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Drosophila Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation

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ABSTRACT

In insects the enzyme phenoloxidase (PO) catalyzes melanin deposition at the wound site and around parasitoid eggs. Its proenzyme prophenoloxidase (proPO) is proteolytically cleaved to active phenoloxidase by a cascade consisting of serine proteases and inhibited by serpins. The *Drosophila* genome encodes 29 serpins, of which only two, Serpin-27A (Spn27A) and Necrotic, have been analyzed in detail. Using a genetic approach, we demonstrate that the so far uncharacterized Serpin-28D (Spn28D, CG7219) regulates the proPO cascade in both hemolymph and tracheal compartments. *spn28D* is the serpin gene most strongly induced upon injury. Inactivation of *spn28D* causes pupal lethality and a deregulated developmental PO activation leading to extensive melanization of tissues in contact with air and pigmentation defects of the adult cuticle. Our data also show that Spn28D regulates hemolymph PO activity in both larvae and adults at a different level than Spn27A. Our data support a model in which Spn28D confines PO availability by controlling its initial release, while Spn27A is rather limiting the melanization reaction to the wound site. This study further highlights the complexity of the proPO cascade that can be differentially regulated in different tissues during development.

Introduction

Several immune mechanisms are induced in insects after wounding and infection. One of the most prominent reactions occurring in wounded arthropods is the activation of prophenoloxidase (proPO) to its active form phenoloxidase (PO), which finally leads to formation of melanin at wound sites and around large intruding organisms in the hemolymph (Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005). Intermediate cytotoxic compounds of the reaction are assumed to be important immune effectors against protozoans and parasitoids, although this notion is currently controversial (Michel et al., 2006; Schnitger et al., 2007). Studies in *Drosophila* have suggested that the melanization reaction, although not essential for resistance to microbial infection, can enhance the effectiveness of other immune reactions (Braun et al., 1998; Leclerc et al., 2006; Tang et al., 2006). In addition to its role in the wound response, PO of the laccase type has been reported to be involved in sclerotization and pigmentation of the cuticle during development (Andersen, 1995; Arakane et al., 2005). However, it is not clear if melanization during infection and during development in the absence of foreign elicitor molecules interacts and if they are similarly regulated.

The proPO cascade consists of several serine proteases which are in turn inhibited by serpins (*ser*ine *p*rotease *in*hibitors) that bind irreversibly to their specific target serine proteases (Kanost and Gorman, 2008). Triggering of the cascade has to be well regulated to avoid activation in the absence of a wound and to ensure a localized reaction. It is assumed that this regulation is mainly achieved by serpins, which is strengthened by the fact that the expression levels of PO genes remain relatively stable after wounding or infection, while several serine proteases and serpins are strongly induced by such treatment (De Gregorio et al., 2001). The fine balance of the factors required for beneficial PO activity may be achieved at both the temporal and the spatial level. The inhibitory mechanism of serpins is characterized as a “suicide substrate-like mechanism”, in which the serpin is cleaved by its target serine protease and remains irreversibly bound to it (Ye and Goldsmith, 2001). Serpins were first described as regulators of blood coagulation and complement activation in humans. Attention was drawn to them, because mutations in these proteins cause diseases such as emphysema, cirrhosis, dementia and blood coagulation disorders (van Gent et al., 2003). Similarly, serpins are also implicated in different aspects of arthropod immunity

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(Kanost, 1999). *Drosophila* is a genetically tractable model system that is currently used to address general questions concerning serpin activities and functions (Carrell and Corral, 2004).

Of the 29 serpin genes encoded by the *Drosophila* genome, 19 are predicted to be inhibitory molecules (Reichhart, 2005). One of them, *serpin-27A* (*spn27A*), is already known to regulate proPO activation and is strongly expressed in larvae and pupae. Spn27A is an ortholog of a serpin that regulates a protease that directly activates proPO in Manduca (Zhu et al., 2003). In *Drosophila* adults, the *spn27A* gene is induced upon microbial infection by the Toll pathway (De Gregorio et al., 2002; Ligoxygakis et al., 2002). The *spn27A* mutant displays a weak but consistent constitutive melanization of internal organs and cuticles of larvae and adults, which mainly affects the wings and leg joints of flies. The mutant is further characterized by an over-reactive PO activity after pricking or wasp infection. In addition, Spn27A has a crucial role in determination of dorsoventral polarity (Ligoxygakis et al., 2003). While Easter was identified as the developmental protease target of Spn27A during development (Hashimoto et al., 2003), the protease target of this serpin in the *Drosophila* melanization cascade is to date not clear. However, a prophenoloxidase-activating enzyme (PPAE) from a coleopteran insect, *Holotrichia diomphalia*, was inhibited *in vitro* by recombinant *Drosophila* Spn27A protein (De Gregorio et al., 2002).

Although melanization has been known for a long time, the molecular identity of the serine proteases and serpins implicated in the activating pathway remains largely unknown, even in the well-established *Drosophila* model system. Here we present evidence for a role of the so far uncharacterized serpin Serpin-28D (Spn28D, CG7219) in melanization. We used a loss-of-function mutation and *in vivo RNAi* to study the function of Spn28D. We show that knockdown of *spn28D* causes an over-reactive PO activity after pricking in larvae, deregulated developmental PO activation, leading to extensive melanization of tissues in contact with air, hemolymph PO exhaustion and a pigmentation phenotype. Our data point towards a function of Spn28D in limiting proPO activation in hemolymph and tissues exposed to air such as the tracheae. Epistatic analysis indicates that Spn28D acts together with Spn27A in the tight control of hemolymph PO activity in *Drosophila*.

Materials and methods

Fly stocks

A deletion of the *spn28D* locus was obtained by homologous recombination (Rong and Golic, 2000). 3.5 kb and 4.5 kb of DNA sequence flanking the 5′ and 3′ parts, respectively, of the *spn28D* locus (see Fig. 2A) were cloned in the p{W25} vector (Gong and Golic, 2004). Flies transformed with p{W25} were used to generate a deletion of *spn28D* using a standard crossing protocol (Rong et al., 2002). We confirmed by PCR that *spn28D* was replaced by the w^+ gene in $spn28D^{\Delta 1}$ and $spn28D^{\Delta 2}$ mutants, and that neighbouring genes were not affected (data not shown). The $spn28D^{\Delta 1}$ and $spn28D^{\Delta 2}$ mutations were balanced with a *CyO GFP* balancer. *Bc*, $spn28D^{\Delta 1}$ and $spn28D^{\Delta 1}$, *MP2/PAE1* fly stocks were generated by recombination for epistatic analysis.

