The melanization reaction in *Drosophila*: More than black or white

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4 SUMMARY

The melanization reaction is a rapid and important immune mechanism in arthropods. It results in the production of melanin at the site of injury and around invading microbes. The enzymes responsible for melanogenesis are phenoloxidases (PO), which catalyze the oxidation of phenols to quinones, which then polymerize into melanin. A by-product of melanogenesis are cytotoxic compounds that can pose a threat to the host organism itself due to their unspecific effects. Melanogenesis is therefore tightly regulated: POs are produced in an inactive form as prophenoloxidases (PPOs), which get activated by the sequential cleavage of an extracellular serine protease (SP) cascade. The aims of this PhD thesis were to better understand the melanization reaction in *Drosophila melanogaster*, at both the effector and the regulation levels. *D. melanogaster* has three *PPO* genes. We generated for the first time a mutant for the third PPO gene, *PPO3*, and analyzed its function. We demonstrated that PPO3 has an important role in the melanotic encapsulation reaction, a defense mechanism against parasitization. Additionally, we extended our knowledge about the other PPOs, PPO1 and PPO2. We confirmed their role in the defense against septic infections and ascribed a new role for PPO2 in the melanotic encapsulation. The use of single or combined mutations allowed us to show that each PPO mutant has a specific phenotype, and that knocking out two of three genes is required to abolish a particular function completely. Thus, *Drosophila* PPOs have partially overlapping functions to optimize melanization. Finally, we demonstrated that *PPO3* is the result of a gene duplication of *PPO2*, restricted to a subgroup of *Drosophila*, and likely evolved as an additional defense mechanism in the cellular encapsulation process, probably due to the evolutionary pressure from parasitoid wasps. In the second part of this thesis, we re-addressed the roles and functions of three SPs involved in the melanization process. We developed a novel screening method for defects in melanization by infecting flies with a low-dose of *S. aureus*. We found that only one of the three SPs, Sp7, is involved in survival upon septic infections. Additionally, we demonstrated that the melanization reaction resulting in the clearance of systemic infections is regulated by extracellular components of the Toll pathway. While a connection between the SPs regulating the melanization and the Toll pathway was found in other insects, our study provides the first demonstration in *D. melanogaster*. We also present evidence of a disconnect between the melanin production at

wound sites and the melanization reaction resulting in the clearance of infections, indicating a role of cytotoxic compounds in the killing of microbes. Finally, we ascribed a new role to Hayan, another SP implicated in melanization. Hayan is dispensable for the clearance of septic infections, but is important for wound melanization. We also demonstrated that Hayan plays an important role in the activation of the Toll pathway. Hayan, together with the SP Persephone, is necessary to activate Toll after infection. We propose that both Hayan and Persephone are the result of a recent gene duplication event. This can explain why they still have overlapping functions, but these genes also show signs of early sub-functionalization. Collectively, our work provides important insights on both melanization and the activation of the Toll pathway.

Keywords: Drosophila – Immunity - Melanization - Toll - Serine-proteases

5 ZUSAMMENFASSUNG

Die Melanisierung ist eine schnelle und wichtige Immunantwort in Arthropoden. Sie resultiert in der rapiden Produktion von Melanin an Verletzungen und an eindringenden Mikroben. Die für die Produktion von Melanin verantwortlichen Enzyme werden Phenoloxidasen (PO) genannt. Sie katalysieren die Oxidation von Phenolen zu Quinonen, welche dann zu Melanin polymerisieren. Ein Seiteneffekt der Melanisierung ist die Produktion von zytotoxischen Stoffen, welche aufgrund ihrer unspezifischen Wirkung auch eine Gefahr für den produzierenden Organismus darstellen. Die Produktion von Melanin ist daher streng reguliert: POs werden als inaktive Proteine produziert, welche Prophenoloxidasen (PPO) genannt werden. Diese werden durch eine Kaskade von proteolytischen Serinproteasen (SP) aktiviert. Die vorliegende Arbeit hatte das bessere Verständnis der Melanisierung im Modellorganismus *Drosophila melanogaster* auf der Ebene der Effektormoleküle (POs) und der regulierenden Ebene (SPs) zum Ziel. Das Genom von *D. melanogaster* enthält drei *PPO*-Gene. Wir generierten in dieser Arbeit zum ersten Mal eine Mutation im dritten *PPO*-Gen (PPO3) und analysierten dessen Funktion. Wir konnten zeigen, dass PPO3 eine wichtige Rolle in der Immunfunktion der melanotischen Einkapselung spielt, welche eine wichtige Immunantwort gegen bspw. Parasitierung darstellt. Zusätzlich zu diesem Ergebnis, erweitern wir das Wissen über die anderen PPOs, PPO1 und PPO2. Wir bestätigten das beide eine wichtige Rolle in der Verteidigung gegen bakterielle und fungale Infektionen spielen, und demonstrieren das PPO2 ebenfalls zur melanotischen Einkapselung beiträgt. Weiterhin stellen wir unsere neuen Ergebnisse in einen evolutionären Zusammenhang: Wir zeigen das *PPO3* das Resultat einer Genduplikation von *PPO2*, welche nur in einer Untergruppe von *Drosophila* präsent ist. Weiterhin liefern wir Indizien, dass diese Genduplikation das Resultat evolutionären Drucks durch parasitoide Wespen ist. Auf der Ebene der Regulation der Melanisierung durch SPs überprüfen wir die Rollen von drei bereits bekannten Melanisierungs-SPs. Wir beschreiben eine neue Infektionsmethode mit niedrig dosiertem *Staphylococcus aureus*, zur Detektion von Defekten in der Melanisierung. Mit dieser Methode zeigen wir, dass nur eine dieser drei SPs, Sp7, für die Eliminierung einer Infektion wichtig ist. Außerdem legen wir eine Verbindung dieser melanisierungsabhänigen Eliminierung mit dem Toll Signalweg offen, die zwar in anderen Insekten bereits bekannt ist, hier jedoch das erste Mal für *Drosophila* beschrieben wird. Weiterhin

separieren wir die Eliminierung einer Infektion durch POs von der eigentlichen Produktion Melanins. Wir liefern Indizien, dass die Abtötung der Mikroben durch zytotoxische Stoffe aber nicht durch Melanin an sich erreicht wird. Abschließend beschreiben wir eine neue Funktion für eine weitere Melanisierungs SP mit dem Namen Hayan. Hayan spielt eine wichtige Rolle in der Produktion von Melanin an Wunden, aber keine Rolle in der Eliminierung von Mikroben. Überraschenderweise trägt Hayan aber zu der Regulation des Toll Signalwegs bei. Zusammen mit der SPs Persephone ist Hayan wichtig für die Aktivierung von Toll nach einer Infektion mit Grampositiven Bakterien, Pilzen oder mikrobiellen Proteasen. Wir zeigen zusätzlich das Hayan und Persephone das Resultat einer kürzlichen Genduplikation sind. Beide können noch die gleichen Funktionen übernehmen, zeigen aber bereits Anzeichen einer Subfunktionalisierung.

