg-serpin active against proteases of tryptic substrate specificity (Han *et al.*, 2000).

Our own study of Spn5 expression by Northern blot and *in situ* hybridization revealed that Spn5 transcripts are produced in the ovary and expressed during the first hours of embryogenesis. This is in agreement with the report of Han *et al.* (Han *et al.*, 2000). The presence of Spn5 mRNA in ovarian nurse cells and oocytes but also in syncytial blastoderm, prior or at the onset of zygotic transcription, suggests that Spn5 functions as a maternal effect gene during the earliest stages of embryogenesis. To test this hypothesis, we targeted Spn5 expression by RNAi, and obtained a clear reduction in Spn5 mRNA levels in ovaries and preblastoderm embryos. However, we detected neither developmental abnormalities nor defects in fertility in the mutant flies. Several explanations may account for this lack of an obvious phenotype. First, the RNAi transgene (UAS-Spn5-IR) did not induce a complete suppression of Spn5 gene expression; in fact, we had to generate flies carrying multiple copies of the UAS-Spn5-IR transgene in order to achieve a detectable effect on Spn5 mRNA levels. In this context, a *Drosophila* mutant line with a transposable element (PBac) insertion into the Spn5 gene has been generated by Exelixis as part of the Gene Disruption Project (Thibault *et al.*, 2004). The homozygous mutant line is not viable; thus, provided that insertion of the transposable element alters exclusively the Spn5 locus, this indicates that Spn5 could play an important role during development, perhaps as a maternal effect gene. By contrast, the heterozygous line is viable suggesting that a partial decrease of Spn5 expression is not sufficient to perturb development, in accord with our own observations. It is possible that other serpin genes (Spn1-4, 6) also expressed in ovary (Han *et al.*, 2000) could compensate for the lack of Spn5. Spn3-6 have the same reactive site residue at the P1 position as Spn5 and thus may also be active against trypsin-like enzymes; since it is known that a single member of the serpin family can inhibit several serine proteases, it is reasonable to envision a process of serpin compensation.

The presence of Spn5 transcripts in a variety of different tissues at the end of embryogenesis and at larval, pupal and adult stages suggests that Spn5 could function during postembryonic development and adult life. In late embryos (stages 16 and 17), the gene encoding Spn5 exhibits a complex expression pattern involving the epidermis, posterior spiracles, foregut and tracheal system; however, the RNAi approach that we used failed to abolish Spn5 expression at these stages and consequently the role of Spn5 in late embryos is still unknown. By contrast, in adult *Drosophila*, serine proteases and serpins appear to have important roles in the regulation of immune responses, and Spn5 may be a relevant player in these processes. In this regard, Lemaitre and colleagues monitored the gene expression profile of adult flies in response to microbial infection, using high density oligonucleotide microarrays encompassing nearly the full *Drosophila* genome. They found that 19 trypsin-like serine protease genes are up-regulated and 16 down-regulated by septic injury while five serpin genes, amongst which Spn5, are up-regulated and three down regulated under the same conditions (De Gregorio *et al.*, 2001; De Gregorio and Lemaitre, 2002). Using the same microarray approach, Irving *et al.* confirmed these results and also noted Spn5 up-regulation during immune responses (Irving *et al.*, 2001). A transcriptional profiling of distinct *Drosophila* blood cell (hemocytes) populations showed that, of the 13000 genes represented on the microarray, over 2500 exhibited significantly enriched transcription according to hemocytes subgroups; among these were genes encoding serine proteases and serpins such as Spn5 (Irving *et al.,* 2005). Karlsson *et al.* (Karlsson *et al.*, 2004) and Irving *et al.* (Irving *et al.*, 2005) have detected of Spn5 in larval hemolymph, while De Gregorio *et al.,* and Ligoxygakis *et al.,* reported in flies deficient for another Serpin called Spn27A constitutive melanization of the cuticle and wings of adult flies, or melanotic tumors in larvae (De Gregorio *et al.*, 2002; Ligoxygakis *et al.*, 2002). In preliminary results, we failed to detect Spn5 mRNA in larval hemolymph cells, or aberrant activation of melanization in flies with a low basal level of Spn5 expression (data not shown).