Fly lines carrying RNAi constructs targeting three different regions of the *spn28D* gene were used in this study. We first generated a *spn28D RNAi* construct by cloning a PCR fragment of 600 bp corresponding to the 402–1001 bp region of the *spn28D* ORF as inverted repeats into the pWIZ vector (Lee and Carthew, 2003). Two transgenic lines, i17 and i28, bearing this construct were used in the present study. The fly lines R2 and R3 (obtained from the National Institute of Genetics in Japan) contain a construct targeting base pairs 1–500 of the *spn28D* ORF. The RNAi line 12377 was obtained from the Vienna RNAi stock center and possesses an insertion targeting the region 966–1309 of the ORF. No off-targets of these RNAi constructs were detected using a web-based search tool (http://www.dkfz.de/signaling/tools.php). The insertions R2 and R3 were located on the second chromosome, and i17, i28 and 12377 on the third chromosome. We used larvae and adults carrying one copy of a *UAS-spn28D-IR* construct combined with one copy of a *GAL4* driver.

If not indicated differently, *act>spn28D-IR* larvae and adults carrying both the ubiquitous driver *actin5C-GAL4* (*act5C-GAL4*) and the *spn28D-IR* R3 insertion were analyzed. Other *GAL4*-drivers used in this study also include the ubiquitously expressed *daughterless (da)-GAL4* driver (Giebel et al., 1997) with a lower RNAi knockdown efficacy. The *lz-GAL4; UAS-GFP* line specifically expresses GAL4 and GFP in larval crystal cells and eye imaginal discs. Experiments with RNAi larvae were performed at 29 °C. *spn28D*-RNAi adults were obtained as follows: *da>spn28D-IR* animals were raised at 25 °C and transferred to 29 °C as flies 4 days prior to experiments. *act>spn28D-IR* flies were initially kept at 25 °C, switched to 23 °C as young pupae until the adult stage (to preclude strong pupal lethality at 25 °C with this GAL4 line and obtain about 10% of adult escapers) and then shifted to 29 °C for 4 days prior to analysis.

Larvae lacking or overexpressing *Notch* were obtained as described earlier and the decrease or increase of crystal cells was confirmed by heating larvae for 10 min at 60 °C, followed by microscopic observation of blackened crystal cells (Duvic et al., 2002). MP2/PAE1 is a mutation affecting the CG3066 gene that encodes a serine protease regulating the proPO cascade (Castillejo-Lopéz and Hacker, 2005; Leclerc et al., 2006; Tang et al., 2006). *Bc* and *spn27A* fly stocks have been described elsewhere (De Gregorio et al., 2002). Fly lines carrying the *spn27A* mutation combined with either *act5C-GAL4* or *spn28D-IR* were constructed and crossed with each other to obtain *act>spn28D-IR* flies with the *spn27A* mutation. Flies carrying one copy of *act5C-GAL4* or *da-GAL4* (*act*>+ and *da*>+) or OregonR flies were used as *wild-type* controls, if not indicated differently. Clean wounding and infections were performed as described (Tzou et al., 2002).

Quantitative real-time PCR

For *spn28D* quantification from whole animals, RNA was extracted using RNA TRIzol™. cDNAs were synthesized using SuperScript II (Invitrogen) and PCR was performed using dsDNA dye SYBR Green I (Roche Diagnostics). Primer pairs for *spn28D* (*CG7219*) (sense 5′-TAG AGT CAG CCA CAC G-3′ and antisense 5′-CCA TTA GCT CCC TCA C-3′), and control primers for *rp49* (sense 5′-GAC GCT TCA AGG GAC AGT ATC TG-3′, and antisense 5′-AAA CGC GGT TCT GCA TGA G-3′) were utilized. SYBR Green analysis was performed on a Lightcycler (Roche). The amount of mRNA detected was normalized to control *rp49* mRNA values to quantify the relative levels of *spn28D* mRNA according to cycling threshold analysis (ΔCt) (Pili-Floury et al., 2004). *Drosomycin* and *Diptericin* expression were monitored as described in (Pili-Floury et al., 2004).

Hemolymph PO activity assay

Larval hemolymph from ten individuals was collected and pooled on an ice-cooled object slide with 40 µl protease inhibitor solution (Roche; one tablet dissolved in 4 ml phosphate-buffered saline, PBS). The hemolymph sample was transferred to a tube on ice; a total volume of approximately 50 µl was obtained from 40–60 larvae. Adult hemolymph was obtained by pricking female flies with a tungsten needle in the thorax. Hemolymph from about 50 flies was collected as described earlier and protein concentrations adjusted after a Bradford test (Tang et al., 2006). The samples were diluted to 800 µl in PBS, and after addition of 200 µl of a L-DOPA solution (20 mM in phosphate buffer pH 6.6) the samples were incubated at 23 °C in the dark. After 30 min, the optical density at 492 nm was measured for each sample against an L-DOPA control containing no hemolymph. Since activation of the proPO system was blocked by the presence of the protease inhibitor, the values reflect the *in vivo* PO activity at the time of wounding. Each experiment was repeated two or three times.

Western blots

For Western blots, hemolymph samples were collected from 10–20 flies in a protease inhibitor solution as described above. Protein concentrations of the samples were determined with a Bradford assay and adjusted to 10 µg/µl prior to gel electrophoresis. Blotting conditions were applied as reported (Ligoxygakis et al., 2002). Blot membranes were incubated overnight at 4 °C with a rabbit antibody against *Anopheles* proPO/PO in a 1:5000 dilution and the next day, after washing with TBS-Tween (0.1%), for 1 h with a goat-anti-rabbit secondary antibody coupled to horseradish peroxidase (Invitrogen) in a 1:2000 dilution. The blots were developed with an ECL system.

Results

spn28D is an injury-induced serpin

The *spn28D* (*CG7219*) gene encodes a protein with a characteristic serpin domain and a putative secretion signal at the N-terminus. Interestingly, we also noted at the C-terminal end of Spn28D the presence of an RGD motif, which is absent from all the other *Drosophila* serpins (Fig. 1A). Spn28D was weakly expressed in naive flies, but strongly induced after an injury in a Toll-dependent manner in both larvae and adults. Importantly, *spn28D* was the strongest induced serpin gene with a peak of about 20-fold induction 1.5 h after injury, which is characteristic of an acute phase profile (De Gregorio et al., 2001) (Fig. 1B). In contrast to many immune genes, the level of *spn28D* expression after pricking was not much enhanced by the addition of microorganisms (Fig. 1C) and thus seemed to be linked to injury rather than microbial infection. In addition, this gene was induced upon septic injury of larvae but not upon natural bacterial infection (Vodovar et al., 2005). An inducible expression of *spn28D* in hemocytes has also recently been demonstrated {Irving et al., 2005). Hence, the expression profile of *spn28D* pointed towards a possible role of this serpin in the wound response rather than in the antimicrobial defense.