Schlüsselwörter: Drosophila – Immunsystem - Melanisierung – Toll - Serinproteasen

6 INTRODUCTION

6.1 BASIC CONCEPTS OF INSECT IMMUNITY

Like many organisms, insects live in a microbe-rich environment. Their survival is impacted by the power of their innate immune system (Lemaitre and Hoffmann, 2007). Insects and humans have a Janus-faced relationship. On one hand, insects can have beneficial roles, e.g. the agricultural industry relies heavily on pollinators. Thus, having a good understanding of insect immunity can help to protect insects which have such positive effects. On the other hand, insect disease vectors or agricultural pests can have a detrimental impact on human health or economic revenues. To improve the efficiency of vector- or pest control, it is crucial to have an understanding on how insects survive constant microbial challenges.

In contrast to vertebrates, which have two types of defense mechanisms, the adaptive and the innate immune system, arthropods rely solely on the latter one. Microbes harbor molecules that can be exclusive for their biological kingdom that hosts can use to distinguish them from themselves. This mode of recognition, called the pattern recognition model, is the backbone of innate immunity (Janeway and Medzhitov, 2002). Invading microbes release a variety of "microbe associated molecular patterns" (MAMPs), which are recognized by host receptors called "pattern recognition receptors" (PRRs) (Akira et al., 2006; Janeway, 1989). Amongst those MAMPs are e.g. the bacterial cell wall component peptidoglycan (PGN) or the fungal cell wall component ß-1,3-glucan (Gillespie et al., 1997). The insect immune system relies then on PRRs like peptidoglycan recognition proteins (PGRPs) or gram-negative bacteria-binding proteins (GNBPs) to identify microbes and subsequently mount an immune response against them (Govind, 2008).

6.1.1 Drosophila immunity

The fruit fly *Drosophila melanogaster* has been a fundamental model to investigate insect immunity and the interaction between hosts and microbes (Buchon et al., 2014; Lemaitre and Hoffmann, 2007). Immune pathways and genes are highly conserved between *D. melanogaster* and higher organisms, ergo the research on fruit fly immunity has revolutionized the understanding of the immune system across taxonomic classes in the last decades.

D. melanogaster flies and larvae live and feed on rotten fruits, which are enriched in microbes. To prevent those microbes from entering their body cavity, they have a first line of defense which are physical barriers, like the cuticle exoskeleton or the peritrophic matrix, a physical barrier separating the food pulp from the gut epithelium (Lemaitre and Hoffmann, 2007). When microbes breach these initial barriers and break through the integument, a systemic immune response is mounted which can be separated into two major arms: i) the cellular and ii) the humoral immune response. This division is somewhat arbitrary, as many humoral factors stem from cells, and vice versa cells can be affected by humoral molecules, which leads to a certain overlap (Lavine and Strand, 2002). The following two paragraphs will introduce the main components of both immune arms.

6.1.2 The cellular immune response of D. melanogaster

The blood cells (hemocytes) of *D. melanogaster* reside in the open circulatory system and are the key participants in the cellular immune response. In *D. melanogaster,* there are three subsets of mature hemocytes.

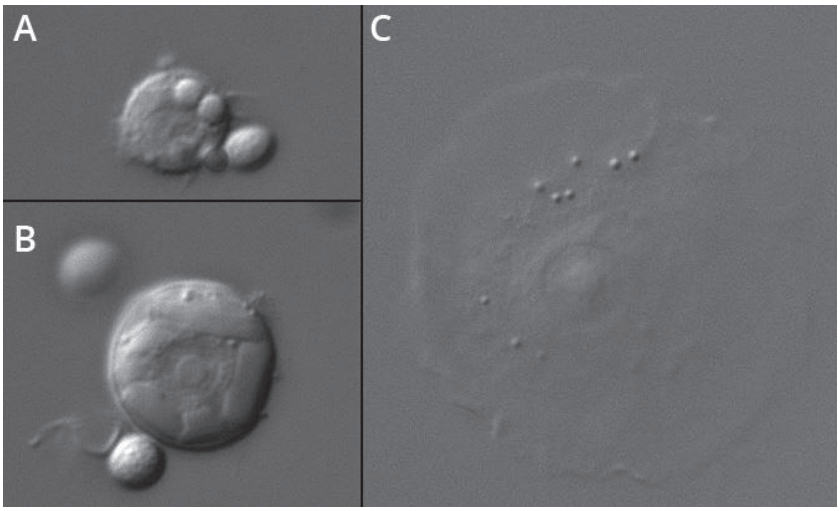

Figure 1: The three hemocyte types of *D. melanogaster*. Plasmatocytes **(A)** are macrophage like cells which participate in e.g. phagocytosis. Crystal cells **(B)** produce and store enzymes involved in the melanization process, which they release after wounding. Lamellocytes **(C)** are large, adhesive cells and play a role in the encapsulation of large foreign objects. Image source: Jan Dudzic.

i) plasmatocytes are macrophage-like cells that are capable of phagocytosis, take part in the recognition of pathogens, and represent ~95% of all hemocytes (Lanot et al., 2001; Rizki, 1957); ii)

crystal cells amount to roughly 5% of total hemocytes in larvae. These fragile cells can rupture upon injury or infection and release enzymes into the hemolymph (blood), which participate in the biosynthesis of melanin (Binggeli et al., 2014; Meister, 2004); iii) lamellocytes are large adhesive cells which play a role in encapsulation, a defense mechanism against foreign objects which are too big to be phagocytosed (Vlisidou and Wood, 2015). Lamellocytes are not present in healthy larvae, but differentiate after injury or infestation with parasites (Rizki and Rizki, 1992). A visual example of the three hemocyte types from *D. melanogaster* is shown in ***Figure 1***.