As described above, the only conspicuous phenotype that we have observed following RNAi-mediated perturbation of Spn5 expression is a defect in wing development, specifically in the unfolding and expansion of the wings. The demonstration of Butler *et al.,* via microarray analysis, that Spn5 is expressed in wild-type imaginal discs, is in accord with our observations (Butler *et al.*, 2003). Moreover, De Gregorio *et al.* have observed very similar wing phenotypes to the ones we have reported, in flies deficient in Spn-27A (De Gregorio *et al.*, 2002). These results suggest that serpin-mediated regulation of serine protease activity plays an important role in wing morphogenesis.

The development of *Drosophila* wings up to the time of eclosion has been well documented (for reviews, see Fristrom and Fristrom, 1993, Murray *et al.*, 1995). The wing disc, which consists initially of a single layer of epithelial cells, grows by cell proliferation during larval development and everts during pupal metamorphosis. As a result, two single-layered epithelial sheets of flattening cells become precisely apposed to each other and differentiate into the dorsal and ventral surfaces of the wing. During pupation, the wing layers go through two rounds of adhering to one another and then separating during metamorphosis. During these processes, it is thought that hemocytes secrete extracellular matrix (ECM) that binds epithelia together and subsequent separations might be caused by proteolysis and phagocytosis of the ECM by hemocytes (Kiger *et al.*, 2001). One of the potential roles of Spn5 during this process could be to control the proteolysis of the ECM so as to preserve the developing tissue from over-degradation.

The events accompanying wing maturation subsequent to eclosion of the adult fly are not as well understood as those involved in earlier steps of wing formation. Shortly after eclosion, nearly all wing cells die and an increase in blood pressure begins to expand the wings which, within one hour, unfold to form a cuticular bilayer devoid of cells. A few earlier studies have identified different genes involved in the regulation of wing inflation, but none of these encoded a serpin. The tracheal system could be implicated in the gas filling of the wing (Hartenstein *et al.*, 1997) and defects in neurosecretory cells producing the peptides that signal the wing inflation process could interfere with normal wing expansion (Bantignies *et al.*, 2000). It has also been showed that hemocytes persist between the wing surfaces after eclosion

and are essential for normal wing maturation (Kiger *et al.*, 2001). From all of these reports it has been suggested that wing maturation could be controlled by the release of a hormone that increases blood pressure, causing wing unfolding, and that activates hemocytes to perform their roles of phagocytosis and ECM synthesis (Kiger *et al.*, 2001). In this context, it is tempting to speculate that the reduction of Spn5 mRNA abundance in the unfolded wings of the RNAi Spn5 mutant adults concerns the hemocytes, which can express Spn5 (Irving *et al.*, 2005), and that this impairs the function of these cells and thereby wing maturation.

The RNA interference approach that we used causes a milder phenotype in comparison to the lethality observed in the homozygous transposable element insertion line specific for Spn5 (Thibault *et al.*, 2004). This has allowed us to observe a more subtle and late phenotype and therefore to reveal a specific function of Spn5 in development which has been, to date, impossible to detect with the transposable element insertion line.

Additional studies will be required to determine the proteolytic and physiological pathways that involve Spn5. The wing phenotype obtained by expressing the *da-Gal4*-driven UAS-Spn5-IR transgene demonstrates that this serpin plays at least one important role in development; it also provides a good model to elucidate one of its essential biological functions. Given the importance of proteases and antiproteases in physiology and pathology, identification of cellular events controlled by a serpin in *Drosophila* may enlighten some of the functions of these proteins in other species.

## Materials and Methods

### Drosophila *stocks*

*Drosophila melanogaster* of Canton-S wild-type strain were used for all experiments concerning the biochemical characterization and purification of Spn5. The flies were grown at 22°C on a standard cornmeal/agar medium and eggs were collected employing standard conditions and procedures (Roberts, 1986; Wieschaus and Nusslein-Volhard, 1986).