Generation and phenotypic analysis of spn28D mutants

To generate a null mutation of *spn28D*, we used a homologous recombination approach to delete 3 kb corresponding to the ORF of the gene. We obtained two independent fly lines, $spn28D^{\Delta 1}$ and $spn28D^{\Delta 2}$, in which the entire *spn28D* locus was replaced by a copy of the *white* gene. We used RT-qPCR to confirm the absence of *spn28D* transcript in homozygous $spn28D^{\Delta 1}$ and $spn28D^{\Delta 2}$ mutants. We checked that the *spn28D* deletion did not affect the expression of three flanking genes, *Wwox* (CG7221), CG12560 and CG34010 (Fig. 2A, data not shown). Both $spn28D^{\Delta 1}$ and $spn28D^{\Delta 2}$ mutations caused 100% lethality at the pupal stage, demonstrating that *spn28D* is an essential gene. Here we only report data obtained with the $spn28D^{\Delta 1}$ mutant, because both alleles produced identical results.

Initially, we checked whether Spn28D acts similarly to Spn43Ac (*necrotic*) (Levashina et al., 1999) as a negative regulator of the AMP response. However, we found that the $spn28D^{\Delta 1}$ mutation had no major effect on the expression of *Drosomycin* (*Drs*) and *Diptericin* (*Dpt*) in both unchallenged and bacterially infected larvae (Fig. 2B), although the *Drs* level was partially reduced in *spn28D* mutants after bacterial challenge. Because the expression of *Drs* and *Dpt* is controlled by the Toll and Imd pathways, respectively, we concluded that Spn28D is not a direct regulator of either pathway.

We observed constitutive melanization in about 80% of $spn28D^{\Delta 1}$ larvae (Figs. 3A and B) and pupae (Figs. 3C and D). Melanization occurred mostly in the tracheae and spiracles, with rare melanotic tumors being observed in the haemocoel, of $spn28D^{\Delta 1}$ larvae and pupae Thus, *spn28D* appears to prevent ectopic melanization of tracheae, a tissue exposed to oxygen important for prophenoloxidase activity. Our phenotypic analysis indicates that *spn28D* is an essential gene required for regulation of melanization.

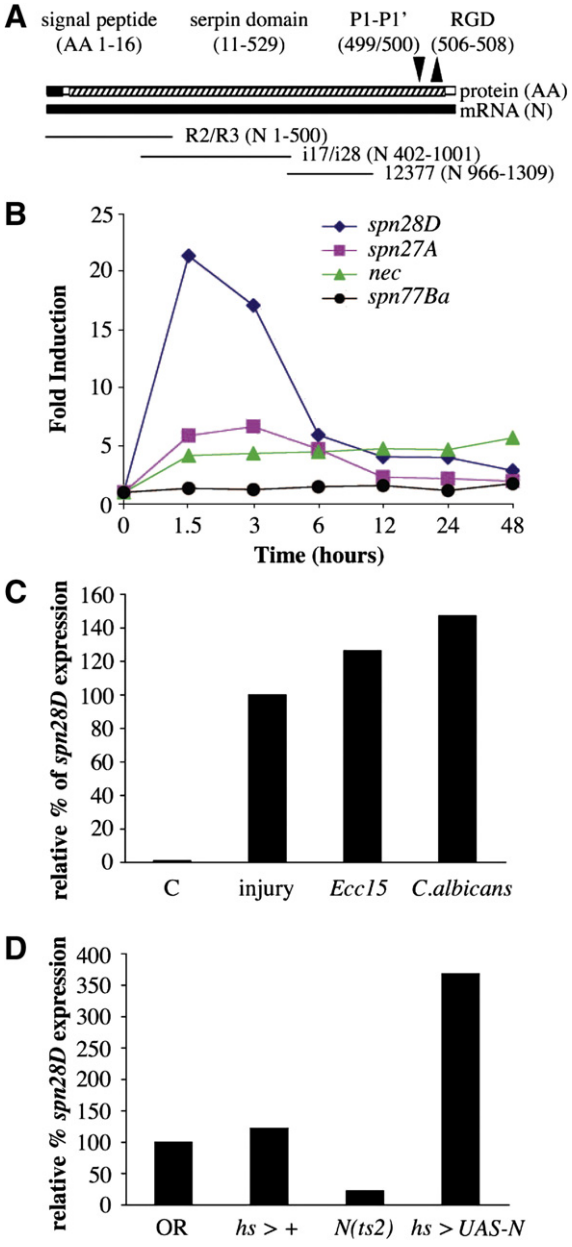

Fig. 1. Spn28D structure and expression in wild-type and *spn28D*-RNAi flies. (A) Spn28D amino acid (AA) sequence with predicted domains, and nucleotide (N) sequence regions targeted by the different IR constructs utilized in the present study; (B) expression profile of *spn28D* and other serpins after microbial challenge (extracted from microarray data in De Gregorio et al., 2001); (C) relative *spn28D* expression in female flies after clean injury or 1.5 h after septic injury with a Gram-negative bacterium (*Erwinia carotovora* 15) or the fungus *Candida albicans* as demonstrated by RT-qPCR, C: unchallenged control; (D) using RT-qPCR, *spn28D* expression was quantified in larvae with normal (OregonR or *hs>+*), lower ($N^{(ts2)}$) or higher (*hs>UAS-N*) numbers of crystal cells. Larvae were wounded with a clean needle and collected 1.5 h later for RNA extraction. The expression of *spn28D* was reduced to about 20% of the control level in a N^{ts2} *Notch* mutant background lacking crystal cells, while heat-shock induced overexpression of *Notch*, leading to supernumerary crystal cells, caused an elevated *spn28D* expression at about 350% of the wild-type amount.