6.1.2.1 Phagocytosis

Phagocytosis is the process of engulfing entities by individual cells, with their subsequent destruction within phagosomes (Lemaitre and Hoffmann, 2007). In *D. melanogaster* plasmatocytes are the main hemocytes which perform phagocytosis (Elrod-Erickson et al., 2000). The target of phagocytosis must be first recognized by the plasmatocyte. Several receptor proteins, notably members of the Nimrod-receptor family, have been described to be critical for this task (Kocks et al., 2005; Rämet et al., 2001). These receptors can either directly recognize foreign entities via conserved MAMPs, or they rely on secreted proteins, opsonins like members of the TEP family, to fulfill their function (Dostálová et al., 2017; Lagueux et al., 2000). By genetically ablating plasmatocytes in flies it was demonstrated, that phagocytosis contributes to the host defense after infection: flies lacking plasmatocytes exhibit a susceptibility against certain types of infectious agents or microbes, e.g. *Salmonella typhimurium* or *Staphylococcus aureus* (Charroux and Royet, 2009; Defaye et al., 2009).

6.1.2.2 Coagulation

Upon breaching through the integument, insects must rapidly close the wound to prevent the loss of hemolymph. The immune response of coagulation (clotting) is critical for this wound closure, but also to prevent pathogens from systemic dissemination by immobilizing them (Theopold et al., 2014). Several factors which contribute to this process have been identified in *Drosophila*. An important clotting factor is the enzyme transglutaminase, which is released from plasmatocytes after wounding and crosslinks proteins (Lindgren et al., 2008; Wang et al., 2010). Transglutaminase activity results in the cross-linking of aggregated clotting-proteins, and the formation of an initial soft clot. Another plasmatocyte-specific protein is hemolectin, which is a large multidomain

protein contributing to the clot. Several reports demonstrated that hemolectin is required for successful clot formation, a knockdown of hemolectin results e.g. in the lack of the formation of clot fibers or in reduced viscosity of the clot (Goto et al., 2003; Scherfer et al., 2004).

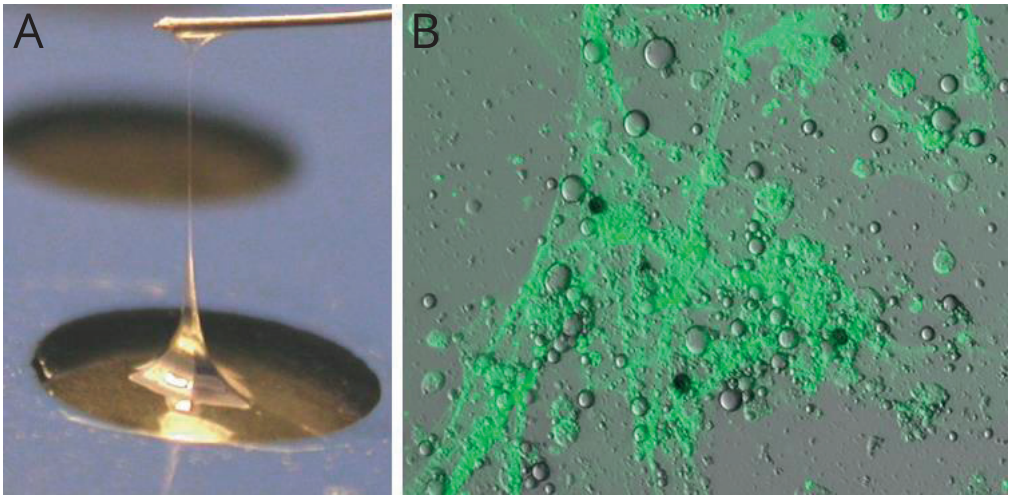

Figure 2: Illustration of the pullout method and the isolated clot. Hemolymph is incubated on a microscope slide until the clot is formed. A needle **(A)** is used to isolate the clot. Micrograph **(B)** of the isolated clot, stained with Peanut agglutinin (PNA-GFP). Visible are the clot fibers in green and melanized crystal cells as black spots. Image source: (A) Scherfer et al., 2004, (B) this work.

An elegant method to identify factors involved in clotting is the pullout method for hemolymph (Scherfer et al., 2004). Harvested hemolymph is incubated on a microscope slide until the formation of a clot is observed, this clot can then be isolated, pulled out, with a needle to investigate it (**Figure 2**). With this method a clotting factor named Fondue was discovered, without which clot fibers are more ductile and longer than in wild-type conditions (Scherfer et al., 2006). Interestingly, phenoloxidases are also found in the clot. These enzymes, which are critical for an immune reaction called melanization, have a crosslinking function that hardens the clot (Karlsson et al., 2004; Scherfer et al., 2004). The observations that *Drosophila* individuals lacking clotting factors are more susceptible to various bacteria, as well as to infection with entomopathogenic nematodes, emphasizes the importance of coagulation as an immune function (Bajzek et al., 2012; Scherfer et al., 2006; Theopold et al., 2014; Wang et al., 2010).

6.1.2.3 Encapsulation

The encapsulation immune reaction is a defense reaction against objects which are too big to be phagocytosed. It will be illustrated here with the example of parasitoid wasps. Parasitoid wasps are insects which lay their eggs inside other insects, where the wasp offspring develops and ultimately consumes the host. *Drosophila* larvae respond to the infestation via the cellular encapsulation reaction (Russo et al., 1996; Salt, 1970). First, the wasp egg will be recognized as foreign, which is then followed by the recruitment and the attachment of plasmatocytes to the egg. Subsequently, an unknown signal then leads to the differentiation of lamellocytes, which are large, adhesive cells. Next, lamellocytes and plasmatocytes adhere to the wasp egg, where they consolidate to form a multi-layered capsule, which physically separates the developing wasp from the hemolymph and therefore limits the access to nutrients (Carton et al., 2008; Keebaugh and Schlenke, 2014). Finally, phenoloxidases produce melanin around the capsule (Binggeli et al., 2014; Salt, 1970). The production of melanin is accompanied by the formation of cytotoxic compounds, which play a role in killing the wasp, while the capsule is thought to protect the host from these toxic compounds (Nappi et al., 2009).