### *Preparation of* Drosophila *embryo extracts*

Embryo extracts were prepared on ice by homogenizing dechorionated embryos in TEI buffer (Tris-HCl 50mM pH 7.4, EDTA 1mM, 5mM iodoacetamide (Sigma, Buchs, Switzerland)), and spinning at 3000 x g for 5 minutes to remove yolk proteins and debris. The supernatants were collected and aliquots were immediately used for the binding assays. Protein concentration was determined by the Bradford assay. Extracts were routinely prepared at concentrations of 10-20 mg/ml.

### *Binding assay*

Trypsin (TPCK treated bovine trypsin, Sigma, Buchs, Switzerland), was iodinated using IODO-GEN and $Na^{125}I$ (Amersham Biosciences, Otelfingen, Switzerland) as described (Fraker and Speck, 1978; Kruithof *et al.*, 1984). The binding assay was performed as described (Charron *et al.*, 2005) except that the incubation of protein extracts with iodinated trypsin was carried out at 37°C for 30 minutes.

### *DEAE-Sepharose chromatography*

A DEAE-Sepharose column (1x5 cm) (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with TEI buffer at 4°C. Samples (protein extracts) were applied to the column at a flow rate of 20 ml/h and the column was washed with three column volumes of the equilibrating buffer. The column was eluted with a 50 ml linear gradient of 0-0.4M NaCl in TEI buffer. Fractions (3 ml) were collected and assayed for complex formation with $^{125}I$-trypsin. The fractions which contained the ligand of interest were pooled and used for further experiments.

### *Protein purification*

The protein extract from 2g of 0-2 hour old embryos in 10 ml of TEI was applied to a DEAE-Sepharose column as described above. 20 ml of pooled DEAE-purified material were incubated for 30 minutes at 37°C following the addition of 10 μg of biotinylated-trypsin (DPCC treated bovine trypsin, Sigma, Buchs, Switzerland) containing a tracer of $^{125}I$-biotinylated-trypsin ($2x10^6$ cpm). 300 μl of tetralink tetrameric avidin resin (Promega, Walliseller, Switzerland) were added and incubation was continued for 2 hours at 4°C with agitation. The sedimented resin was then washed extensively with TEI buffer. Elution was either performed with 2% SDS / 20%–β-mercaptoethanol in a boiling water bath to release the complex, the sample being subsequently treated with $NH_4OH$ 2M, or the resin was directly treated with $NH_4OH$ 2M to release the ligand by dissociation of the complex. Measurement of radioactivity was used to estimate the efficiency of binding or elution. Samples were analyzed by SDS-PAGE under reducing conditions followed by silver staining or blotting on a PVDF membrane (Immobilion-P, 0.4 μm, Millipore). The 45 kDa ligand band, visible after amidoblack staining, was subjected to N-terminal amino acid sequencing in an Applied Biosystems sequencer model 473 according to the manufacturer's instructions.

### *Cloning of* Spn5 *cDNA*

4 μg of total RNA extracted from 0-2 hours embryos with Trizol (Invitrogen, Basel, Switzerland) were mixed with 0.5 μg oligo dT (16T), denatured 10 minutes at 65°C and used in a reverse transcription reaction following the manufacturer's instructions (Invitrogen, Basel, Switzerland). The full length Spn5 cDNA was then amplified with primers designed on the basis of the EST sequence LD02956 (GenBank accession no. AA202738) similar to the purified peptide (5'-GGAATTCGGCACGAGCGGACTTGCAGTCGTTCCAAG-3', and 5'-GCTCTAGAGCAAAGTTGCAGCACCTTAAAGC-3'), using Expand™ High Fidelity PCR System (Roche, Basel, Switzerland) according to standard reaction conditions except for the addition of 5mM $MgCl_2$. 10 cycles were performed, each consisting of 1 minute at 94°C, 1 minute at 60°C (-1°C/cycle), and 1 minute at 72°C, followed by 20 cycles each consisting of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C. The 1.4 kb PCR product was cleaved with *Eco*RI and *Xba*I and ligated into the *Eco*RI-*Xba*I sites of the pBSKS vector to yield the plasmid pBSKS-Spn5-cDNA. DNA sequencing analysis confirmed the presence of the full length Spn5 cDNA sequence.

