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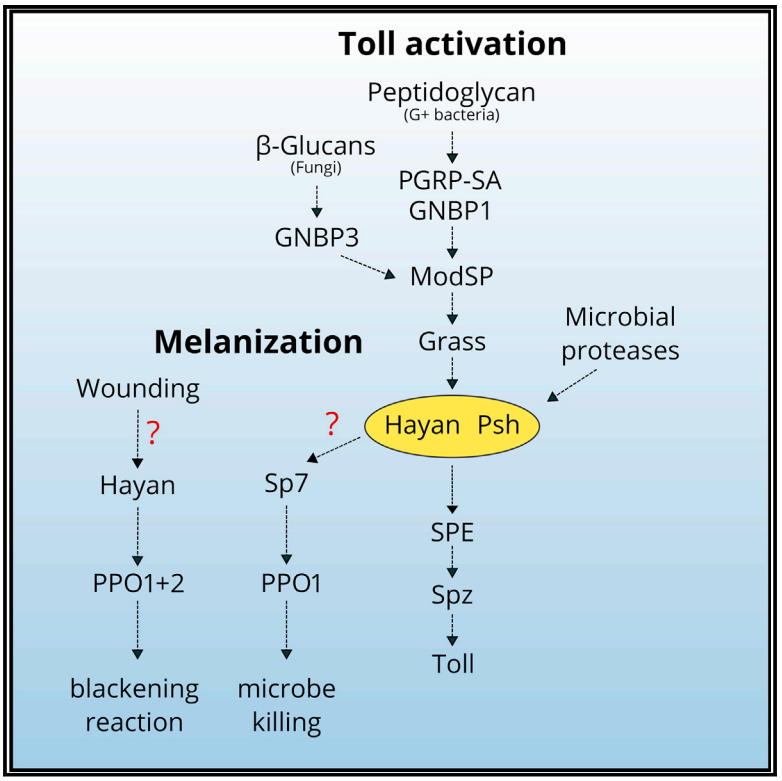
More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in *Drosophila*

Graphical Abstract



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In Brief

Dudzic et al. investigate the regulation of the melanization response in *D. melanogaster* with compound mutants of serine proteases. They find two distinct melanization pathways and a connection between melanization and the Toll pathway. Additionally, two related serine proteases, Hayan and Persephone, regulate the Toll pathway downstream of pattern-recognition receptors.

Highlights

- Two distinct pathways activate the melanization reaction in *Drosophila*
- Serine proteases regulating the Toll pathway also regulate melanization
- Hayan and Psh redundantly regulate the Toll pathway downstream of pattern recognition

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More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in *Drosophila*

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SUMMARY

The melanization response is an important defense mechanism in arthropods. This reaction is mediated by phenoloxidases (POs), which are activated by complex extracellular serine protease (SP) cascades. Here, we investigate the role of SPs in the melanization response using compound mutants in *D. melanogaster* and discover phenotypes previously concealed in single-mutant analyses. We find that two SPs, Hayan and Sp7, activate the melanization response in different manners: Hayan is required for blackening wound sites, whereas Sp7 regulates an alternate melanization reaction responsible for the clearance of *Staphylococcus aureus*. We present evidence that Sp7 is regulated by SPs activating the Toll NF-κB pathway, namely ModSP and Grass. Additionally, we reveal a role for the combined action of Hayan and Psh in propagating Toll signaling downstream of pattern recognition receptors activating either Toll signaling or the melanization response.

INTRODUCTION

The sequential activation of extracellular serine protease (SP) cascades regulates important aspects of insect innate immune reactions, notably the activation of the Toll pathway and the melanization response. These proteolytic cascades have a functional core, consisting of several SPs that undergo zymogen activation upon cleavage by an upstream protease. This sequential cleavage shapes the immune response by providing a link between recognition and the induction of effectors (Lemaitre and Hoffmann, 2007). In *Drosophila* immunity, the Toll pathway mediates resistance to Gram-positive bacteria and fungi by regulating a subset of effectors in the fat body. Unlike mammalian Toll-like receptors, which function as pattern recognition receptors (PRRs), the *Drosophila* Toll receptor does not interact directly with microbial products and is instead activated by a cleaved form of the cytokine Spätzle (Spz) (Lemaitre et al., 1996; Weber et al., 2003). The SPs that regulate the cleavage of Spz during the immune response are different from those regulating Spz in the dorsoventral patterning of the early embryo (Valanne et al., 2011). The SP Spz processing enzyme (SPE), which contains a CLIP-domain, has been identified as the terminal SP that cleaves Spz (Jang et al., 2006). Genetic analysis supports the existence of two complex cascades that link microbial recognition to activation of SPE: the PRR and Persephone (Psh) pathways. In the PRR pathway, PRRs involved in the sensing of Gram-positive bacteria (GNBP1 and PGRP-SA) or fungi (GNBP3) bind to their respective microbial ligands to activate an upstream SP, ModSP, leading to the activation of the SP Grass, followed by the maturation of SPE (Buchon et al., 2009; El Chamy et al., 2008; Gobert et al., 2003; Gottar et al., 2006; Pili-Floury et al., 2004). In the Psh pathway, infectious agents are detected directly via cleavage of the Psh protease bait region by microbial proteases, leading to Spz cleavage by SPE (El Chamy et al., 2008; Issa et al., 2018).

Melanization is one of the most spectacular immune reactions in insects. It is an arthropod-specific immune response resulting in the rapid deposition of the black pigment melanin at wound or infection sites (Cerenius et al., 2008; González-Santoyo and Córdoba-Aguilar, 2012; Tang, 2009). This process relies on enzymes called prophenoloxidases (PPOs), which catalyze the oxidation of phenols, resulting in the polymerization of melanin. The mechanism by which melanization contributes to the killing of bacteria, fungi, or parasitoid wasp larvae remains elusive, although reports indicate a role for reactive oxygen species (ROS) and other metabolic intermediates of the melanin synthesis pathway (reviewed in Nappi et al., 2009). In *D. melanogaster*, three PPOs have been identified: PPO1 and PPO2 are produced by crystal cells and contribute to hemolymph melanization, whereas the role of the lamellocyte-derived PPO3 is confined to encapsulation (Binggeli et al., 2014; Dudzic et al., 2015; Nam et al., 2008). To date, three SPs have been implicated in activating PPOs in the hemolymph: MP1, Sp7, and Hayan (Castillejo-López and Häcker, 2005; Nam et al., 2012; Tang et al., 2006). Whereas a null mutation in *Hayan* abolishes melanization in adults, a *Sp7* null mutation results in only a slight reduction (Dudzic et al., 2015). The positions of these SPs in the melanization cascade have not been fully established, although Hayan and Sp7 can cleave PPO1 *in vivo* and *in vitro* (An et al., 2013; Nam et al., 2012). It is still unclear whether PPO1 and PPO2 are differentially activated by distinct SP cascades.

In many insects, the Toll and melanization pathways are activated by the same SPs, diverting only at the terminal steps (Kan et al., 2008; Park et al., 2006; Volz et al., 2006). In contrast, SPs regulating the Toll pathway upon infection in *Drosophila* are not mandatory for the melanization cascade

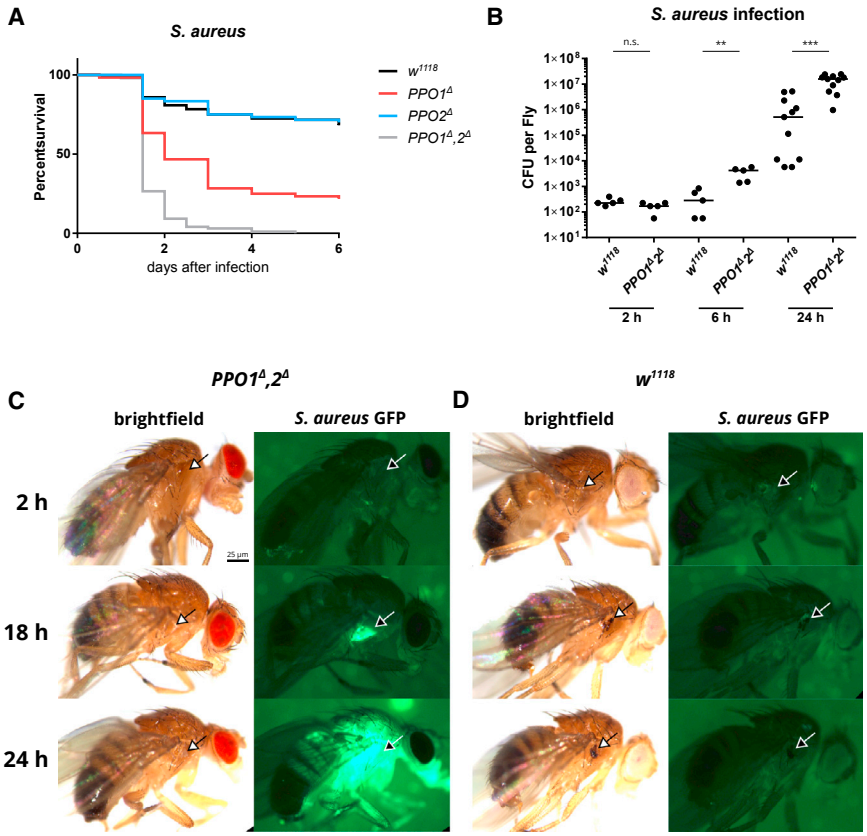

Figure 1. PPOs Contribute to the Resistance of *Staphylococcus aureus*

(A) Survival rates of flies following septic injury with *S. aureus*. Flies lacking *PPO1* ($p < 0.0001$), but not *PPO2* (not significant [n.s.]), alone are less resistant than wild-type flies (w^{1118}). Flies lacking two PPO genes ($PPO1^{\Delta},2^{\Delta}$) ($p < 0.0001$) show less resistance than flies lacking only *PPO1*.