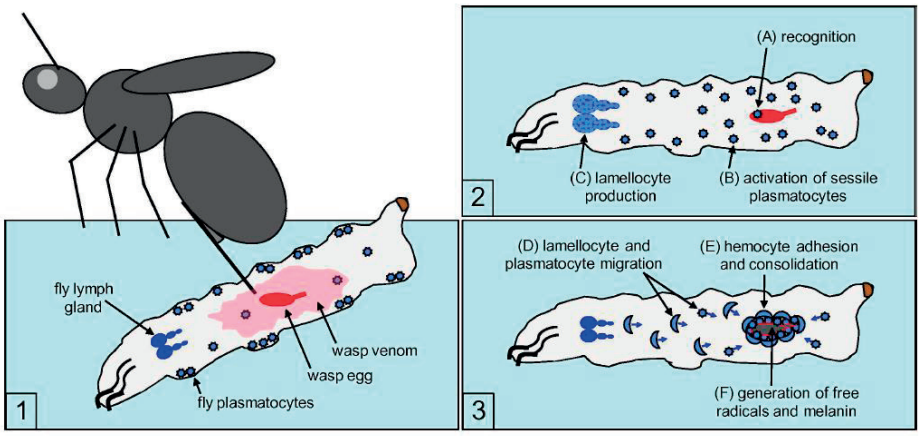

Figure 3: Schematic overview of the interactions between *Drosophila* and endo-parasitoid wasps. The developing wasp egg gets first recognized by the larva as foreign and then the cellular immune response of encapsulation is mounted. Often the wasp simultaneously injects a venom, which can contain various effectors to modulate the larva's immune response. Figure from Keebaugh and Schlenke, 2014.

There are two potential outcomes of a wasp infestation. The host can either i) effectively encapsulate and kill the wasp, or the wasp ii) will counter the defense mechanisms and slowly

consume the host from within. This relationship led to an arms-race to evolve defense mechanisms from the host side, or counter-measures from the wasp side. An example for the latter is the simultaneous injection of wasp venom during the egg laying, which can contain various virulence factors to e.g. interfere with hemocytes or suppress melanization (Asgari et al., 2003; Labrosse et al., 2005; Mortimer et al., 2013). An overview of the interactions between *Drosophila* larvae and endo- parasitoid wasps is shown in **Figure 3**.

6.1.2.4 The melanization reaction

The melanization reaction is an arthropod specific immune response which involves the biosynthesis of melanin via specialized enzymes called phenoloxidases (PO) (Lemaitre and Hoffmann, 2007). The attribution of the melanization reaction towards the cellular or the humoral immune response can be debated. While the source of the enzymes responsible for the melanization reaction are hemocytes (crystal cells), those enzymes are released into the hemolymph to fulfill their function (Bidla et al., 2007; Binggeli et al., 2014). As the topic of this thesis, the melanization reaction will be addressed in a separate chapter in detail.

6.1.3 The humoral immune response of D. melanogaster

Humoral immunity (or systemic immunity) is an umbrella term for immune defenses that are found in extracellular fluids, which addresses mainly the hemolymph in arthropods. Humoral defenses include the production and secretion of antimicrobial peptides, the production of reactive oxygen or nitrogen intermediates, as well as the enzymatic cascades which regulate e.g. the production of melanin or the activation of the clotting factors (Gillespie and et al., 1997; Meister et al., 2000; Muta and Iwanaga, 1996; Vass and Nappi, 2001). The following paragraphs will introduce the role of antimicrobial peptides in immunity, and their regulation via the Toll and IMD pathways.

6.1.3.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small peptides which are induced to extremely high levels upon infection in *Drosophila* (Lemaitre and Hoffmann, 2007). The first insect AMP was discovered in and isolated from the cecropia moth almost four decades ago, and was named cecropin (Steiner et al.,

1981). Up to now more than 1200 peptides with antimicrobial characteristics have been isolated throughout all living organisms (Nakatsuji and Gallo, 2012). Taken the sheer number together with the fact that AMPs are already found in simple multicellular organisms like Cnidaria and even microbes themselves, illustrates that AMPs are an ancient and very important immune mechanism (Bosch, 2013; Mygind et al., 2005). In *Drosophila* AMPs are mainly produced by the fat body (an organ similar to the mammalian liver) and they are subsequently secreted into the hemolymph. Additionally, AMPs are also produced by epithelial surfaces like trachea or the gut (Lemaitre and Hoffmann, 2007). AMPs are typically cationic due to the presence of arginine and lysine residues, and in many cases their three-dimensional structure results in one hydrophobic and one hydrophilic side (Tossi et al., 2000). While their exact mode of function is not fully understood in many cases, it is suggested that they can interact with negatively charged microbial membranes due to their amphipathic features. Defensins, for example, can form pores in microbial membranes and are likely to interact with negatively charged molecules like DNA or RNA inside the pathogen (Wimley et al., 1994). Next to their critical role in immunity, insect AMPs have recently become the target of applied science for their potential use in medical and agrochemical applications (Tonk and Vilcinskas, 2017; Yi et al., 2014). *Drosophila melanogaster* has 14 identified AMP genes which are classified in 7 families: Drosomycin, Metchnikowin, Diptericin, Drosocin, Defensin, Cecropin and Attacin. While Attacins and Diptericins show activity against Gram-negative bacteria, Defensins show antibacterial activity primarily against Gram-positive bacteria. In contrast, Drosomycins and Metchnikowins show antifungal activity, and Cecropins are active against both fungi and bacteria (Imler and Bulet, 2005). In *Drosophila*, AMPs are massively up-regulated at the transcriptional level upon infection. This induction is regulated by two NF-κB signaling pathways, the Imd and the Toll pathway. Both pathways can be activated by the recognition of MAMPs. The PRRs, PGRP-LC and PGRP-LE, which activate the Imd pathway, can recognize DAP-type peptidoglycan, a cell wall component exclusive for Gram-negative bacteria and some Gram-positive bacteria like bacilli (Kaneko et al., 2004).