### *Northern blots*

Total RNA was extracted from staged embryos, larvae, pupae, adults and wings with Trizol (Invitrogen, Basel, Switzerland), and 1, 2, 5 or 10 μg of RNAs were denatured in glyoxal, electrophoresed in 1% agarose gels, transferred to positively charged Nylon membranes (Roche, Basel, Switzerland), and stained with methylene blue to verify the quality of the RNA. Hybridizations were performed with $^{32}P$-labeled riboprobes complementary to a 457 bp fragment of Spn5 cDNA (positions 1-457 of cDNA clone CG18525, GenBank accession no. NM_080215) and to rp49 cDNA (a PCR fragment of 211 bp generated between two oligonucleotides designed from the rp49 coding sequence) (O'Connell and Rosbash, 1984), under standard conditions.

### *Whole mount* in situ *hybridizations*

Whole mount *in situ* hybridizations to ovaries and embryos were performed as described (Lehmann and Tautz, 1994) using the same probe as in Northern blots, labeled with digoxigenin UTP (Boehringer Mannheim, Rotkreuz, Switzerland).

### *Bacterial expression and affinity purification of Spn5*

Poly histidine-tagged Spn5 fusion protein was generated using the

QIAexpress bacterial expression system (Qiagen, Basel, Switzerland). A *Nde*I-*Not*I cDNA coding for Spn5 was amplified with Expand$^{TM}$ High Fidelity Polymerase PCR System (Roche, Basel, Switzerland) according to standard reaction conditions and using the following oligonucleotides: 5'-GGAAAATCCATATGGGTCTCTGTGGG-3' and 5'-ATAGTTTAGCGGCCGCCTGCTTTATTG-3'. The PCR product was cloned via the *Nde*I and *Not*I sites into the expression vector pET-29b (Novagen, Madison, USA). This yielded a 422-amino acid recombinant protein without signal sequence at the NH2-terminus and comprising six additional histidines (His-tag) at the C-terminus. The correct structure of the construct was confirmed by DNA sequencing.

Over-expression using pET-29b-Spn5-6XHIS was found to be optimal in Escherichia coli strain BL21 (DE3) pLys using LB medium (Sambrook and Russel, 2001). Recombinant protein was expressed and purified under native conditions by affinity chromatography on a Ni-NTA agarose resin essentially as described by the manufacturer (Qiagen, Basel, Switzerland).

***Caseinolytic assay***

100 ng of trypsin containing a tracer of $^{125}$I-trypsin (1x10$^5$ cpm; 6 ng) were incubated at 37°C in a 96 wells plate with 0, 6, 12, 18, 30, 60, 120 and 240 ng of recombinant Spn5 in 100mM Tris/HCl, pH 8.1, containing 1% casein. Absorbance at 410 nm was determined with a plate reader after 0, 30, 60, 120 and 240 minutes of incubation; hydrolysis of casein by trypsin causes a decrease in absorbance which is proportional to enzyme activity.

An aliquot of each sample was collected after 30 minutes of incubation and analysed by SDS-PAGE and autoradiography. The fixed gel was sliced at the position of free trypsin and of the complex and the slices counted in a gamma counter. The proportion (%) of complexed $^{125}$I-trypsin was calculated.