(B) Persistence of *S. aureus* in w^{1118} or $PPO1^{\Delta},2^{\Delta}$ flies at 2, 6, or 24 h post-infection. Increased *S. aureus* counts are found in $PPO1^{\Delta},2^{\Delta}$ flies after 6 and 24 h. The number of colony-forming units (CFU) per fly is shown on a logarithmic scale.

(C and D) Growth of GFP expressing *S. aureus* in $PPO1^{\Delta},2^{\Delta}$ (C) or w^{1118} (D) flies after 2, 18, and 24 h. *Wild-type* flies melanize the wound area (arrows), whereas $PPO1^{\Delta},2^{\Delta}$ do not. No GFP signal is observed in w^{1118} flies, whereas $PPO1^{\Delta},2^{\Delta}$ exhibit a local GFP signal after 18 h and systemic GFP signal after 24 h. A minimum of three independent experiments with 20 flies per genotype was conducted for all survival experiments.

*$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$.

RESULTS

Melanization Is Important to Survive *S. aureus* Infection

While reviewing survival data from Binggeli et al. (2014), we confirmed that survival of adult flies against *S. aureus* is strongly dependent on a functional melanization response. Flies lacking *PPO1* rapidly succumb to infection with a low dose of *S. aureus* (optical density 0.5 [$OD_{0.5}$], 1–100 colony forming units [CFU]/fly), whereas the lack of *PPO2* alone is not critical. A synergistic effect can be observed in flies lacking both *PPO* genes simultaneously; in this case, no fly survives the infection (Figure 1A). Analysis of the bacterial load of $PPO1^{\Delta},2^{\Delta}$ flies reveals that melanization-deficient flies already have a higher bacterial burden 6 h after infection compared with their wild-type (w^{1118}) counterparts (Figure 1B). Their inability to control *S. aureus* growth is even more prominent 24 h after infection. Following injection of *GFP*-expressing *S. aureus,* $PPO1^{\Delta},2^{\Delta}$ flies did not melanize the wound (Figure 1C, arrows) and exhibit a local GFP signal after 18 h, indicating *S. aureus* growth at the wound area (Figure 1C). After 24 h, most $PPO1^{\Delta},2^{\Delta}$ flies exhibit a strong, systemic GFP signal indicating that they were unable to suppress *S. aureus* growth and spread (Figure 1C). This contrasts with wild-type flies, which deposit melanin at the wound area and control bacterial growth over 24 h (Figure 1D). The importance of the melanization response to resist *S. aureus* is further illustrated by the observation that $PPO1^{\Delta},2^{\Delta}$ flies show no defect in Toll or immune deficiency (IMD) pathway activation after *S. aureus* infection (Figures S1A and S1B). Compared with wild-type flies, they even show an approximately seven times higher expression of the Toll-activity readout *Drosomycin* (*Drs*), as well as approximately eight times higher expression of the IMD-activity readout *Diptericin* (*Dpt*) (Figures S1A and S1B), likely because of unconstrained bacterial growth. To test the and vice versa. This led to the assumption that both immune processes are regulated by distinct sets of SPs (Kanost and Jiang, 2015; Veillard et al., 2016). Nevertheless, the melanization and Toll pathways interact, as Toll signaling regulates the expression of many genes encoding SPs and serpins involved in melanization (De Gregorio et al., 2002a, 2002b; Ligoxygakis et al., 2002). Moreover, there is evidence that PRRs upstream of Toll, such as PGRP-SA and GNBP1, can impact Toll-independent responses, notably melanization (Matskevich et al., 2010).

In this article, we introduce a model of systemic infection using a low dose of *Staphylococcus aureus* where the survival of flies relies on the melanization response, but not on Toll signaling nor phagocytosis. Using this sensitive assay, we show that resistance to *S. aureus* correlates with the melanization response, but surprisingly not the deposition of melanin itself. We also reveal specific roles for the SPs Sp7 and Hayan in the melanization pathway: Hayan is specifically tied to the blackening of the cuticle, whereas Sp7-dependent melanization is connected to the upstream part of the Toll PRR signaling. Meanwhile, a small deficiency removing both *Hayan* and *psh* reveals an unexpected role for these two SPs in the canonical Toll pathway. We provide evidence that *Hayan* and *psh* arose from a recent gene duplication, and that these genes currently have both overlapping and distinct functions in the melanization and Toll pathways. Globally, we describe the existence of two distinct pathways leading to melanization and reveal a role for both Hayan and Psh in Toll signaling and the melanization response.

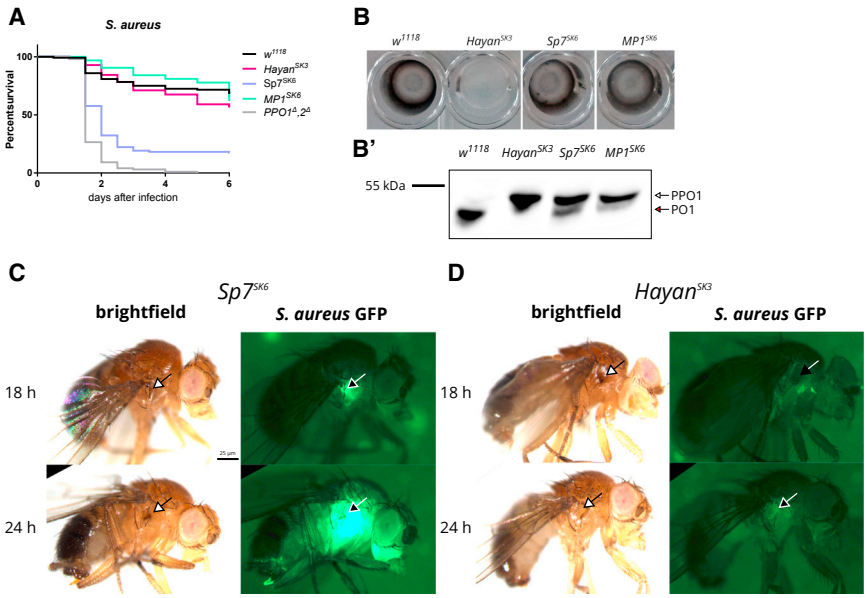

Figure 2. *Sp7*, but Not *Hayan* or *MP1*, Is Critical to Resist *S. aureus* Infections

(A) Survival rates of flies following septic injury with *S. aureus*. Flies lacking *Sp7* ($p < 0.0001$), but not *Hayan* or *MP1*, are less resistant than wild-type flies (w^{1118}). $PPO1^{\Delta},2^{\Delta}$ flies were used as a positive control.

(B) Capability of L3 larval hemolymph to produce black melanin after incubation at room temperature. Hemolymph from w^{1118}, $MP1^{SK6}$, and $Sp7^{SK6}$ flies shows melanization, whereas $Hayan^{SK3}$ hemolymph fails to melanize.

(B′) Cleavage of PPO1 in hemolymph of larva illustrated via western blot. Whereas cleavage is observed in hemolymph of w^{1118}, $MP1^{SK6}$, and $Sp7^{SK6}$ larvae, $Hayan^{SK3}$ fails to cleave PPO1.

(C and D) Growth of GFP-producing *S. aureus* in $Sp7^{SK6}$ (C) or $Hayan^{SK3}$ (D) flies after 18 and 24 h. $Sp7^{SK6}$ flies show a local GFP signal at the wound site (arrows), whereas $Hayan^{SK3}$ flies show a strong reduction. In *Sp7* mutants, this develops into a systemic GFP signal after 24 h.

role of hemocytes in the resistance to *S. aureus* infection, we produced hemoless flies using a plasmatocyte-specific Gal4 (hml^{Δ}-*Gal4*) to express the pro-apoptotic gene *bax* (Defaye et al., 2009), and also examined *eater*-deficient flies who exhibit a defect in *S. aureus* phagocytosis (Bretscher et al., 2015). In both cases, hemoless and *eater*-deficient flies survived similar to the wild type (Figure S1C). Our results contrast with previous reports that show hemocytes are critical to survive *S. aureus* infections (Defaye et al., 2009; Garg and Wu, 2014; Nehme et al., 2011). We attribute this discrepancy to using a low dose of *S. aureus* that is controlled specifically by the melanization response, but not phagocytes, because flies lacking hemocytes succumb to *S. aureus* when a higher dose is used (Figures S1F and S1G). Cytotoxic by-products of the melanization reaction include ROS (Nappi et al., 2009). We investigated the role of ROS during *S. aureus* infections by measuring H_2O_2 levels in whole fly lysates using a fluorimetric approach. We did not see a change in H_2O_2 levels over the 6 h following *S. aureus* infection in wild-type flies. Interestingly, $PPO1^{\Delta},2^{\Delta}$ flies show a mild but consistent reduction in H_2O_2 levels compared with wild-type flies (Figure S1D). Taken together, these results demonstrate that the melanization response is critical to resist a low dose of *S. aureus* infection consistent with Binggeli et al. (2014). Furthermore, our *S. aureus* infection model provides a sensitive assay to characterize the role of melanization in resistance to infection.

Sp7, but Not Hayan nor MP1, Is Required to Resist *S. aureus* Infection

Using this low-dose *S. aureus* infection model, we analyzed the role of three SPs (MP1, Sp7, and Hayan) previously described as involved in melanization. For this, we used a newly generated CRISPR null mutant for *MP1* ($MP1^{SK6}$) and null mutants for *Sp7* and *Hayan* ($Sp7^{SK6}$, $Hayan^{SK3}$) (Dudzic et al., 2015). Contradicting a previous report that used an *in vivo* RNAi approach (Tang et al., 2006), we did not observe any overt defect in Toll and PPO activation in *MP1*-deficient flies, whose melanization and fitness phenotype are further described in Figure S2. We therefore focused our attention on the respective roles of Hayan and Sp7. Surprisingly, only *Sp7* mutants phenocopy the susceptibility of $PPO1^{\Delta},2^{\Delta}$ flies against *S. aureus*, whereas *Hayan*- and *MP1*-deficient flies behave as wild type (Figure 2A). By injecting GFP-producing *S. aureus* in flies lacking these SPs, we observed that $Sp7^{SK6}$ flies fail to restrict *S. aureus* growth first locally after 18 h, then systemically after 24 h (Figure 2C, arrows). In contrast, $Hayan^{SK3}$ flies show no increase in GFP signal over time, indicating that *S. aureus* proliferation is controlled as in the wild type (Figure 2D, arrows).