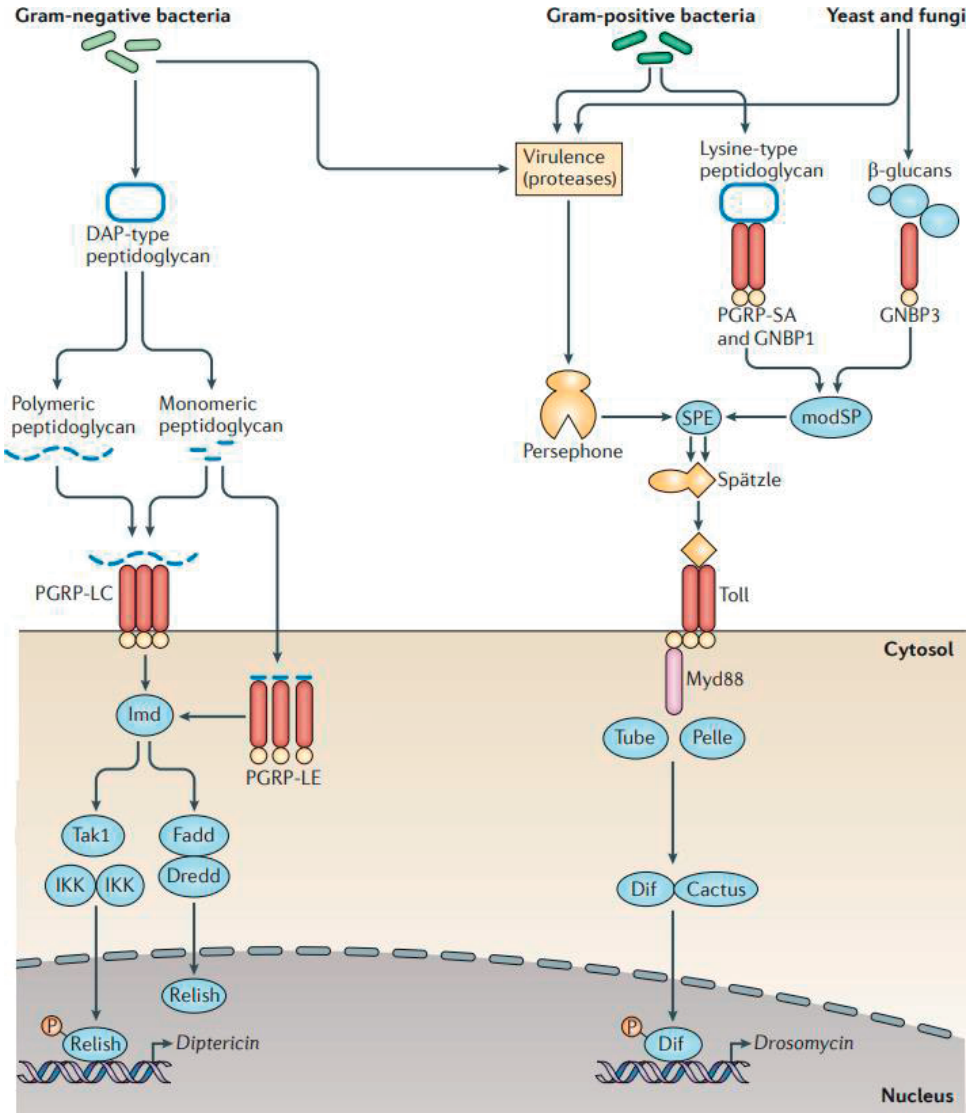

Figure 4: Activation of the Imd and the Toll pathways in *D. melanogaster*. Microbe associated molecular patterns, distinct for Gram-negative-, Gram-positive bacteria or fungi, are detected by pattern recognition receptors specific for either the Imd or Toll pathway. The detection of DAP-type peptidoglycan by PGRP-LC or -LE leads to the activation of intracellular factors of the Imd pathway. The recognition of Lys-type peptidoglycan or β-glucans by PGRP-SA/GNBP1 resp. GNBP3, initiates an extracellular proteolytic cascade which leads to the cleavage of the cytokine Spätzle, which then binds to the membrane receptor Toll to activate intracellular factors. Toll can be additionally activated through a separate "danger" associated arm, where Persephone can sense irregular protease activity from microbes. Both Imd and Toll activation leads to a transcriptional response, resulting in e.g. the expression of antimicrobial peptide genes. Adapted from (Buchon et al., 2014).

In contrast, the PRRs, PGRP-SA and GNBP3, which trigger the Toll pathway can detect Gram-positive specific Lys-type peptidoglycan or the fungal cell wall component β-(1,3)-glucan (Gottar

et al., 2006; Leulier et al., 2003). This leads to a specific transcriptional response depending on what pathogen group is detected (see **Figure 4**)**.** The function of both pathways will be addressed in detail in the following chapters, with an emphasis on the Toll pathway due to its implication in this thesis.

6.1.3.2 The Imd pathway

The Imd pathway is named by the *immune deficiency* (*imd*) mutation, which controls the expression of several AMPs mainly induced by DAP-type PGN from Gram-negative bacteria (Lemaitre et al., 1995a). The activation of the Imd pathway relies on the detection of peptidoglycan via peptidoglycan recognition proteins (PGRPs) (Leulier et al., 2003). The main transmembrane receptor which leads to the activation of the Imd pathway in the systemic immune response is PGRP-LC (Choe et al., 2002; Gottar et al., 2002). PGRP-LC can recognize both monomeric and polymeric DAP-type peptidoglycan depending on its isoforms (Kaneko et al., 2004; Neyen et al., 2016). The function of PGRP-LC is assisted by another PGRP, the secreted PGRP-SD, which enhances the localization of peptidoglycan to the cell surface, and promotes Imd signaling (Iatsenko et al., 2016). A third sensor for peptidoglycan is PGRP-LE, which can detect monomeric peptidoglycan intracellularly and acts mainly in the gut (Bosco-Drayon et al., 2012; Kaneko et al., 2006; Takehana et al., 2004). Flies mutant for both PGRP-LC and -LE show no induction of Imd specific AMPs, indicating that they are the major receptors for the immune response against Gram-negative bacteria mediating the Imd pathway (Kaneko et al., 2006). The Imd pathway is also negatively regulated by a battery of factors which prevent its overactivation. Enzymatically active PGRPs, such as PGRP-LB, can degrade peptidoglycan into small fragments which do not elicit the Imd pathway anymore. Those PGRPs, named amidase PGRPs, downregulate the Imd pathway extracellularly via the scavenging of PGN and therefore reducing PRR activation (Paredes et al., 2011; Zaidman-Rémy et al., 2006). Additional to this extracellular regulation, there is also the possibility to regulate the Imd pathway intracellularly. The activation of Imd leads to the expression of Pirk, which negatively regulates the PRR PGRP-LC at its intracellular tail, providing a negative feedback loop of Imd (Kleino et al., 2008). This negative regulation is important to adjust the immune reaction according to the severity of infection, or shut down Imd activity after an infection is cleared.