Spn28D controls the melanization process in response to injury

The induction of *spn28D* expression by injury and the *spn28D* mutant phenotype suggested a role of this serpin in melanization at wound sites. Larval crystal cells express two out of three proPO genes

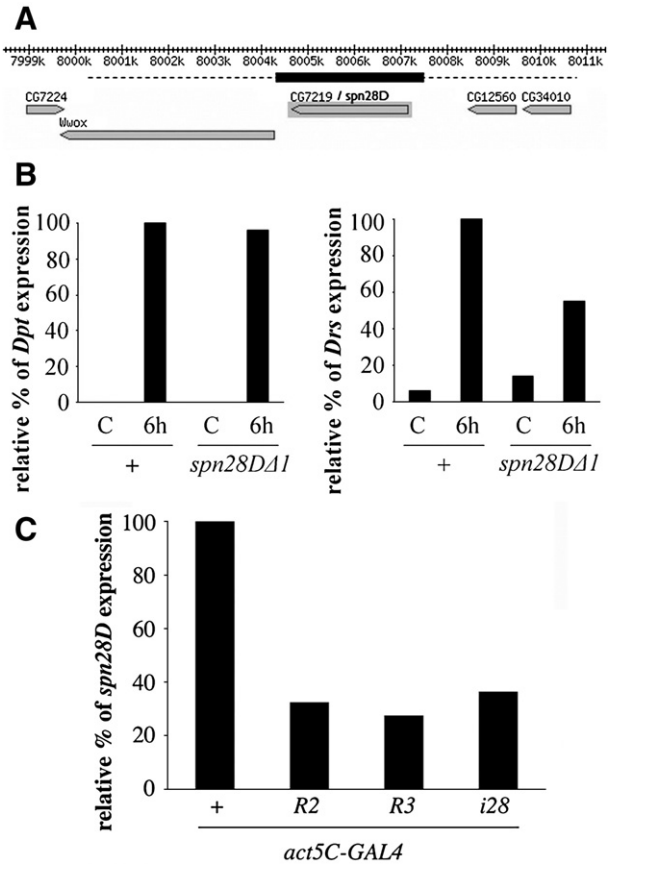

Fig. 2. Generation of *spn28D* mutants. (A) Schematic representation of the *spn28D* deletion. The gene map was adapted from FlyBase and includes *spn28D* and the neighbouring genes. The deleted segment replaced by the *w* gene (black box) as well as the flanking sequences used for recombination (dotted lines) is indicated. (B) Expression of *Dpt* and *Drs* in *spn28D* mutant larvae. Total RNA was extracted from wild-type (+) and *spn28D*$^{\Delta 1}$ larvae, either uninfected (C) or 6 h after infection with Gram-negative *E. carotovora*. Shown are the relative expression levels of *Dpt* (left panel) and *Drs* (right panel) in relation to *rp49*. (C) Relative *spn28D* expression after RNAi using the *act5C-GAL4* driver in combination with different *spn28D-IR* constructs (RT-qPCR). Female flies were pricked with a clean tungsten needle 1.5 h prior to collection of the fly samples. RT-qPCR was performed as described previously (Pili-Floury et al., 2004). Relative *spn28D* expression in relation to *rp49* expression is shown.

(Irving et al., 2005) and are the main source of hemolymph PO activity at wound sites (Rizki and Rizki, 1959). We therefore investigated the possible role of *spn28D* in injury-induced melanization, starting with an analysis of *spn28D* expression in crystal cells. It was not possible to directly isolate crystal cells for RT-qPCR analysis, since these cells are fragile and form only a minor fraction of hemocytes. We thus chose an indirect approach and compared *spn28D* expression in larvae carrying either a *Notch* mutation or overexpressing *Notch*, which lack or contain supernumerary crystal cells, respectively (Duvic et al., 2002). *spn28D* transcripts were less abundant in *Notch* deficient larvae lacking crystal cells and higher in *Notch* overexpressing larvae with supernumerary crystal cells (Fig. 1D). These results suggested a major contribution of crystal cells to the total amount of *spn28D* transcripts in larvae.

Injury to wild-type larvae using a sharp needle induces melanization at the wound site, while *spn27A* mutant larvae and adults have excessive PO activity at the injury site after pricking (De Gregorio et al., 2002). Interestingly, in *spn28D* homozygous mutant larvae, integumental injury led to a stronger melanization reaction visible within 2 h after pricking (Fig. 4C) compared to wild-type larvae (Canton S, Oregon R or flies with a genetic background similar to *spn28D*$^{\Delta 1}$ mutants) (Fig. 4A). In contrast to the *Spn27A* mutant, in which the melanization reaction is diffuse throughout the larval body cavity, in *spn28D*$^{\Delta 1}$ mutants the elevated PO activity was localized exclusively to the wound site. Surprisingly, we observed that the melanization intensity at the wound site was also stronger in *spn28D*$^{\Delta 1}$/+ heterozygous flies compared to wild-type larvae (Fig. 4B). A random analysis of differences in melanization strength between *spn28D*$^{\Delta 1}$/*spn28D*$^{\Delta 1}$ and *spn28D*$^{\Delta 1}$/+ larvae revealed a significant increase of *spn28D*$^{\Delta 1}$/*spn28D*$^{\Delta 1}$ mutants among the most melanized larvae: 36 of 68 *spn28D*$^{\Delta 1}$/*spn28D*$^{\Delta 1}$ larvae exhibited strong melanization at the wound site compared to 23 of 75 *spn28D*$^{\Delta 1}$/+larvae ($P<0.05$). Although melanization at the wound site is variable, our data support the idea that Spn28D regulates melanization at the wound site.

Ectopic melanization and pigmentation defects in spn28D-RNAi adults

We also investigated the role of Spn28D in adults, since it is easier to monitor hemolymph PO activity at this stage. To circumvent the requirement of *spn28D* at the pupal stage, we decided to use RNAi to knockdown its expression *in vivo* and therefore analyzed transgenic fly lines that could express double stranded RNA targeting *spn28D* under the control of the UAS sequence element. Three distinct inverted repeat constructs targeting different regions of the *spn28D* mRNA were tested in this study (Fig. 1A, Materials and methods). *act5C-GAL4* (*act>*) and *da-GAL4* (*da>*), in which the GAL4 protein is broadly expressed under the *actin5C* or *daughterless* gene promoters, induce UAS-coupled genes throughout development in virtually all tissues. When we used *act5C-GAL4* for ubiquitous *spn28D*-RNAi, RT-PCR analysis indicated that *spn28D* expression at 1.5 h post injury was specifically knocked down to 30–40% of the wild-type level (Fig. 2C). The *spn28D* knockdown efficacy was weaker in *da>spn28D-IR* flies (about 60% of the wild-type amount; data not shown). The use of three independent RNAi constructs (*spn28D-IR*) and the observation that all the insertions tested caused a similar melanotic phenotype to that of *spn28D*$^{\Delta 1}$ mutants (see below), with its relative strength well correlated to the observed knockdown efficacy, indicated that the RNAi effects reported in this study are due to *spn28D* knockdown.