These results were surprising, because *Hayan*, but not *Sp7*, mutants have reduced melanization at the wound site after clean injury (CI) (Figure S2A) and *S. aureus* (Figures 2C and 2D) (Dudzic et al., 2015; Nam et al., 2012). This revealed that the resistance to infection conferred by melanization SPs does not correlate with the blackening reaction at the wound site. We therefore further investigated the respective role of Sp7 and Hayan in the melanization of adult and larval hemolymph. Extracting hemolymph with subsequent incubation at room temperature leads to blackening because of phenoloxidase (PO)-dependent melanin production. Surprisingly, hemolymph from both $Hayan^{SK3}$ mutant adults and larvae fails to turn black, whereas hemolymph of $Sp7^{SK6}$ mutants blackens over time (Figure 2B for larval hemolymph; data not shown for adults). Consistent with this, we observed a cleaved form of PPO1 in hemolymph samples derived from wild-type and *Sp7* larvae, but not *Hayan*-deficient larvae (Figure 2B′).

Collectively, our results suggest that Sp7, but not Hayan, controls *S. aureus*, and that the underlying resistance mechanism does not involve melanin production, a terminal step in the melanization cascade. Thus, our results disconnect the blackening of the hemolymph from a melanization reaction-dependent clearance of *S. aureus*. This suggests that by-products of the melanization reaction, such as ROS or other metabolic intermediates, might be the active molecules controlling bacterial growth. This

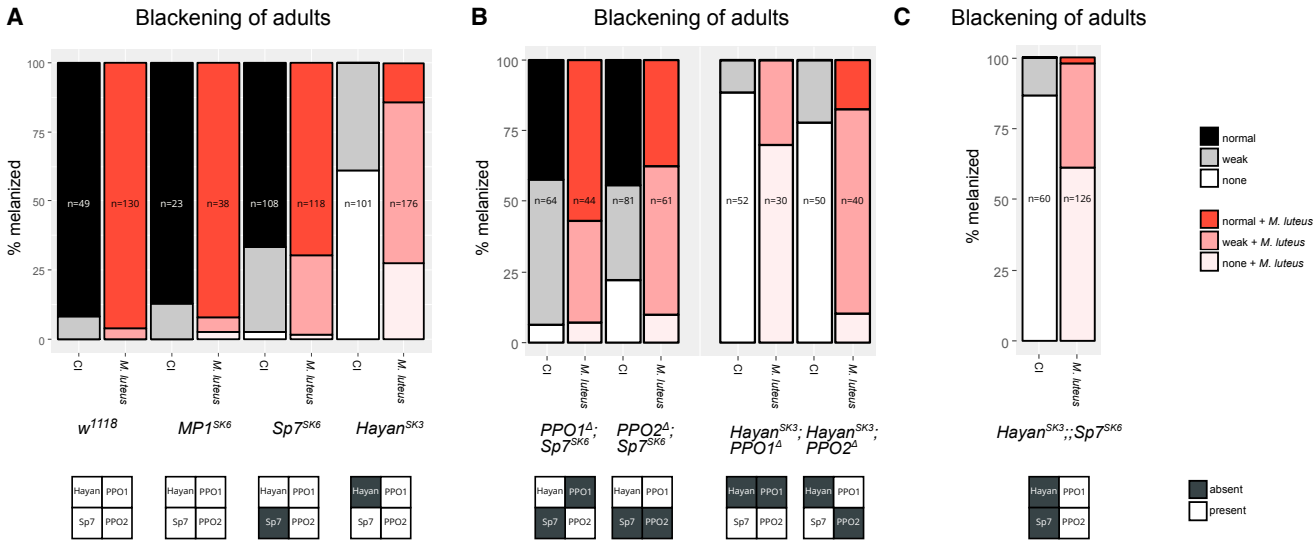

Figure 3. Sp7 and Hayan Regulate PPOs Differently

Flies were either injured with a clean needle (black bars) or with a needle previously dipped in *M. luteus* solution (red bars). Blackening was assessed in three categories: normal, weak, or none (see Figure S3). CI: clean injury. *M. luteus*: septic injury with *M. luteus*.

(A) $MP1^{SK6}$ flies do not exhibit any defect in blackening compared with w^{1118} flies ($p = 0.8219$). $Sp7^{SK6}$ flies show a reduction in normal blackening, although almost all flies blacken at least weakly ($p = 0.0083$). $Hayan^{SK3}$ flies show a major defect in blackening after clean injury ($p < 0.0001$), which can partially be rescued by wounding with *M. luteus* ($p < 0.0001$).

(B) *Sp7* mutants still blacken the wound area to a certain extent with a simultaneous mutation in either *PPO1* or *PPO2*. In contrast, the partial blackening of $Hayan^{SK3}$ flies after infection with *M. luteus* relies on the presence of *PPO1*.

(C) $Hayan^{SK3};;Sp7^{SK6}$ mutants lose most, but not all, blackening. Percentages of total flies (n) are displayed. Sample size (n) is indicated in each respective bar.

also reveals the complexity of PPO cascades in *Drosophila*, pointing to the existence of multiple branches involving either Sp7 or Hayan. To disentangle the deposition of melanin from the melanization reaction as a whole, we will use "blackening reaction" to refer to melanin deposition and "melanization response" to refer to PPO-derived activities as a whole, which includes the blackening reaction.

Sp7 and Hayan Regulate PPOs Differently

Melanization is a reaction that can be triggered by clean injury (CI) or by the presence of microbial products. To further understand how Hayan and Sp7 contribute to melanization, we compared the blackening reaction at the wound site upon clean injury and septic injury using the avirulent Gram-positive bacterium *Micrococcus luteus*. This lysine-type peptidoglycan-containing bacterium has been shown to activate Toll signaling by the PRRs PGRP-SA and GNBP1 (Leulier et al., 2003). We categorized the blackening of the cuticle into three levels: strong, weak, and none; an example for each category is given in Figure S3. After clean injury, 91.8% of wild-type w^{1118} flies blacken strongly and 8.2% blacken weakly. This ratio shifts slightly for *Sp7* mutants (66.6% strong, 30.6% weak, 2.8% none; $p = 0.008$; Figure 3A, gray columns), whereas *Hayan* mutants were almost deficient for blackening (0% strong, 38.9% weak, 61.1% none; $p < 0.0001$). Interestingly, some cuticle blackening was recovered in *Hayan* mutants upon septic injury with *M. luteus* (16.2% strong, 64.6% weak, 19.2% none; $p < 0.0001$; Figure 3A, red columns). Thus, Hayan is not solely responsible for the blackening reaction, because the presence of bacteria can induce cuticle blackening in a Hayan-independent manner.

In *Drosophila,* two PPOs, PPO1 and PPO2, are primarily responsible for hemolymph PO activity, including blackening (Binggeli et al., 2014). However, it is unclear whether these PPOs are activated by the same SP or are differentially regulated. In addition, we do not know whether Hayan and Sp7 are involved in the cleavage of one specific PPO or both PPOs. To address this question, we generated double mutants for *Hayan* or *Sp7* in combination with *PPO1* or *PPO2*, and subsequently analyzed their blackening capabilities upon clean injury and *M. luteus* infection. It was expected that if one SP and one PPO function in the same pathway, single mutant and double mutants should have the same phenotype. Previous reports have shown that mutations in *PPO1*, and to a lesser extent *PPO2*, reduce melanization upon both clean and septic injury (Binggeli et al., 2014). Here, we observed that $PPO1^{\Delta},Sp7^{SK6}$ and $PPO2^{\Delta},Sp7^{SK6}$ retain a blackening reaction (Figures 3A and 3B), which was only slightly reduced compared with $PPO1^{\Delta}$ or $PPO2^{\Delta}$ alone (data not shown). This indicates that Sp7 is not essential for the blackening reaction, and that Hayan (or another SP) can activate either PPO1 or PPO2 in the absence of Sp7. In contrast, $Hayan^{SK3};PPO1^{\Delta}$ double mutants fail to blacken upon septic injury with *M. luteus*, with a level of melanization being lower than $Hayan^{SK3}$ alone. Interestingly, $Hayan^{SK3};PPO2^{\Delta}$ flies retain the same level of blackening upon *M. luteus* infection than $Hayan^{SK3}$ single mutants (Figure 3B). This indicates that another SP (possibly Sp7) can promote melanization via PPO1 after exposure to Gram-positive bacteria.

Finally, we observed that double-mutant flies for both *Hayan* and *Sp7* have further reduced (but still detectable) levels of the blackening reaction (Figure 3C). This suggests the existence of a third, although minor, branch for activating PPO that leads to blackening of the wound site.

Together, these results demonstrate that the blackening reaction after clean injury relies mostly on the presence of Hayan, acting through both PPO1 and PPO2. Upon septic injury with the Gram-positive bacterium *M. luteus*, cuticle blackening can be observed even in the absence of Hayan, and this residual melanization requires both PPO1 and Sp7. Thus, our study uncovers an Sp7- and PPO1-dependent pathway that is activated in the presence of bacteria that can lead to the blackening reaction in the absence of Hayan.