***RNAi plasmid construction***

A 917 bp fragment containing the last 305 codons of Spn5 (GenBank accession no. NM_080215, positions 458-1373), flanked by the restriction sites *Xba*I and *Not*I on the 5' side and *Kpn*I and *Xho*I on the 3' side, was amplified from the pBSKS-Spn5-cDNA vector using the following primers: 5'-ATATCTAGAGCGGCCGCTTCTCCTCAGCC-3' and 5'-TGTGGTACCCTCGAGCTGCTTTATTGTTTTGG-3'. The PCR product was cloned into the pGEM-T easy vector (Promega, Walliseller, Switzerland) to yield the plasmid pGEM-Spn5-0.9. The 900 bp Spn5 fragment was then cut out of this plasmid by digestion with *Not*I and *Xho*I, and ligated into *Not*I-*Xho*I double-digested P-element vector, pUAST1 (containing a 223 bp intron from the *trachealess* (*trh*) gene; Ghabrial A. and Schupbach T., personnal communication), to give pUAST1-Spn5. A second 900 bp Spn5 fragment was cut out of the plasmid pGEM-Spn5-0.9 by digestion with *Xba*I and *Kpn*I, and ligated into *Xba*I-*Kpn*I double-digested pUAST1-Spn5 vector, to produce the inverted repeat construct pUAST1-Spn5-IR. Finally, the 2053 bp *Not*I-*Not*I fragment containing the two Spn5 inverted repeats separated by the 223 bp *trh* intron was cloned into the *Drosophila* P-element transformation vector pUASP (Rorth, 1998) to give pUASP-Spn5-IR. The pUASP-derived vector UAS-Spn5-IR was transformed into the w$^{1118}$ strain. The 917 bp Spn5 fragment was found by Blast analysis to have 14,7% homology with another serpin-encoding gene, CG6687 (GenBank accession no. NM 142170). To investigate possible off-target effects we performed RT-PCR analysis of different RNA samples from mutant and control larvae, using the following CG6687-specific primers: 5'-AGGAGATCACACCCCACACC-3' and 5'-GATGTTGGGCGTCATCGATG-3'.

***P-element mediated transformation***

Transgenic flies were generated by standard P-element transformation procedures into the w$^{1118}$ recipient strain as previously described (Karess and Rubin, 1984; Spradling, 1986).

Eighteen independent transformant lines were obtained with autosomal insertions, the chromosome harboring the UAS-Spn5-IR insert(s) was determined with standard genetic crosses and the stocks were established using either TM3 or Cyo balancer chromosomes. To produce the UAS-Spn5-IR recombinant strains, we used genetic recombination between the seven independent third chromosome UAS-Spn5-IR transgenic insertions that had been obtained. All transformant and recombinant lines were analyzed for their ability to trigger dsRNAi upon activation of transgene transcription by crossing to *daughterless-Gal4* (*da-Gal4*), *Actin5c-Gal4* (*Act5c-Gal4*), *GMR-Gal4*, *nanos-Gal4:VP16* (*nos-Gal4:VP16*) and *nanos-Gal4-tubulin* (*nos-Gal4-NGT$^{40}$*) driver strains.

The *nanos-Gal4:VP16* (Doren *et al.*, 1998) and *nanos-Gal4-NGT$^{40}$* (Tracey *et al.*, 2000) drivers were used to express pUASP-transgenes in the germline. The *daughterless-Gal4* (Wodarz *et al.*, 1995) and *Actin5c-Gal4* (Ito *et al.*, 1997) drivers were used to ubiquitously express pUASP-transgenes during development. Transgenic RNAi flies were all homozygous and Gal4 driver flies were all homozygous with the exception of the *Actin5c-Gal4* that carries a single copy of the *Actin5c-Gal4* transgene balanced on chromosome II. Descriptions of markers, Gal4-expressing lines, and chromosomes can be accessed using the Flybase databases (Consortium, 1999).

***Wing histology***

Wings were dissected and fixed in freshly prepared 4% formaldehyde for 2 to 8 hours at room temperature. Fixative was washed out with six to ten changes of phosphate-buffered saline or $H_2O$ over a 3 hours period. The tissue was then impacted in agar, dehydrated, infiltrated with xylol and embedded in paraffin. 10 μm sections were cut with knives and the material was stained with hematoxylin and eosin.

***X-ray microradiology***

X-ray analysis was performed with the technique of phase contrast imaging based on the spatially coherent x-rays emitted by a synchrotron source. This approach yields very good levels of spatial and time resolution and is particularly appropriate for microradiology analysis. The technique and the procedure are described in Hwu *et al.,* (Hwu *et al.*, 2002).

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