Ubiquitous *spn28D* knockdown occasionally led to small black or brown spots on the cuticle of *act>spn28D-IR* larvae. These flies died at the pupal stage when raised at 25 °C or 29 °C, the latter considered optimal for the UAS/GAL4 system. When young *act>spn28D-IR* pupae were moved to 23 °C to preclude pupal lethality, about 10% adult escapers were obtained that could further be studied at 29 °C. Strikingly, almost no melanized spots were present in young *act>spn28D-IR* flies at the time of eclosion, but developed several hours after eclosion. In

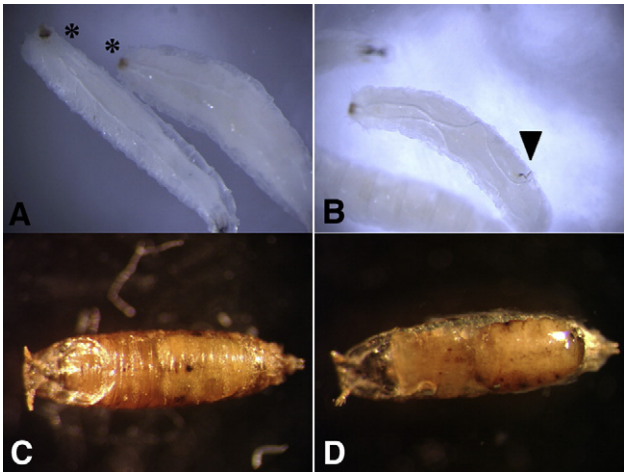

Fig. 3. Ectopic melanization of trachea in *spn28D* mutant larvae and pupae. Phenotypes of *spn28D*$^{\Delta 1}$ unchallenged third instar larvae (A, B) and pupae (C, D) are shown in this figure. Melanization of the tracheae (arrowhead) and spiracles (asterisks) are observed in larvae. Frequently lines of black dots were found at the lateral sides of the pupae.

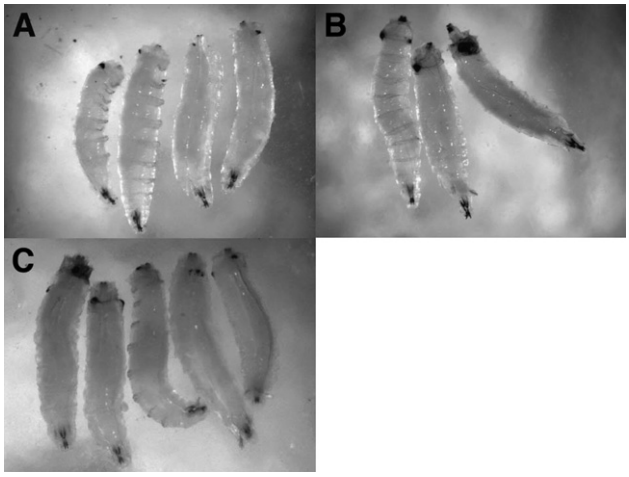

Fig. 4. *spn28D* regulates PO activity at wound site. Effects of a clean injury on *wt* (A), $spn28D^{\Delta 1}$/+ mutant (B) and $spn28D^{\Delta 1}$/+ larvae (C). Larvae were pricked at the posterior end (top of picture) with a clean needle and analyzed 2 h afterwards. Melanization at the wound site was more intense in *spn28D* homozygous larvae.

addition to dispersed melanotic spots and extensive blackening of large cuticle areas, specific body parts were frequently found melanized in 50–70% of all *act>spn28D-IR* flies. Such blackening was never located in the body cavity, but rather in proximity to the cuticle and especially in areas potentially in contact with air such as thoracic and abdominal spiracles, with melanization often extending from the spiracles to the surrounding cuticle (Fig. 5B); melanization was also observed on the foramen of the halteres (Figs. 5A and B) and occasionally oocelles (not shown). Dissection of melanized tracheae from *act>spn28D-IR* flies revealed that only the trunk in close proximity to the spiracle had blackened (Fig. 5C). Most strikingly, the ptilinal suture and the surrounding area between the eyes was melanized to variable extent in about 80% of all *act>spn28D-IR* flies (Fig. 5D), which could be linked to the ptilinum itself, since young flies showed strong blackening of this structure (Fig. 5E). The ptilinum is a hemolymph-filled sac above the antennal base and is everted by eclosing *Drosophila* flies that emerge from the pupal case. Importantly, hemolymph is pumped into this area under high internal pressure and is in close vicinity to air through a thin and soft cuticle layer (Demerec, 1950). In *da>spn28D-IR* flies, melanization of spiracles was frequent though weaker, but blackening of the ptilinum or the oocelles was not observed.

In addition to melanotic spots, about 50% of all *act>spn28D-IR* flies displayed an insufficiently pigmented abdomen, which was most apparent in males (Fig. 5F), but also present in females. Lack of pigmentation was often associated with the presence of large melanotic spots, suggesting that this pigmentation defect is a secondary consequence of depletion of PO or of a melanization substrate, possibly due to its utilization in melanotic spot formation.

Collectively, the melanotic phenotype observed in *act>spn28D-IR* flies appears to result from strong over-activation of melanization, which occurs predominantly in air-exposed body parts and at a developmental stage when the cuticle is sclerotized. This phenotype is clearly distinct from that observed in *spn27A* mutants (De Gregorio et al., 2002), which exhibit melanotic spots in the hemolymph and different body areas such as wings and leg joints while presenting wild-type adult cuticle pigmentation. This and the observation that *spn28D-RNAi* adults had a wild-type expression level of antimicrobial peptide genes following bacterial challenge (data not shown) confirmed a role of Spn28D in the melanization cascade of larvae and adults.