Extracellular Components of the Toll Pathway Are Required to Survive *S. aureus* Infections

The Toll pathway is the most responsive pathway to infections by Gram-positive bacteria in *D. melanogaster*, notably *M. luteus* (Leulier et al., 2000; Rutschmann et al., 2000). This raises the possibility that the Sp7- and PPO1-dependent melanization pathway, which is induced upon *M. luteus* infection, is activated by upstream components of the Toll PRR pathway. Consistent with this, a previous study has already suggested a link between the melanization and Toll PRR pathways (Matskevich et al., 2010). However, how the Toll pathway can impact melanization is not well understood. This prompted us to investigate the role of the Toll PRR pathway in melanization. First, we monitored the survival of flies lacking upstream or downstream components of the Toll pathway using our melanization-sensitive *S. aureus* survival assay. We observed that flies lacking GNBP1 or PGRP-SA, two PRRs implicated in the recognition of Gram-positive bacteria, are as susceptible to *S. aureus* infections as $PPO1^{\Delta},2^{\Delta}$ and *Sp7* mutant flies (Figure 4A). In contrast, flies lacking GNBP3, a PRR for fungal β-glucans, resist as the wild type. Importantly, *spätzle* (spz^{rm7}) flies lacking intracellular Toll signaling exhibit wild-type resistance to *S. aureus* infection (Figure 4B). This indicates that the susceptibility of flies lacking PGRP-SA and GNBP1 is not linked to the intracellular Toll pathway and its transcriptional output. Our results rather suggest a direct input of PRRs upstream of Toll in the melanization response. We therefore analyzed the role of the three SPs functioning upstream of Spz in the Toll PRR pathway, ModSP, Grass, and SPE, for their role in resistance to *S. aureus*. Among them, only $ModSP^{1}$ and $Grass^{Herrade}$ mutant flies die at similar rates to $PPO1^{\Delta},2^{\Delta}$, $GNPB1^{Osiris}$ and $PGRP\text{-}SA^{seml}$ flies (Figure 4C). We conclude that a subset of the extracellular components functioning upstream of the Toll ligand, namely, PGRP-SA, GNBP1, ModSP, and Grass, regulate a mechanism of resistance to *S. aureus* that is independent of intracellular Toll signaling.

ModSP/Grass and Hayan Contribute Independently to Different Types of Melanization

A hypothesis to reconcile our data is that the PRRs GNBP1 and PGRP-SA activate ModSP and Grass, which branch out of the PRR pathway to activate the melanization response via Sp7. Thus, ModSP and Grass could also contribute to the blackening reaction and notably contribute to Hayan-independent cuticle blackening observed upon septic injury by *M. luteus*. To further analyze the role of ModSP in the melanization pathway and its relationship with Hayan and Sp7, we generated $Hayan^{SK3};;ModSP^{1}$ and $Sp7^{SK6},ModSP^{1}$ double-mutant flies and compared the blackening reaction at the wound site upon clean and *M. luteus* injury. In agreement with previous studies (Buchon et al., 2009), $ModSP^{1}$ mutant flies exhibit a wild-type blackening reaction in both conditions. Interestingly, $Hayan^{SK3};;ModSP^{1}$ mutants failed to show cuticle blackening regardless of treatment (Figure 4D). The observation that $Hayan^{SK3};;ModSP^{1}$ have no blackening upon *M. luteus* is consistent with our hypothesis that ModSP regulates an alternate melanization pathway independently of Hayan. Interestingly, $Sp7^{SK6}, ModSP^{1}$ mutant flies showed a similar level of blackening reaction found in *Sp7* mutants alone (Figure 4D), consistent with the notion that ModSP regulates melanization through Sp7. We conclude that at least two independent pathways contribute to cuticle blackening in *D. melanogaster* adults: one involving Hayan, and another involving Toll signaling through PGRP-SA, GNBP1, ModSP, Grass, and Sp7.

Evidence of a Close Evolutionary Relationship between Hayan and Psh

In addition to PRRs, Toll signaling can be activated by the Psh pathway in response to microbial proteases (El Chamy et al., 2008). After having studied the SPs functioning upstream of the Toll PPR pathway, we investigated a possible role of Psh in the melanization reaction. An implication of Psh in melanization was supported by the observation that a mutation affecting the serpin *necrotic*, which inhibits Psh activity, not only leads to Toll pathway activation but also to a constitutive melanization phenotype (Levashina et al., 1999; Ligoxygakis et al., 2002). Interestingly, *Hayan* and *psh* are only 751 bp apart on the *D. melanogaster* X chromosome and form a closely related monophyletic lineage among CLIP-domain SPs (Figure 5A; Data S1), suggesting that they evolved from a gene duplication. By using a bioinformatic approach, we provide evidence that *psh* is a lineage-restricted duplication found only in Melanogaster group flies, arising from an ancestral *Hayan* gene conserved across the genus *Drosophila* (Figure 5B; Figure S4A; Data S1). We also found that the Psh protease "bait region" (Issa et al., 2018), a region prone to cleavage by pathogen proteases, is present in Hayan (Figure S4B). Additionally, we realized that the transcript structures of *psh*-RA and *Hayan*-RA were extremely similar (Figure 5C). Thus, alternative splicing can produce *Hayan* transcripts very similar to *psh*, and this feature is conserved throughout the *Drosophila* lineage (Figure S5; Data S1). This led us conclude that *psh* is a recent duplication of *Hayan*, and transcripts from both genes can encode highly similar proteins.

Hayan and Psh Together Are Required for the Melanization Reaction

The gene duplication event producing *psh* from the ancestral Hayan is quite recent, suggesting both genes could have redundant functions otherwise masked in single-mutant analyses. To further characterize the role of *psh* in the melanization pathway, we generated a psh^{SK1} null mutant, a small deficiency

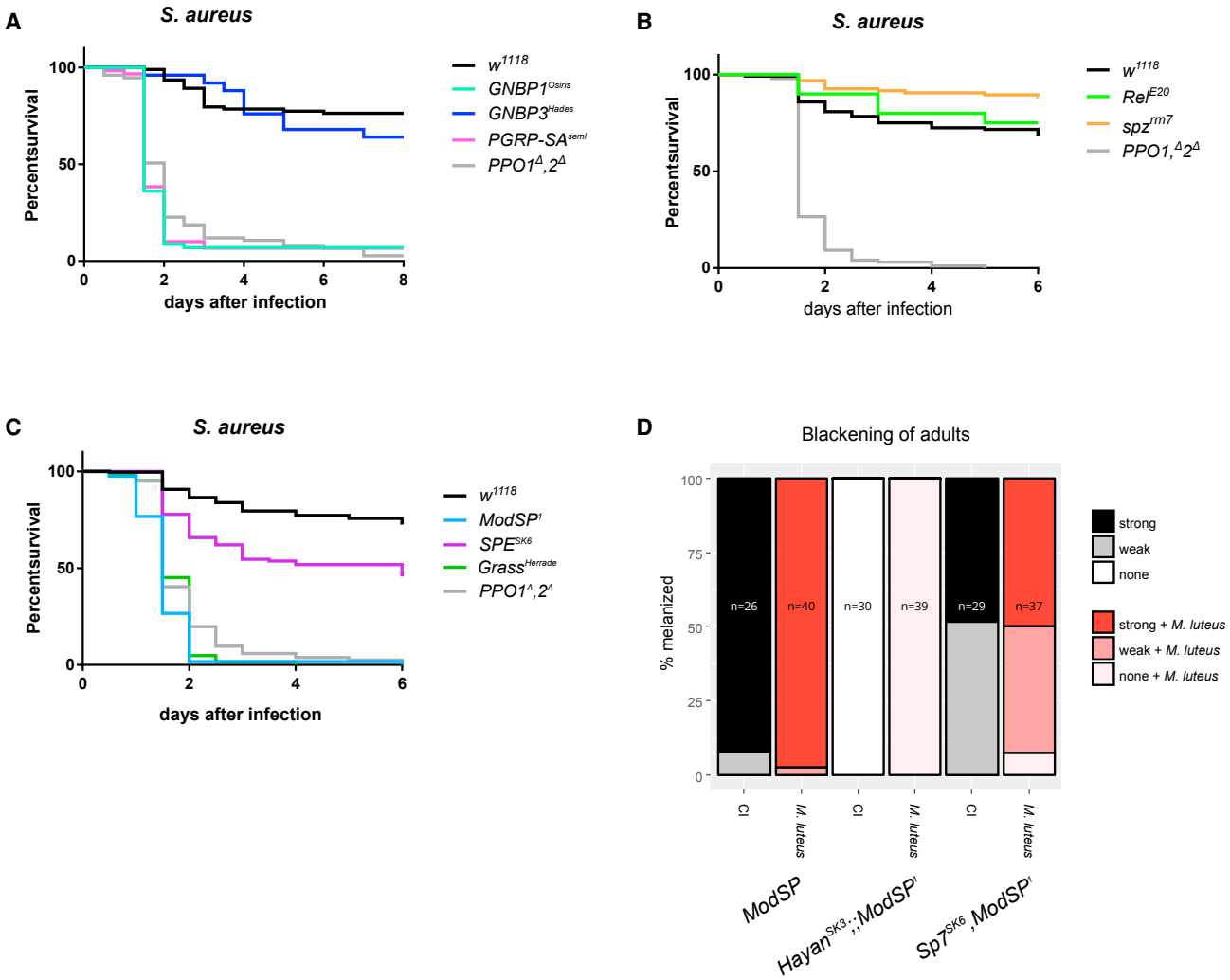

Figure 4. PRRs Regulating Toll Signaling Are Critical to Resist *S. aureus*

(A) Survival rates of flies following septic injury with *S. aureus*. Flies lacking *GNBP1* ($p < 0.0001$) or *PGRP-SA* ($p < 0.0001$), but not *GNBP3* ($p = 0.3141$), are less resistant than wild-type flies (w^{1118}).

(B) Flies lacking the Toll ligand *Spätzle* or IMD-component *Relish* resist *S. aureus* infection similar to wild-type flies (w^{1118}).

(C) Survival rates of flies following septic injury with *S. aureus*. Flies lacking $ModSP^{1}$ ($p < 0.0001$) or *Grass* ($p < 0.0001$) exhibit high susceptibility compared with wild-type flies (w^{1118}). The loss of *SPE* ($p < 0.0001$) results in a minor survival defect.