6.1.3.3 The Toll pathway

The Toll pathway is named by the transmembrane receptor Toll, first identified in *Drosophila* for its role in dorsal-ventral polarity establishment during development (Anderson et al., 1985). Subsequently, Toll was identified to play a role in immunity, initially with the observation that a gain of function allele produces melanotic bodies and therefore affects melanization in flies (Gerttula et al., 1988). Afterwards, it was discovered that Toll plays a crucial role in the defense against fungi (Lemaitre et al., 1996) and Gram-positive bacteria (Leulier et al., 2000; Rutschmann et al., 2000). The *Drosophila* Toll pathway is activated by extracellular PRRs, that initiate an extracellular cascade of serine proteases (SPs) which finally cleave the cytokine Spätzle (Spz). The mature form of Spz can then bind to Toll to activate the intracellular pathway (Morisato and Anderson, 1994; Schneider et al., 1994; Weber et al., 2003). The intracellular cascade culminates in the nuclear translocation of the NF-κB transcription factors Dif and Dorsal, which leads to the induction of a variety of immune genes, e.g. AMPs (Belvin and Anderson, 1996; Lemaitre and Hoffmann, 2007).

Three circulating PRRs are known to be involved in the extracellular activation of Toll in immunity: i) PGRP-SA can mainly bind to Lys-type PGN from Gram-positive bacteria, and to a lesser extent to DAP-type PGN (Leulier et al., 2003; Michel et al., 2001). ii) GNBP-1 can promote the function of PGRP-SA, serving as an adapter and forming a complex with the latter (Buchon et al., 2009; Gobert, 2003). iii) GNBP3 is a PRR for fungal ß-1,3-glucans (Gottar et al., 2006). In addition to the PRR dependent activation of Toll, there is a PRR independent pathway that is activated by the SP Persephone (Psh). Psh can activate the extracellular Toll cascade without the use of PRRs. Aberrant protease activity, e.g. due to secreted microbial proteases, is detected via the proteolysis of the Psh bait region, a special protein domain prone to protease cleavage (Issa et al., 2018). The activation of Psh leads to the activation of extracellular Toll SPs and therefore the activation of Toll (Chamy et al., 2008; Ligoxygakis et al., 2002).

After the initial activation of either the PRR- or the Psh-pathway, complex cascades of SPs are triggered. The PRR complex PGRP-SA/GNBP can recruit the Modular Serine Protease (ModSP), which then activates the downstream SP Grass (Gram-positive-specific serine protease), which itself activates the Spätzle-processing enzyme SPE, which finally cleaves Spätzle to its mature form

(Buchon et al., 2009; Gobert, 2003; Gottar et al., 2006; Jang et al., 2006). Psh can directly activate SPE without the need of ModSP or Grass (Chamy et al., 2008). The extracellular Toll pathway can also be negatively regulated at the level of the SP cascade, via serine protease inhibitors (serpins, spn) (Reichhart et al., 2011). The functional mechanism of SPs and serpins will be addressed in more detail in 6.2.4.

6.2 THE MELANIZATION REACTION OF *D. MELANOGASTER*

An important and rapid immune response of arthropods is the melanization reaction. It results in the production of melanin (melanogenesis) via enzymes called phenoloxidases (PO). PO activity results in the deposition of black melanin e.g. around wound sites. The melanization reaction participates in several immune functions like wound healing, the encapsulation reaction and the defense against systemic infection by Gram-positive bacteria and fungi (Binggeli et al., 2014; Cerenius et al., 2008; Tang, 2009). While the source of POs are crystal cells, the melanization reaction itself takes place in the hemolymph. The final attribution of the melanization reaction to either the cellular or the humoral immune response can therefore be debated. Examples of the melanization reaction in *D. melanogaster* are illustrated in **Figure 5**.

POs catalyze the oxidation of phenols to quinones, which then subsequently polymerize to melanin. Melanogenesis also causes the production of reactive oxygen species and other cytotoxic intermediates, which are thought to play a role in the microbicidal mechanism, although the defined mechanisms taking part in the killing of microbes are unknown (Nappi et al., 2009). Due to the unspecific nature of the cytotoxic intermediates, and therefore the potential danger of the host to damage itself, the melanization reaction must be tightly regulated. One regulatory mechanism is the fact that POs are produced in an inactive zymogen form called prophenoloxidases (PPOs), which need to be activated by SPs via proteolytic cleavage (Cerenius and Soderhall, 2004; Söderhäll and Cerenius, 1998). Another mechanism for regulating POs is the inhibition of the activating SPs via serpins (Silverman et al., 2001). The aspects of the melanization reaction will be introduced in detail in the following paragraphs.

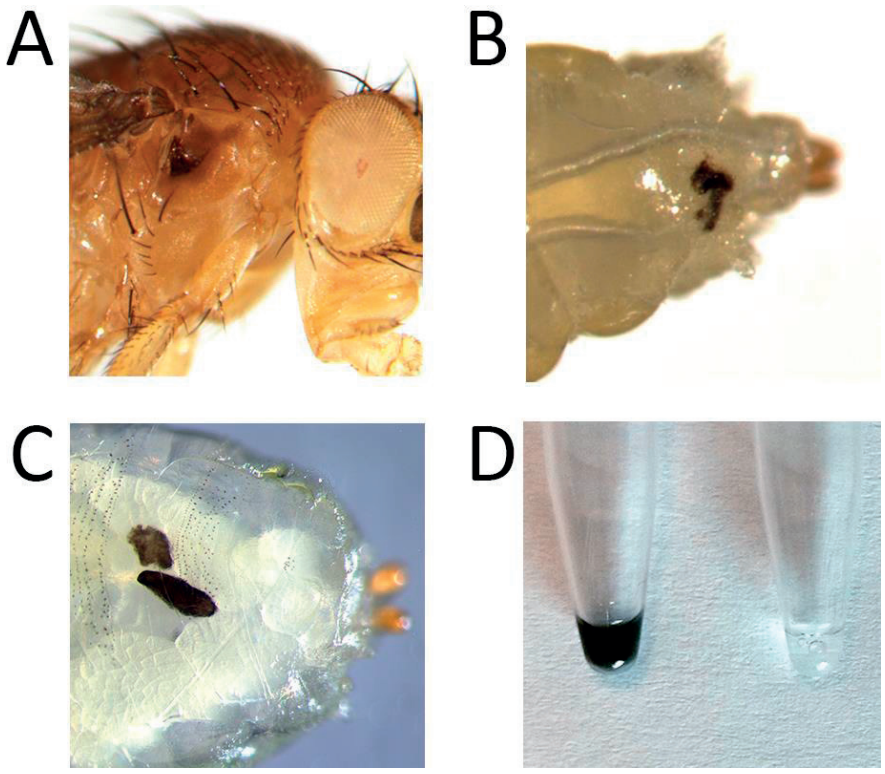

Figure 5: Examples of the melanization reaction in *D. melanogaster*. Deposition of melanin around the wound area after injury with a needle in adults **(A)** or larvae **(B)**. Melanin deposition around an encapsulated egg of the parasitoid wasp *Leptopilina boulardi* inside a *D. melanogaster* larva **(C).** Melanin production in larval plasma **(D)** is found in wild-type (left) but not in phenoloxidase mutant animals (right). (A) and (B) adapted from Dudzic et al., 2018, (C) and (D) this work.