Exhaustion of hemolymph PO activity in spn28D-RNAi flies

To analyze in more detail the melanotic phenotype of *spn28D*-RNAi adults, we examined whether Spn28D regulates PO by measuring PO enzymatic activity in adult hemolymph samples (Fig. 6). In wild-type flies, only a low level of PO activity was detected in the absence of any challenge. However, 4 h after septic injury, a higher level of PO activity was observed as previously reported (Ligoxygakis et al., 2002). When *act>spn28D-IR* flies were analyzed, no significant PO activity was detected in the hemolymph of either naive flies or flies collected 4 h after septic injury (Fig. 6A). In agreement with this observation, a strongly reduced melanization reaction was observed at the wound site of *act>spn28D-IR* flies compared to control flies (Fig. 7). This phenotype was similar albeit weaker than that induced by overexpression of *spn27A* and correlates with the low PO activity observed in the hemolymph of *act>spn28D-IR* flies (Fig. 7). In sharp contrast, wounds of freshly eclosed *act>spn28D-IR* adults melanized normally (data not shown).

To determine the protein levels of circulating proPO and PO in *spn28D*-RNAi adults, we performed a Western blot analysis with hemolymph extracts using an anti-*Anopheles* PPO2 serum that crossreacts with *Drosophila* proPO (Leclerc et al., 2006). Leclerc et al. previously reported that this antibody recognizes a single band of about 75 kDa in the absence of wound-induced activation and an additional band of about 72 kDa following wounding, which correlates with the calculated molecular weights for *Drosophila* proPO and PO (Leclerc et al., 2006). Fig. 6B shows a Western blot analysis using the anti-*Anopheles* PPO2 with hemolymph samples from naive *Bc*, *spn27A*, and *act>spn28D-IR* flies. As expected, in wild-type adults only the band corresponding to proPO was identified. We detected no (or occasionally only a faint) band in the hemolymph of the *Black cells* (*Bc*) mutant, which is devoid of hemolymph PO activity (Rizki et al., 1980), while a faint band corresponding only to the activated form of PO was observed in *spn27A* mutant flies. Importantly, a lower total amount of

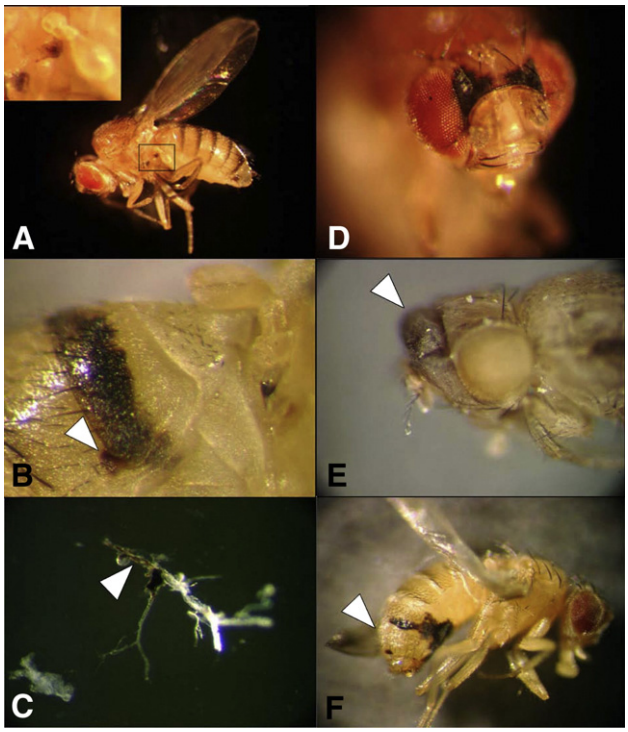

Fig. 5. Melanotic phenotype of *spn28D*-RNAi flies. Phenotypes of 4–7 day old *act>spn28D-IR* flies are shown. Spiracles were also melanized in *da>spn28D-IR* flies, while the other phenotypes were only occasionally observed in this background. (A) Melanization of the second thoracic spiracle and the foramen of the haltere (see inset for magnification of the affected area); (B) blackening of abdominal spiracle (arrowhead) and the surrounding cuticle; (C) dissected trachea with melanized tracheal trunk (arrowhead); (D) melanized area in proximity to ptilinal suture; (E) blackened ptilinal sac (arrowhead) in a fly about 1–2 h after eclosion; (F) male fly showing deficient abdominal pigmentation (arrowhead) combined with extensive melanization of the neighbouring abdominal region.

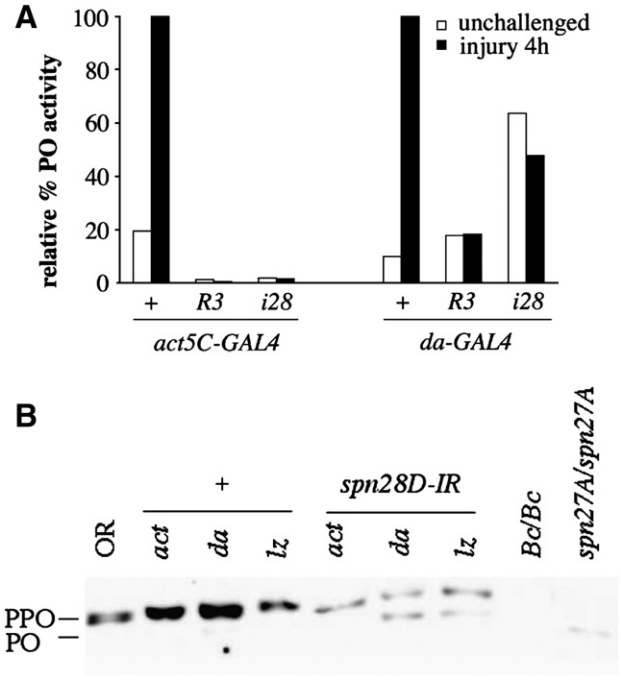

Fig. 6. Hemolymph PO activity flies is exhausted in *spn28D*-RNAi flies. (A) Pricking of wild-type flies (*act5C-GAL4/+*) leads to an increase of hemolymph PO activity. No PO activity was observed in *act>spn28D-IR* flies. Knockdown of *spn28D* using the weaker *da-GAL4* driver induced a constitutively elevated PO activity compared to control flies. Each panel represents an independent experiment. Pricked *act-GAL4/+* and *da-GAL4/+* wild-type controls were set to 100% to account for the different genetic background. The PO activity tests were repeated 2 times and yielded similar results (raw PO activities are given in Supplementary Table 1). A representative result is shown. (B) Decrease of proPO in hemolymph of *spn28D*-RNAi flies. Adult hemolymph was recovered in protease inhibitor-containing buffer to prevent PO activation after wounding and to obtain unactivated hemolymph. The concentration of the samples was adjusted using a Bradford assay prior to electrophoresis and Western blotting. The anti-PO antiserum recognized two bands corresponding to the inactive (75 kDa, proPO) and active form (72 kDa) of PO. Hemolymph samples from *spn28D*-RNAi flies using three different GAL4 lines showed an overall reduction of the proPO and PO content. *Bc/Bc* flies contained little or no hemolymph PO, while *spn27A/spn27A* mutants were devoid of the inactive form and contained only a low level of active PO. OregonR (OR) or flies carrying one copy of the GAL4 driver were used as *wild-type* controls. *act*: *act5C-GAL4*; *da*: *da-GAL4*; *lz*: *lz-GAL4;UAS-GFP*.

proPO and PO was present in the hemolymph of *spn28D*-RNAi adults compared to wild-type flies, in agreement with the strongly decreased PO activity in these flies.