(D) More than 90% of $ModSP^{1}$ flies blacken normally. In contrast, flies mutant for $Hayan^{SK3};;ModSP^{1}$ do not blacken at all. This effect is dependent on the simultaneous absence of *Hayan* and $ModSP^{1}$ because $Sp7^{SK6},ModSP^{1}$ flies retain a blackening phenotype similar to $Sp7^{SK6}$ alone.

Hayan-psh^{Def} removing both genes, as well as $psh^{SK1};;Sp7^{SK6}$ flies. All flies were perfectly viable. psh^{SK1} flies showed a wild-type level of blackening upon injury and only a mild susceptibility to *S. aureus* (Figures 6A and 6B). Strikingly, we observed that *Hayan-psh*^{Def} flies failed to blacken upon clean and septic injury with *M. luteus* to the same extent as *Hayan;;ModSP* and $PPO1^{\Delta},2^{\Delta}$ mutant flies (Figure 6A). Additionally, *Hayan-psh*^{Def} flies also succumb rapidly to *S. aureus* infections similar to $PPO1^{\Delta},2^{\Delta}$ flies (Figure 6B). In contrast, $psh^{SK1};;Sp7^{SK6}$ show similar levels of the blackening reaction and succumb to *S. aureus* similar to $Sp7^{SK6}$ single-mutant flies ($p = 0.1517$) (Figures 2A and 6B). Collectively, these results uncover a role of Psh together with Hayan in the melanization reaction, with both contributing to the blackening reaction, as well as to resistance against *S. aureus* infections.

Hayan and Psh Redundantly Regulate Toll Signaling

We found that components of the Toll pathway directly regulate the melanization response. Conversely, we then tested whether the two SPs involved in the melanization pathway, Sp7 or Hayan, are involved in Toll pathway activation by the PRR or the Psh pathway. We observed that $Sp7^{SK6}$, $Hayan^{SK3}$, psh^{SK1}, single-mutant and $Hayan^{SK3};;Sp7^{SK6}$ and $psh^{SK1};;Sp7^{SK6}$ double-mutant flies activate the Toll pathway normally after *M. luteus* infection (Figures 6C, 6D, and S6A) (Nam et al., 2012; Tang et al., 2006). Of note, we observed a mild over-activation of the

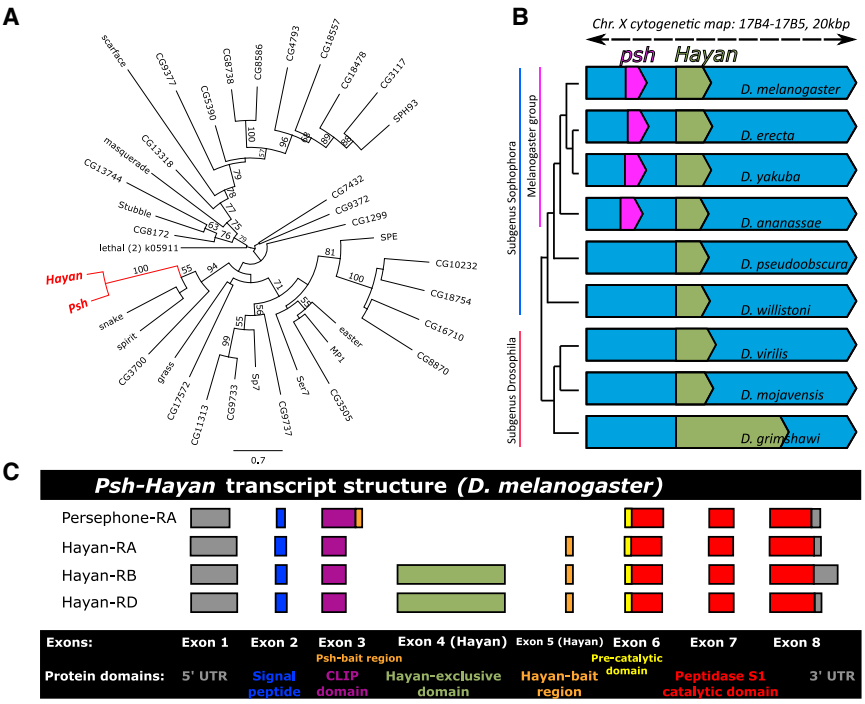

Figure 5. Evolutionarily Conserved Hayan Isoforms Are Either Similar to Psh or Unique to Hayan

(A) Maximum likelihood phylogeny of catalytic domains from *Drosophila* CLIP-domain SPs from Veillard et al. (2016). Support values represent 100 bootstraps. Hayan and Psh form a monophyletic lineage within *D. melanogaster* CLIP-domain SPs.

(B) *psh* is a gene duplication restricted to Melanogaster group flies, derived from ancestral *Hayan* (also see Figure S4A). Annotations represent CDS gene regions. *Drosophila grimshawi*'s *Hayan* region is elongated due to a 4,000-bp intron between Dgri＼GH12343-RB exons 3 and 4.

(C) *Hayan*-RA and *psh*-RA bear a striking resemblance in transcript structure, whereas the *Hayan* transcript isoforms *Hayan*-RB and *Hayan*-RD include a *Hayan*-exclusive domain on exon 4 (in green) not found in other CLIP-domain SPs in *D. melanogaster*, described further in Figure S5. Possible roles for these alternate Hayan transcripts are annotated in Figure 7.

Toll pathway in *Sp7* mutants upon *M. luteus* infection compared with wild-type (Figure 6C). Unexpectedly, we observed a very low level of Toll activation in *Hayan-psh*^{Def} mutants following *M. luteus* infection; the levels of *Drs* expression in the double mutant was similar to those observed in *spz*^{rm7} flies (Figure 6D). Re-introduction of the *Hayan-psh* genomic region in double mutants using a Flyfos transgene (Sarov et al., 2016) rescued *Drs* expression to wild-type levels after exposure to *M. luteus* (Figure 6E). Similarly, *Hayan-psh*^{Def} mutants failed to activate Toll after *C. albicans* infection (Figure S6B). We conclude that Hayan and Psh redundantly regulate the Toll pathway downstream of PRRs. Consistent with this observation, the expression of an activated form of *Hayan* using a fat body Gal4 driver (*c564*) leads to the upregulation of *Drs* in the absence of infection (Figure 6F), as previously shown for the expression of *psh* (Jang et al., 2006).

The injection of microbial proteases activates the Toll pathway through the SP Psh (El Chamy et al., 2008). Consistent with this, *psh*^{SK1} flies retained only ~35% of wild-type *Drs* expression upon injection of *Bacillus* sp. protease, whereas *spz*^{rm7} flies showed a very reduced *Drs* induction. Although *Hayan*^{SK3} flies showed wild-type levels of *Drs* expression after injection of microbial proteases, the simultaneous absence of both *Hayan* and *psh* resulted in very low level of *Drs* expression, similar to *spz*^{rm7} levels (Figure S6C). We conclude that both Hayan and Psh redundantly regulate the Toll pathway downstream of PRRs, and that Hayan together with Psh also contribute to Toll activation by microbial proteases.

DISCUSSION

The melanization reaction in insects is arguably one of the most striking immune reactions, resulting in a visible black spot at the site of infection. Although the *Drosophila* immune response has been studied intensely, our knowledge of the melanization reaction has lagged behind. This is in part due to the overwhelming complexity of SP cascades, which has prevented a clear understanding. Indeed, insect genomes harbor an incredible diversity of SPs, whose functions remain largely uncharacterized (Cao et al., 2015; Ross et al., 2003). In this study, we provide insights on SPs of the PO cascade and uncover a relationship between the Toll and melanization pathways.

Melanization Is More Than the Blackening of the Wound Area

Although the importance of the melanization response has been demonstrated in other insects (e.g., *Manduca sexta*) (Eleftherianos et al., 2007; Lu et al., 2008), the precise relevance of PPOs in *D. melanogaster* host defense was disputed until recently (Ayres and Schneider, 2008; Leclerc et al., 2006; Tang et al., 2006). The use of null mutations in *Drosophila PPO* genes clearly demonstrated that melanization-deficient flies lack resistance against microbes, mainly Gram-positive bacteria and fungi (Binggeli et al., 2014). In this study, we developed an adult infection model using a low-dose inoculation of the Gram-positive bacterium *S. aureus*, which we find is especially appropriate to study melanization. Strikingly, flies deficient for PPOs, but not flies lacking hemocytes or Toll effectors, rapidly succumb to this challenge. The melanization response restricts the growth of *S. aureus*, preventing its systemic dissemination. Furthermore, our study reveals a disconnect between resistance to infection and the blackening of the wound site. Although a mutation in *Hayan* leads to the almost complete loss of the blackening reaction in adults, *Hayan* mutants do not share the susceptibility of *PPO1*$^{\Delta}$,*2*$^{\Delta}$ flies against *S. aureus*. In contrast, *Sp7* mutant flies do not survive *S. aureus* infection, despite almost wild-type levels of cuticle and hemolymph blackening. This indicates that it is not the