6.2.1 Biochemistry of melanin and melanin synthesis

Melanins (melanos = Greek for dark) are a group of light-absorbing copolymers containing indoles, quinones and other intermediates originating from the oxidation of tyrosine. Melanins are found in plants, animals and protists. The most common melanin is eumelanin, which produces a dark brown or black color and is formed by dihydroxyindoles. Also common is pheomelanin, which produces a more reddish color and is formed from benzothiazine polymers (Napolitano et al., 2000). Melanins have a plethora of biological functions, ranging from photon shielding (e.g. protection from ultraviolet light), structural properties (e.g. increased rigidity of insect cuticles), antibiotic effects (e.g. cytotoxic byproducts of insect melanogenesis) to chemoprotection (e.g. acting as a free radical sink) (Riley, 1997). Melanogenesis in animals is performed by the enzymatic oxidation of the amino acid tyrosine to either dopaquinone or to dopamine-quinone via POs.

Dopaquinone can then spontaneously cyclisize into dopachrome and is then decarboxylated by the dopachrome conversion enzyme to form 5,6-Dihydroxyindole (DHI). Alternatively, there is a second pathway to produce DHI from Dopamine (Beerntsen et al., 2000). DHI can again be oxidized by POs to form Indole-5,6-quinone which finally polymerizes into melanin (Cerenius and Soderhall, 2004; González-Santoyo and Córdoba-Aguilar, 2012; Riley, 1997; Whitten and Coates, 2017). While quinones can polymerize to form melanin, the same process can also lead to the production of spontaneously formed semiquinones. Semiquinones are highly unstable and may produce free radicals that have cytotoxic properties (Cerenius and Soderhall, 2004; Zhao et al., 2011). An overview of the synthesis pathways is given in **Figure 6**.

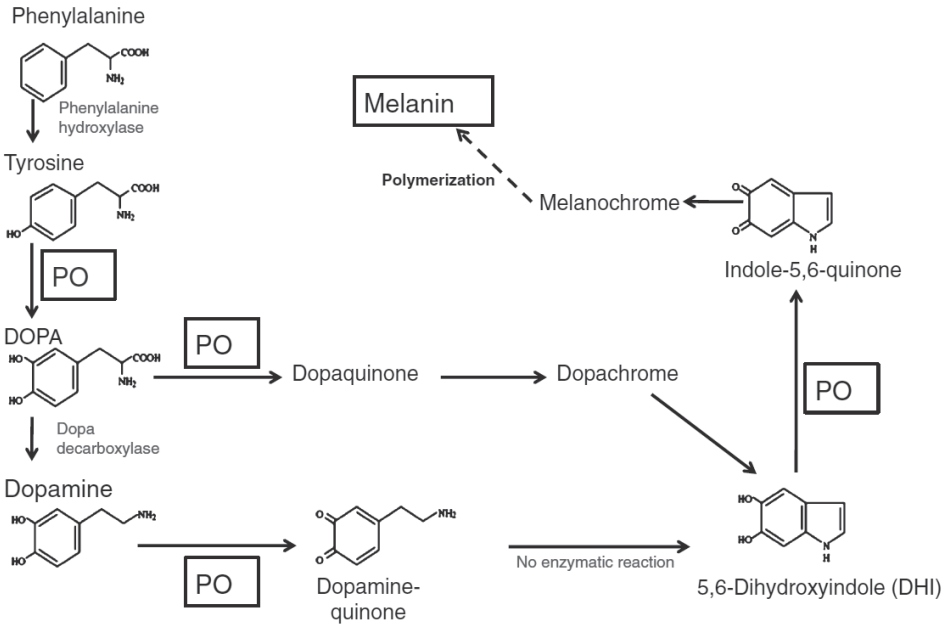

Figure 6: Schematic view of insect melanogenesis via phenoloxidase (PO). Tyrosine can be transformed into DOPA by PO. DOPA or Dopamine can be further transformed into Dopa- resp. Dopamine-quinone through oxidation via PO. Both synthesis pathways result in the formation of 5,6-Dihydroxyindole (DHI). The oxidation of DHI by PO leads to the formation of Indole-5,6-quinone, which finally polymerizes into melanin (González-Santoyo and Córdoba-Aguilar, 2012; Whitten and Coates, 2017). Figure source: González-Santoyo and Córdoba-Aguilar, 2012.

6.2.2 Properties and function of insect phenoloxidases

The main role of PO in the production of melanin is the conversion of phenols to quinones via oxidation. Insect POs are type-III copper metalloenzymes. They contain a pair of copper centers

(CuA and CuB), each coordinated by three histidine residues, to enable the binding of oxygen to the enzyme (Solomon et al., 2014). In general, insect POs have a molecular weight of ~70-80 kDa and consist of three domains: i) an *N*-terminal domain, which is preceded by a pro-region in the zymogenic PPO form, ii) an α-helical domain containing the copper centers and iii) a *C*-terminal domain (Kanteev et al., 2015). The PO substrate tyrosine enters the dicopper center and is deprotonated into a phenolate intermediate. This phenolate intermediate is further directed to the CuA region where it reacts with a peroxide to form a catecholate. The catecholate is then finally oxidized and released as a quinone (Kanteev et al., 2015; Whitten and Coates, 2017).

Insect POs are produced in an inactive PPO form. To become enzymatically active, they are cleaved at the position of a conserved Arg-Val or Arg-Phe bond in the pro-region by a protease, usually around 50 residues from the *N*-terminus of the protein (Chen et al., 2012). Prior to the activation, the active site of PPOs is blocked by a residue with usually aromatic or aliphatic properties. This residue is displaced after the activation, allowing the substrate to enter the dicopper center (Cong et al., 2009; Whitten and Coates, 2017).