In summary, inactivation of *spn28D* in *act>spn28D-IR* flies leads to a significant reduction of proPO protein levels, to the absence of detectable PO activity in the hemolymph and to incomplete wound melanization. This observation is puzzling at first sight, since *act>spn28D-IR* flies frequently displayed large and dense melanotic spots, indicating that the PO reaction was not inhibited. A possible explanation for this paradox would be that, in the absence of Spn28D, PO is immediately sequestered for the formation of melanotic spots in young flies, resulting in exhaustion of a component of the PO cascade (either the substrates or PO itself) and thus reduced hemolymph PO activity available for injury-induced melanization. This hypothesis is further supported by the observation that very young but not yet pigmented *act>spn28D-IR* flies did not exhibit melanotic spots, and melanized normally at the injury site. To test the above hypothesis, we monitored the level of hemolymph PO activity in flies carrying the *spn28D-IR* construct under the control of the *da-GAL4* driver that produces a weaker *spn28D* knockdown than *act5C-GAL4*. *da>spn28D-IR* flies exhibited less and smaller areas of melanotic spots (mainly at the thoracic spiracles; see Fig. 5A), and no pigmentation defects. Interestingly, unchallenged *da>spn28D-IR* flies displayed a constitutively elevated hemolymph PO activity compared to hemolymph of unchallenged wild-type flies (Fig. 6A). In addition, hemolymph samples derived from unchallenged *da>spn28D-IR* flies contained the activated form of PO at a similar level as seen in *spn27A* deficient mutants (Fig. 6B). This suggests that depletion of *spn28D* increases hemolymph PO activity similar to the *spn27A* mutation.

Altogether, these data suggest that Spn28D functions as an inhibitor of the proPO cascade. However, unlike Spn27A, strong depletion of Spn28D (as observed with the *act5C-GAL4* driver) leads to complete exhaustion of available hemolymph PO.

Relationship of Spn28D with other components of the proPO cascade

Our results indicate that Spn28D controls the hemolymph melanization reaction and suggest a specific role for this serpin in limiting the availability of PO originating from larval crystal cells. To better understand how Spn28D regulates proPO activation, we analyzed the interaction of Spn28D with other previously identified components of the proPO cascade. *Bc* mutants are devoid of circulating PO activity originating from crystal cells, but show neither lethality nor a tracheal melanization defect (Rizki et al., 1980). We found that Bc^1 did not suppress lethality caused by $spn28D^{\Delta 1}$, which indicates that this lethality was not due to PO activity from crystal cells. However, *Bc*; $spn28D^{\Delta 1}$ double mutant larvae did not melanize at the wound site, similar to *Bc* mutants. Since *Bc* also fully suppresses the melanization phenotype induced by a *spn27A* mutation at the wound site (De Gregorio et al., 2002), we concluded that both Spn27A and Spn28D control hemolymph PO activity but that Spn28D has an additional essential role, which is presumably independent of crystal cells.

To analyze the functional relationship between *spn27A* and *spn28D*, we generated *spn27A* deficient flies in combination with *act>spn28D-IR*. This led to a severely aggravated phenotype compared to the *spn27A* mutant or the *spn28D*-RNAi flies, both in terms of

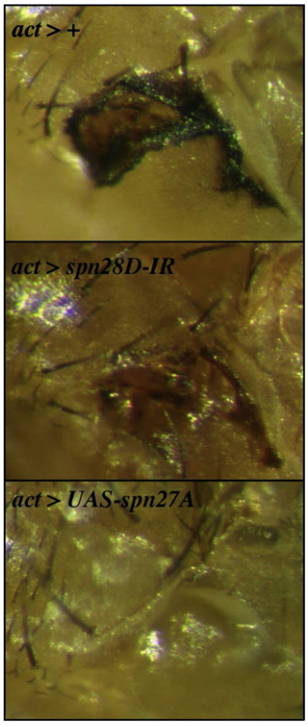

Fig. 7. Wounds of *spn28D*-RNAi flies are not efficiently melanized. Flies were wounded with a tungsten needle and blackening of the wound was recorded 16 h later. In response to wounding, *act>spn28D-IR* flies showed a reduced melanization efficiency compared to *act>+* control flies. Reduced melanization was also observed in flies overexpressing *spn27A*. *act: act5C-GAL4*. The wounding experiment was repeated three times with 20 flies for each genotype. A representative picture is shown for each genotype.

melanotic spots and lethality, with only few escaper flies at 23 °C (data not shown). Interestingly, overexpression of *spn27A* could only partially rescue the lethality of *act>spn28D-IR* flies, while the melanotic phenotype of these flies remained unaffected or was maybe slightly reduced. Thus, Spn27A cannot prevent the ectopic activation of the proPO system in *spn28D*-RNAi flies. This result indicates that Spn28D regulates PO activity differently than Spn27A.

MP2/PAE1 is a serine protease thought to be expressed in crystal cells and required for the activation of hemolymph PO following microbial challenge (Castillejo-Lopéz and Hacker, 2005; Leclerc et al., 2006; Tang et al., 2006). We generated *spn28D*$^{\Delta 1}$; *MP2* double mutants and observed that the *MP2* mutation did not suppress the melanotic phenotype and lethality caused by *spn28D* (data not shown).

Together, the results of the epistatic analysis demonstrate the existence of two distinct functions of Spn28D. The first function is essential for pupal viability and disruption of this function is associated with ectopic melanization of trachea. This function is not affected by *MP2, spn27A* and *Bc* mutations. The second function is to control hemolymph PO in concert with Spn27A. This function appears to involve crystal cells since it is suppressed by the *Bc* mutation.