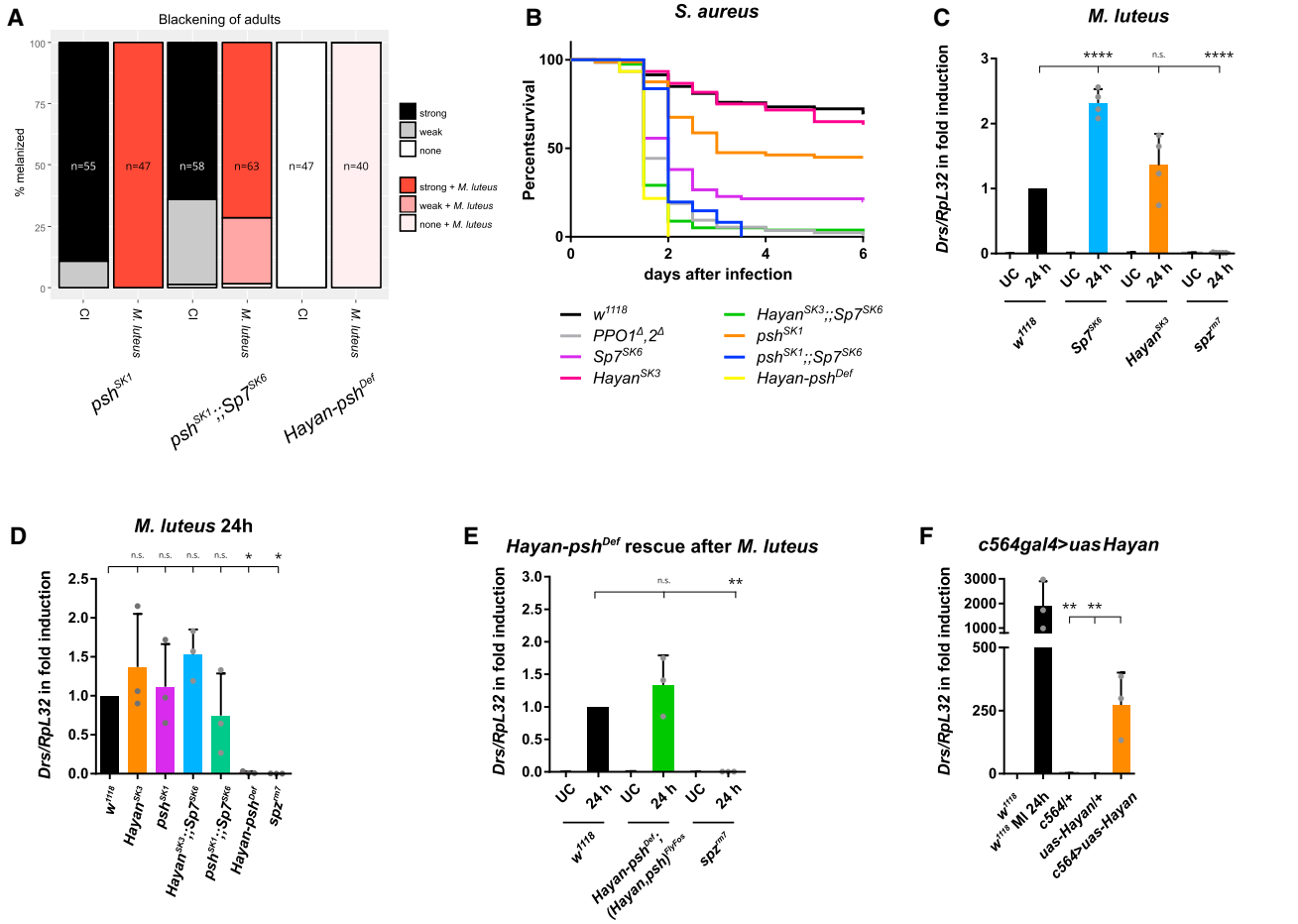

Figure 6. *Hayan* and *Psh* Contribute to the Melanization Reaction and Toll Pathway Activation

(A) Almost 90% of psh^{SK1} flies blacken normally. Flies mutant for psh^{SK1};;$Sp7^{SK6}$ show slightly reduced cuticle blackening levels similar to $Sp7^{SK6}$ alone (see Figure 3). In contrast, flies mutant for both *Hayan* and *psh* show no cuticle blackening. CI: clean injury. UC: unchallenged.

(B) Survival rates of flies following septic injury with *S. aureus*. Flies lacking *Sp7* ($p < 0.0001$) alone or in combination with *Hayan* or *psh* ($p < 0.0001$) exhibit high susceptibility compared with wild-type flies (w^{1118}). The loss of *Hayan* ($p = 0.47$) or *psh* ($p < 0.0001$) alone results in no or a minor survival effect, respectively. In contrast, flies mutant for both *Hayan* and *psh* ($p < 0.0001$) exhibit high susceptibility.

(C) Single mutants for *Sp7* and *Hayan* show no loss of Toll pathway activity (*Drs* expression) after *M. luteus* infection.

(D) Flies lacking both *Hayan* and *psh* fail to induce *Drs* similar to spz^{rm7} flies, whereas single mutants for *Hayan*, *psh,* or both combined to *Sp7* show *Drs* levels similar to w^{1118} flies.

(E) Insertion of a *Hayan, psh* transgene (FlyFos) rescues to wild-type levels of *Drs* expression in the $Hayan\text{-}psh^{Def}$ double-mutant background after *M. luteus* infection.

(F) Expression of a constitutively active form of *Hayan* in the fat body is sufficient to induce *Drs* in otherwise unchallenged flies.

(C–F) Shown are the expression levels of *Drs* relative to *RpL32*. w^{1118} levels after 24 h (C–E) or unchallenged (UC; F) are set to 1. spz^{rm7} flies act as a negative control. A minimum of three independent experiments with 10 flies per genotype was conducted for all expression analyses, with two technical replicates.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ****$p < 0.00005$.

blackening per se that is involved in the control of *S. aureus*, but rather other reactions downstream of PO activity. For instance, the melanization response is associated with the production of cytotoxic molecules like ROS ((Nappi et al., 2009). Consistent with a possible role of ROS, we observed that *Sp7* mutants that are susceptible to *S. aureus* have reduced H_2O_2 levels in total fly lysates, but this difference was not significant (Figure S1E; $p = 0.1$). It is tempting to speculate that ROS or other cytotoxic intermediates contribute to host resistance to microbial infection, while melanin deposition is involved in host protection by acting as a ROS sink as proposed by other authors (Nappi et al., 2009; Riley, 1997).

The Toll Pathway Branches Downstream of Hayan and Psh to Regulate the Melanization Pathway

Biochemical studies in other insects such as *Manduca sexta* and *Tenebrio molitor* have shown that melanization and the Toll pathway share common upstream activation mechanisms that separate only at near-terminal components of the cascade (An et al., 2009; Kan et al., 2008; Kim et al., 2008). Prior to our

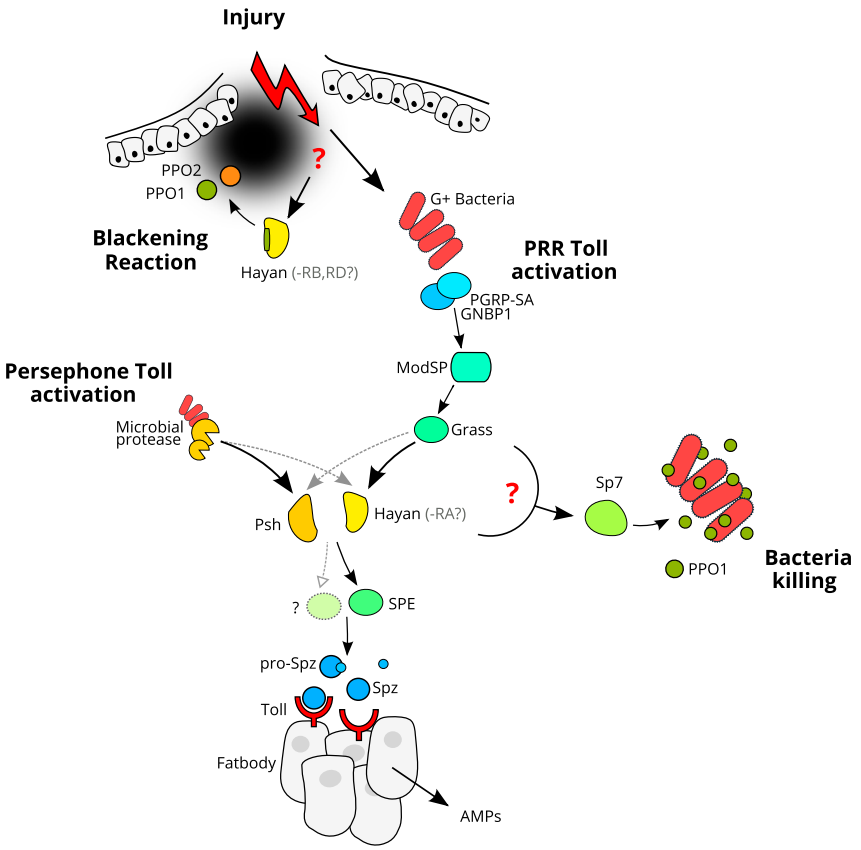

Figure 7. A Revised Model of SPs Regulating the Toll Pathway and the Melanization Reaction

After the introduction of a wound through the cuticle and underlying epithelium, Hayan can be activated by an unknown mechanism that results in the deposition of melanin around the wound area (top left). This Hayan-dependent blackening reaction can be achieved through both PPO1 and PPO2. If Gram-positive bacteria enter through the wound, peptidoglycan can be recognized by the PRRs PGRP-SA, and GNBP1, initiating the sequential activation of the SPs ModSP, Grass Psh/Hayan, and SPE. This leads to the cleavage of Spz and the activation of Toll signaling in the fat body (middle). This extracellular SP pathway likely branches at the position of Hayan and Psh to Sp7, activating PPO1 to combat invading bacteria, possibly via the production of cytotoxic intermediates, but not necessarily melanin itself (right). Microbial proteases can activate the Toll pathway through the Psh-SPE-Spz extracellular pathway. Although suggested by sequence homology and *Drs* expression following protease injection, it is unclear whether microbial proteases can also activate Hayan. However, both Hayan and Psh regulate the Toll pathway downstream of Grass, ModSP, and PRRs. A previous study (Yamamoto-Hino and Goto, 2016) and our data suggest the existence of another SP capable of cleaving Spz beyond SPE.

results, there was only indirect evidence in *D. melanogaster* that the Toll pathway activates melanization (Matskevich et al., 2010). However, Toll regulates many SPs and serpins involved in the melanization cascade at transcriptional levels (De Gregorio et al., 2001; Ligoxygakis et al., 2002), and overexpression of SPs functioning upstream of Spz or gain-of-function activation of the Toll pathway leads to spontaneous melanization (An et al., 2013; Gerttula et al., 1988; Lemaitre et al., 1995; Tang et al., 2006). Some reports also show that Toll PRRs can activate a host defense reaction independent of Toll intracellular signaling, possibly through melanization (Bischoff et al., 2004; Matskevich et al., 2010). Despite these sporadic observations, if and how the Toll pathway branches to PPO activation remained unknown. Here we show that PGRP-SA, GNBP1, ModSP, and Grass, but not SPE, regulate the melanization response through Sp7 and PPO1 after exposure to Gram-positive bacteria. Additionally, we discovered that Psh also contributes to the melanization reaction together with Hayan, notably by impacting both the blackening of the cuticle and the resistance to *S. aureus* infections. Thus, our study demonstrates a direct connection between the extracellular SPs regulating the activation of Toll and the melanization response as observed in other insects. The most parsimonious interpretation of our data is that the Toll PRR cascade diverges downstream of Hayan and Psh to activate Sp7 and PPO1 (see Figure 7 and see also below). Surprisingly, we found no clear role for MP1 in melanization nor Toll pathway activity, which contradicts a previous RNAi screen that positioned MP1 downstream of Sp7 in the PO cascade (Tang et al., 2006). Results obtained with RNAi may be the consequence of off-target effects that shut down other SPs. However, we cannot exclude that MP1 contributes to melanization in a redundant way.

Hayan and Psh Redundantly Regulate Toll Signaling Downstream of the PRR Pathway

One of the most important observations of our study is that a small deficiency removing both *Hayan* and *psh* blocks Toll pathway activation regardless of the type of challenge. This points to a crucial, although partially redundant, function for these two SPs in the activation of Toll in both the protease and PRR pathways. Thus, we uncover a role of the melanization SP Hayan in Toll signaling, and extend the function of Psh beyond the sensing of microbial proteases by revealing that this SP is also involved in the PRR pathway. Interestingly, both Hayan and Psh are phylogenetically related to MsHP6, which propagates Toll signaling and controls melanization in the lepidopteran insect *M. sexta* (An et al., 2009; Cao and Jiang, 2018). In our study, we also found that *Hayan* and *psh* arose from a recent duplication of an ancestral *Hayan* gene, supporting the idea that these SPs perform redundant functions. Indeed, Hayan and Psh share striking similarities in a regulatory region upstream of the catalytic domain, which strongly suggests that they can be cleaved by the same upstream SPs. A previous study has shown that the Psh bait region motif ^{107}GRVDVPTFGS^{116} is critical for

protease-dependent Psh cleavage in *Drosophila* S2 cells (Issa et al., 2018). This bait region is also present but truncated in Hayan (Figure S4). Because mutations in this motif strongly affect cleavage efficiency by *B. subtilis* proteases, the sequence differences in the bait regions of Hayan and Psh could explain their unequal contributions to Toll activation, notably the more important role of Psh in sensing microbial proteases. Thus, these disparate bait regions alongside the loss of the Hayan-exclusive domain in *psh* (Figure S5C) may represent the early beginnings of a process of sub-functionalization for these two SPs (He and Zhang, 2005). Because both SPs also share strong similarities in their catalytic domain, they likely cleave similar downstream targets. During embryonic dorsoventral signaling, the SP Snake regulates Easter, which is the terminal SP that cleaves the Toll ligand Spz (Dissing et al., 2001; LeMosy et al., 2001; Lindsay and Wasserman, 2014). Taking into consideration that Hayan and Psh cluster phylogenetically with Snake (Figure 5A) and that SPE clusters with Easter, it is likely that Hayan and Psh act upstream of SPE as described in the model depicted in Figure 7. Consistent with this, the ubiquitous overexpression of SPE results in early larval lethality, which was not rescued in a *Hayan-psh^{Def}* background. Conversely, the overexpression of *ModSP* resulted in pupal lethality and melanotic tumors, with both phenotypes being rescued in a *Hayan-psh^{Def}* background (Figure S6E). Additionally, the ubiquitous overexpression of *ModSP* leads to a strong activation of the Toll pathway, which is also completely suppressed in a *Hayan-psh^{Def}* background (Figure S6F). This epistatic analysis is consistent with the position of Hayan and Psh being upstream of SPE but downstream of ModSP. Because Psh and Hayan contribute to both Toll signaling and melanization, they can be viewed as a platform integrating various signals associated with infection and damage (microbial proteases and cell wall components, proteolytic activity) to activate downstream immune and repair effectors. We notice that *SPE* mutants still express a residual level of *Drs* upon septic injury with *M. luteus* (Figure S6D). Thus, there is likely another SP with the ability to process Spz, as first proposed by Yamamoto-Hino and Goto (2016). Other CLIP-domain SPs related to Easter and SPE are promising candidates to fulfill this role.

Melanization and Toll: More Than Black and White

Altogether, we propose a revised model of the extracellular SP cascades regulating melanization and the Toll pathway in *D. melanogaster* that takes the three main findings of this work into consideration: (1) the existence of two different pathways activating melanization, (2) the involvement of the extracellular PRRs in the melanization reaction, and (3) the implication of both Hayan and Psh downstream of the extracellular PRR and the Psh pathway in the regulation of both Toll activity and the melanization response (Figure 7). According to our model, cuticle injury activates Hayan by an unknown pathway, which results in the deposition of melanin (blackening reaction) at the wound site through both PPO1 and PPO2. After an infection with Gram-positive bacteria, peptidoglycan can be recognized by the PRRs PGRP-SA/GNBP1, leading to an SP cascade involving ModSP, Grass, Hayan and Psh, and SPE, resulting in Spz cleavage. In our model, the Toll PRR pathway branches at the position of Hayan and Psh to Sp7, activating PPO1 to combat invading bacteria. Alternatively, microbial proteases and endogenous elicitors (Issa et al., 2018) can activate the Toll pathway independently of PRRs by cleaving Psh, and possibly Hayan, directly. Thus, two SPs, Hayan and Psh, merge signals from both the PRR and Psh pathways to activate a common extracellular pathway upstream of Toll. Future biochemical and genetic studies are required to further clarify the complex SP cascades regulating the immune response.

The analysis of proteolytic cascades regulating the *Drosophila* immune response has been hampered by the large number of SP genes, often found in clusters in the genome. Although the biochemical approaches carried out in large insects have allowed a comprehensive understanding of SP signaling cascades in moths and beetles (Kanost and Jiang, 2015), genetic approaches using single-gene mutant analysis were unable to determine these cascades in *Drosophila* (Binggeli, 2013). Functional redundancy, as exemplified in our study of *Hayan* and *psh*, clarifies the shortcomings of single-gene genetic approaches. Coupling phylogenetic analysis approaches with double- (or triple-) compound mutant analysis could pave the way to better characterize these cascades.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.03.101.

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AUTHOR CONTRIBUTIONS

J.P.D. and B.L. designed the study. J.P.D. performed the experiments. M.A.H. performed bioinformatic analyses. J.P.D., M.A.H., and B.L. analyzed the data

and wrote the manuscript. I.I. and S.K. supplied critical reagents. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-PPO1	Nam et al., 2012	N/A
Goat Anti-Rat IgG H&L (HRP)	Abcam	ab97057; RRID:AB_10680316
Bacterial and Virus Strains		
Staphylococcus aureus	Bruno Lemaitre	N/A
Staphylococcus aureus GFP	Needham et al., 2004	N/A
Micrococcus luteus	Bruno Lemaitre	N/A
Bacillus subtilis	Bruno Lemaitre	168
Protease of *Bacillus sp*.	Sigma	P0029
Candida albicans	Bruno Lemaitre	N/A
Critical Commercial Assays		
Fluorimetric Hydrogen Peroxide Assay Kit	Sigma	MAK165
Deposited Data		
Supplemental Data File for sequence data	this study	https://docs.google.com/spreadsheets/d/1jYC9Vj9budiMEAkgc_lZfbMTB8ZXNQZU0qbYrOtgUfA/edit#gid=395553455
Experimental Models: Organisms/Strains		
PPO1$^{\Delta}$	Binggeli et al., 2014	N/A
PPO2$^{\Delta}$	Binggeli et al., 2014	N/A
Relish^{E20}	Hedengren et al., 1999	N/A
spätzle^{rm7}	Lemaitre et al., 1996	N/A
ModSP^{1}	Buchon et al., 2009	N/A
Grass^{Herrade}	Bloomington Drosophila Stock Center	BL: 67099
Hayan^{SK3}	Dudzic et al., 2015	N/A
SPE^{SK6}	Yamamoto-Hino and Goto, 2016	N/A
SP7^{SK6}	Dudzic et al., 2015	N/A
eater^{1}	Bretscher et al., 2015	N/A
hml $^{\Delta}$*-Gal4*	Sinenko and Mathey-Prevot, 2004	N/A
c564-Gal4	Bloomington Drosophila Stock Center	BL: 6982
UAS-bax	Blanchardon et al., 2001	N/A
UAS-hayan	Nam et al., 2012	N/A
uas-SPE	Jang et al., 2006	N/A
uas-ModSP	Buchon et al., 2009	N/A
psh^{SK1}	This paper	N/A
MP1^{SK6}	This paper	N/A
Hayan-psh^{Def}	This paper	N/A
GNBP1^{osiris}	Gobert et al., 2003	N/A
GNBP3^{hades}	Gottar et al., 2006	N/A
PGRP-SA^{seml}	Bloomington Drosophila Stock Center	BL: 55761
Oligonucleotides		
Diptericin F: GCTGCGCAATCGCTTCTACT	Neyen et al., 2014	N/A
Diptericin R: TGGTGGAGTGGGCTTCATG	Neyen et al., 2014	N/A
Drosomycin F: CGTGAGAACCTTTTCCAATATGAT	Neyen et al., 2014	N/A
Drosomycin R: TCCCAGGACCACCAGCAT	Neyen et al., 2014	N/A
RpL32 (Rp49) F: GACGCTTCAAGGGACAGTATCTG	Neyen et al., 2014	N/A
RpL32 (Rp49) R: AAACGCGGTTCTGCATGAG	Neyen et al., 2014	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bruno Lemaitre (bruno.lemaitre@epfl.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects Stocks