Discussion

The insect melanization reaction is a well-described phenomenon, initially analyzed for its developmental function in the sclerotization process (Graubard, 1933) and more recently investigated for its involvement in immunity. The activation steps of the PO system have been described in other arthropods (Kanost and Gorman, 2008; Sugumaran and Kanost, 1993). However, the molecular identity of the proteins in the *Drosophila* proteolytic cascade leading to proPO activation have only recently been successfully investigated (Castillejo-Lopéz and Hacker, 2005; Leclerc et al., 2006; Tang et al., 2006). Importantly, of the 29 serpin genes in the *Drosophila* genome, to date only one, *spn27A*, has been linked to regulation of the proPO cascade (De Gregorio et al., 2002; Ligoxygakis et al., 2002).

In this study we present evidence for the involvement of a novel serpin, Spn28D, in the melanization reaction. A role for this serpin was initially suggested by the observation that it is strongly induced upon challenge by injury rather than by infection and exhibits an acute phase expression profile. We demonstrated here that disruption of Spn28D function causes a striking melanotic phenotype with melanization of tissues exposed to air in both larvae and adults and variable pigmentation in adults. Our results also indicate that Spn28D negatively regulates hemolymph PO activity in both larvae and adults. This is supported by experiments showing that Spn28D regulates PO activity at the wound site of larvae and by indirect evidence that *spn28D* is expressed in crystal cells that represent a major source of hemolymph PO. Our study reveals that complete loss of Spn28D function is lethal to flies. This is consistent with previous studies showing that tight regulation of PO activity is crucial for survival, since high PO activity is toxic for the animal, as revealed by the lethality of *spn27A* mutant flies (De Gregorio et al., 2002; Ligoxygakis et al., 2002). RNAi of *spn28D* lowered hemolymph PO activity in adults in contrast to larvae, as demonstrated by *in vitro* PO activity tests and direct PO detection using a specific antiserum. The decreased level of PO activity seemed counter-intuitive at first sight for a serpin knockdown, but the use of weaker RNAi conditions (*da>spn28D-IR*) suggested that extensive over-activation of the PO system at the time of fly eclosion may lead to PO exhaustion.

Our epistatic analysis reveals that a loss-of-function mutation affecting the clip-domain serine protease MP2 or the *Bc* allele did not rescue the lethality and tracheal melanization phenotypes induced by a mutation in *spn28D*, while *Bc* suppressed the wound site melanization seen in *spn28D*-RNAi flies. Altogether, our study suggests distinct functions of Spn28D in two tissue compartments, tracheae and hemolymph. The first function would be to limit ectopic melanization of tissues exposed to air such as the tracheae. Our results obtained with the *Bc* mutation indicate that tracheal melanization is not mediated by hemolymph PO, pointing towards the existence of another source of PO activity in this tissue. Recently, we identified another serpin, Spn77Ba, that controls tracheal melanization (Tang et al. submitted). Specific expression of a *spn77Ba-IR* construct in the tracheae using the *breathless (btl)-Gal4* driver leads to larval lethality and melanization of tracheae. Thus, two serpins, Spn77Ba and Spn28D, control melanization in the respiratory tract. Since oxygen is required by PO as a substrate, it might be critical to control PO activity in tissues that are in direct contact with air. Nevertheless, the function of Spn28D in the trachea may be indirect, as specific knockdown of *spn28D* in this tissue with *btl*-GAL4 did not induce either lethality or melanization (not shown).

The second function of Spn28D would be to regulate hemolymph melanization. Our analysis reveals distinct functions and/or spatial and temporal requirements for *spn27A* and *spn28D* in the control of hemolymph PO activity. On the basis of our experiments, it is tempting to propose a model in which Spn28D prevents spontaneous activation of PO in the hemolymph after its release from crystal cells. After the disappearance of crystals cells during metamorphosis, this serpin would function directly in the hemolymph of adults to inhibit PO activity. Although Spn27A also regulates PO in the hemolymph, it would function at a later step to spatially limit PO activation to wound sites. This would explain the over-activation (and subsequent exhaustion) of PO in *spn28D*-RNAi flies despite the presence of Spn27A. Interestingly, an RGD cell attachment motif is present in the Spn28D sequence close to the C-terminal reactive centre loop, which is responsible for the entrapment of the target serine protease (van Gent et al., 2003). The RGD sequence constitutes a key recognition sequence for cell adhesion mediated through integrins. It has also been found in the extended C-terminus of alpha(2)-antiplasmin, a human serpin related to Spn28D. The RGD motif contributes to binding of alpha(2)-antiplasmin to endothelial cells, a step required in the control of localized cellular fibrinolysis (Thomas et al., 2007). Spn28D is the only one among all *Drosophila* serpins having the RGD motif. The presence of this motif might thus indicate that Spn28D, in contrast to other *Drosophila* serpins, can directly interact with cells via this integrin binding site. We speculate that circulating Spn28D may be relocated to cell membranes and bind its serine protease substrate, thereby limiting PO activation and enzymatic activity. Future studies should clarify the relationship between Spn28D and proteases involved in PO activation in the hemolymph compartment.

The *spn28D*-RNAi phenotype highlights an intriguing crosstalk between the proPO system and pigmentation of the adult cuticle. The pigmentation phenotype of *spn28D*-RNAi flies shows striking similarities with flies mutated for copper-transporting proteins, which have defects in cuticular tyrosinase or laccase PO that require the cofactor copper for activity (Zhou et al., 2003) and which exhibit insufficient cuticular sclerotization in the absence of PO-induced quinone tanning (Sugumaran, 2002). Pigmentation defects in *act>spn28D-IR* flies are correlated with the presence of melanotic masses, suggesting that this phenotype may be due to PO substrate depletion as a consequence of a strong PO activation in young *act>spn28D-IR* flies. This reveals a crucial role of Spn28D in the control of the proPO cascade at the emergence of flies, when sclerotization of the cuticle occurs. It has been reported that components required for sclerotization are mainly produced in larvae and stored during the pupal stage for melanin production after eclosion (Mitchell, 1966). This suggests that the effect observed in *act>spn28D-IR* flies may be due to a defect in proPO control at the larval stages, which later affects the physiological balance between proPO and factors regulating its activity in emerging flies.

In summary, we present here functional data for a novel *Drosophila* serpin that regulates the proPO cascade in tissues exposed to air and in

hemolymph in a manner different from the already characterized Spn27A. This study further highlights the complexity of the proPO cascade that can be differentially regulated in different tissue compartments during development. Future studies will be needed to identify the target serine protease of Spn28D and its specific interactions with different proPOs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.08.030.

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