Unless indicated otherwise, w^{1118} flies were used as wild-type controls. The $PPO1^{\Delta}$, $PPO2^{\Delta}$ and $PPO1^{\Delta},2^{\Delta}$, $Relish^{E20}$ (Rel^{E20}), $spätzle^{rm7}$ (spz^{rm7}), $ModSP^{1}$, $Grass^{Herrade}$ (BL: 67099), $Hayan^{SK3}$, SPE^{SK6}, $Sp7^{SK6}$, $eater^{1}$, hml^{Δ}-Gal4, c564-Gal4 (BL: 6982), UAS-bax, UAS-Hayan, $GNBP1^{osiris}$, $GNBP3^{Hades}$, $PGRP\text{-}SA^{seml}$ (BL: 55761) lines are described previously or obtained from Bloomington *Drosophila* Stock Center (Binggeli et al., 2014; Bretscher et al., 2015; Buchon et al., 2009; Dudzic et al., 2015; Gobert et al., 2003; Gottar et al., 2006; Lemaitre et al., 1996; Nam et al., 2012; Neyen et al., 2014; Sinenko and Mathey-Prevot, 2004; Yamamoto-Hino and Goto, 2016). The $MP1^{SK6}$, psh^{SK1} and $Hayan\text{-}psh^{Def}$ mutant lines were generated by CRISPR/Cas9 as described in Kondo and Ueda (2013). $MP1^{SK6}$ harbors a 13 bp deletion from position 4308655 to 4308668 on the third chromosome. psh^{SK1} harbors a 10 bp deletion from position 18485863 to 18485873 on the X chromosome. A visual overview of both mutants is given in Figure S2D. The $Df(1)Hayan,psh^{SK5}$ deficiency has a 6816 bp deletion from position 18480206 to 18487022 on the X chromosome (referred to as $Hayan\text{-}psh^{Def}$ in the text). To rescue the $Hayan\text{-}psh^{Def}$ mutant flies, transgenic flies were produced by injecting embryos with a Hayan,psh transgene from FlyFos (Clone CBGtg9060C0781D, Sarov et al. [2016]). This transgene contains ~10 kb of genomic DNA from gene CG15046 to Hayan). *Drosophila* stocks were maintained at 25°C on standard fly medium.

Microorganism culture and infection experiments

The bacterial strains used and the respective optical densities of their pellets (OD) at 600 nm were: the DAP-type peptidoglycan-containing Gram-positive bacteria *Bacillus subtilis* (*B. subtilis*, O.D 5), and the Lys-type peptidoglycan-containing Gram-positive bacteria *Micrococcus luteus* (*M. luteus*, OD 200), and *Staphylococcus aureus* (*S. aureus*, OD 0.5). Strains were cultured in Luria Broth (LB) at 29°C (*M. luteus*) or 37°C (other species). The yeast *Candida albicans* (*C. albicans*, OD 400) was cultured in YPG medium at 37°C. Pellets were diluted in distilled water. The *S. aureus*-GFP strain is described in Needham et al. (2004). Systemic infections (septic injury) were performed by pricking adults in the thorax with a thin needle previously dipped into a concentrated pellet of bacteria. Infected flies were subsequently maintained at 29°C (*M. luteus, C. albicans, B. subtilis*) or at 25°C (*S. aureus*, injection of *B. subtilis* protease). At least 20 flies were used for each genotype in each survival experiment and survival was scored once or twice daily. Survival experiments were performed at least three individual times and data of all experiments was combined (unless otherwise indicated). For lifespan experiments, flies were kept on normal fly medium at 25°C and were flipped every two days. 18 nL of protease of *Bacillus sp*. (Sigma P0029) diluted 1:1500 in PBS was injected into the thorax for qRT-PCR experiments.

Bacterial load of flies

Flies were infected with *S. aureus* as described above. At indicated time points, flies were surface-sterilized by washing them in 70% ethanol. Ethanol was rinsed off with sterile PBS and groups of five flies were homogenized by adding glass beads and the use of a PRECELLYS™ homogenizer in 0.2 mL PBS. The homogenate was serially diluted and plated on LB agar. After incubation at 37°C overnight, colonies were counted and calculated to single fly CFUs; no colonies were recovered from uninfected flies using this approach.

METHOD DETAILS

Wounding experiment

Clean injury (CI) refers to an injury performed with an ethanol sterilized needle. A low level of bacterial contamination is still possible since the surface of the insect was not sterilized. For imaging of the blackening reaction upon pricking, the thoraces of adults or dorsal posterior ends of third instar larvae was pricked (as described in infection experiments) using a sterile needle (diameter: ~5 μm). Pictures were taken 16 hours post-pricking. Pictures of melanized larvae were taken one hour post-injury. Pictures were captured with a Leica M 205 FA microscope, a Leica DFC7000FT camera and the Leica Application Suite. For publication purposes, brightness and contrast were increased on some images.

Melanization assessment

Flies or larvae were pricked as described and the level of blackening at the wound site, estimated by the size and color of the melanin spot, was examined 16-18 h later in adults and 3 h later in larvae. For observing the general capacity of hemolymph to melanize, hemolymph was collected from third instar (L3) larvae by dissection and transferred to a 96 well microtiter plate. After incubation at room temperature blackening was recorded. To assay cleavage of PPO1, hemolymph of 10 L3 larvae was collected at 4°C and incubated for 10 min at 95°C in Laemmli SDS-PAGE sample buffer with β-mercaptoethanol. Samples were separated using 10% Invitrogen Novex gels and western blot was performed according to Binggeli et al. (2014). After the transfer, the membrane was

incubated at 4°C overnight with the primary antibody (anti-PPO1,1:3000, Nam et al., 2012) in 4% milk powder 0.1% Tween20 PBS. After washing 3x in 0.1% Tween20 PBS, the membrane was incubated for 2 hours at room temperature with the secondary antibody (anti-Rat-HRP, 1:15000, Abcam). The signal was developed with Amersham ECL Western Blotting Detection Reagent and digitally imaged with Fusion Solo (Vilber Lourmat) and the presented images are inverted.

H_2O_2 assay

H_2O_2 levels were assessed with a Fluorimetric Hydrogen Peroxide Assay Kit (Sigma MAK165) according to the manufacturer's protocol. Briefly, seven adult flies were homogenized in 0.12 mL PBS with a PRECELLYS^{TM} homogenizer and then centrifuged at 4°C and 13,000 RPM. Afterward, 0.1 mL of the supernatant was transferred to a new tube. Sample volumes of 30 μl were used for the assay and measurements were done in duplicate. Protein concentration of the samples was determined via Bradford assay and results were normalized to the respective protein levels.

Quantitative RT-PCR

For quantification of mRNA, whole flies or larvae were collected at indicated time points. Total RNA was isolated from a minimum of 10 adult flies by TRIzol reagent and dissolved in RNase-free water. 0.5 μg of total RNA was then reverse-transcribed in 10 μL reactions using PrimeScript RT (TAKARA) with random hexamer and oligo dT primers. Quantitative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using the Applied Biosystems SYBR Select Master Mix. Primers were as follows: *Diptericin* forward 5′- GCTGCGCAATCGCTTCTACT-3′, reverse 5′-TGGTGGAGTGGGCTTCATG-3′; *Drosomycin* forward 5′-CGTGAG AACCTTTTCCAATATGAT-3′, reverse 5′- TCCCAGGACCACCAGCAT-3′; *RpL32* (*Rp49*) forward 5′-GACGCTTCAAGGGACAG TATCTG-3′, reverse 5′-AAACGCGGTTCTGCATGAG-3′.

QUANTIFICATION AND STATISTICAL ANALYSIS

SP sequence analysis

A list of *Drosophila melanogaster* CLIP-domain serine proteases was generated from Veillard et al. (2016), and all *D. melanogaster* transcript isoforms were extracted from FlyBase v2018_02 (Gramates et al., 2017). Translated catalytic domains for these SPs were aligned using MAFFT (Katoh and Standley, 2013) for maximum likelihood phylogenetic analysis using PhyML in Geneious 10.2.3 (Guindon et al., 2010; Kearse et al., 2012). Using the FlyBase genome browser, 100kb gene regions surrounding Hayan orthologs were extracted from various *Drosophila* species, aligned using Hayan as a frame of reference, and manually searched for conserved SP motifs (e.g., ''LTAAHC'') common to all *D. melanogaster* CLIP-domain SPs. Following Hayan and Psh characterization, annotated *Hayan* transcripts were extracted from FlyBase and recent immune annotations of subgenus *Drosophila* flies (Hanson et al., 2016). Using these genomic and transcriptomic data, a Hayan-exclusive domain was extracted from diverse *Drosophila* and analyzed for signatures of selection using FEL and SLAC analyses implemented in datamonkey.org (Delport et al., 2010; Kosakovsky Pond and Frost, 2005). We also performed these analyses for comparisons of Hayan to Psh. Sequences and output of those analyses are provided in Data S1.

Statistical analysis

Each experiment was repeated independently a minimum of three times (unless otherwise indicated), error bars represent the standard deviation (s.d.) of replicate experiments (unless otherwise indicated). Statistical significance of survival data was calculated with a log-rank test compared to wild-type flies. Statistical significance of qPCR or ROS data was calculated with One-way ANOVA with Tukey's multiple comparisons test. Blackening strength (normal, weak, or none) of the adult cuticle was analyzed using Pearson's Chi-square test. CFU counts were analyzed using the Mann-Whitney test. P values of < 0.05 = *, < 0.005 = **, and < 0.0005 = *** were considered significant. P values are indicated in figure legends.