6.2.3 PPOs in D. melanogaster

The genome of *D. melanogaster* contains three *PPO* genes. Two of them, PPO1 and PPO2, are produced in a specialized hemocyte type called crystal cells (see **Figure 7**). Crystal cells are fragile hemocytes which rupture after immune stimulation to release PPO (Bidla et al., 2007). Neither PPO1 nor PPO2 was detected in the hemolymph of *Drosophila* larvae devoid of crystal cells, indicating them as the sole source for PPOs (Binggeli et al., 2014). The site of expression of the third *Drosophila* PPO, PPO3, is debated. While some reports indicate that PPO3 is also produced in crystal cells (Ferjoux et al., 2007; Waltzer et al., 2003), other reports showed evidence that PPO3 stems from lamellocytes, a large adhesive cell type only present after parasitoid wasp infestation (Irving et al., 2005; Nam et al., 2008).

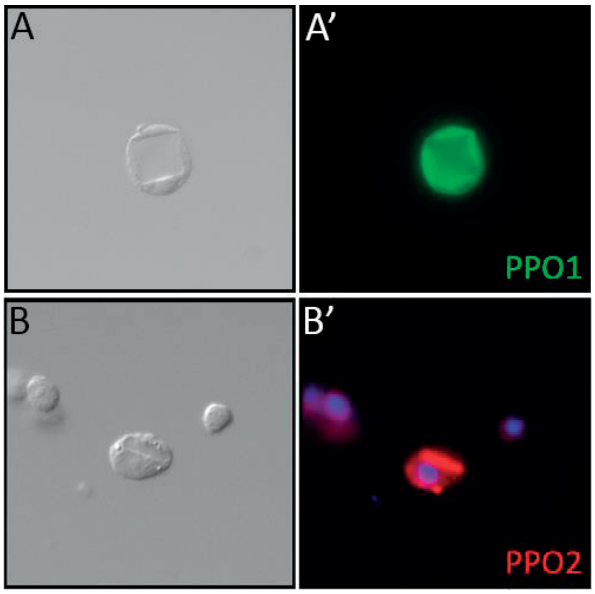

Figure 7: *D. melanogaster* crystal cells contain PPO1 in the cytoplasm and PPO2 in crystalline structures. **(A)** Brightfield micrograph of a crystal cell. **(A')** The use of a PPO1-GFP reporter transgene (Sarov et al., 2016) reveals PPO1 correlated GFP signal mainly in the cytoplasm, but hardly at the crystalline structure. **(B)** Brightfield micrograph of a crystal cell (middle) and surrounding plasmatocytes. **(B')** Immunostaining with a monoclonal antibody against PPO2 (Willott et al., 1994) shows a strong red fluorescence signal at the crystalline structure but not in the cytoplasm or plasmatocytes. This led to the conclusion that both PPO1 and PPO2 are produced by crystal cells, where PPO1 is most likely secreted into the hemolymph, while PPO2 is stored in the crystals for later use. Immunostaining was performed as described in Binggeli et al., 2014.

The importance of the melanization reaction as an immune mechanism was clearly demonstrated in other insects, but it was only recently proven that it also plays a critical role in *D. melanogaster* (Ayres and Schneider, 2008; Binggeli et al., 2014; Leclerc et al., 2006; Tang et al., 2006). Flies deficient for melanization exhibit an increased susceptibility to systemic infections with some Gram-positive bacteria and fungi. It was also demonstrated that PPO1 is involved in the early production of melanin in the hemolymph, while PPO2 is stored in the crystals of crystal cells and deployed over time (Binggeli et al., 2014). For both PPO1 and PPO2 it was validated that they need proteolytic cleavage to become active. In contrast, there is evidence that PPO3 is produced in an already active form: the overexpression of PPO3 *in vitro* or *vivo* readily leads to the production of melanin, while this is not the case for PPO1 and PPO2 (Chen et al., 2012; Nam et al., 2008).

6.2.4 Serine proteases as activators of immune functions

SPs are named after the catalytic serine residue in their active site, which is responsible for the nucleophilic attack of a susceptible peptide bond (Hedstrom, 2002). They belong to the group of chymotrypsin-like proteases (S1 family) (Rawlings et al., 2012). SPs play critical roles in many biological processes e.g. in blood coagulation, digestion, embryonic development and immunity (Veillard et al., 2015). Typically, SPs are produced as zymogens which need proteolysis by another protease to get activated. The sequential activation of one SP through another SP can lead to complex cascade pathways. These cascade pathways evolved in animals to trigger fast responses against e.g. tissue damage or microbes (Krem and Di Cera, 2002). Proteolytic cascade pathways also provide a mechanism for the amplification of an initially small signal, and the possibility of regulation at several levels (Kanost and Jiang, 2015). Prior or during the activation of such a cascade, the *N*-terminal pro-regions of SPs play important roles via protein-protein interactions. These interactions allow the fine tuning of the proteolytic activity or can guide the SPs to their proper locations (Veillard et al., 2015). Genomes of insects encode for a plethora of SPs and their inactive homologs (SPHs), ranging from ~50 to 300 genes (Christophides et al., 2002; Ross et al., 2003; Zhao et al., 2010). Most of them have been identified through prediction algorithms but only a few have been characterized in detail. The *Drosophila melanogaster* genome contains 147 SPs and 57 SPHs currently identified, indicating the importance and the potential complexity of their cascades (Ross et al., 2003). An important and arthropod-exclusive regulatory pro-region of SPs is the clip domain. The clip domain is named after an exposed protein domain, structurally resembling a paper clip and first identified in the pro-clotting enzyme of the horseshoe crab (Kanost and Jiang, 2015; Piao et al., 2005; Smith and Delotto, 1992). Since then, clip domain SPs (clip-SPs) have been implicated in many processes in insects, especially immune related functions like hemolymph coagulation, the induction of AMPS via the activation of the Toll pathway and the activation of the melanization reaction (An et al., 2009; Kan et al., 2008; Muta and Iwanaga, 1996; Tang et al., 2006). Additionally, clip-SPs are also involved in other biological processes, like the dorso-ventral patterning of *Drosophila* during embryonic development (Krem and Di Cera, 2002). The *D. melanogaster* genome encodes for 28 clip-SPs and 14 inactive homologs (Kanost and Jiang, 2015; Veillard et al., 2015).

The clip domain is usually connected *N*-terminally to the active domain of the SP via a 23-92 residues-long linker. Clip domains are around 35-55 residues long and harbor a conserved disulfide bond pattern (Pfam PF12032). Additionally, at least one of the cysteines in the clip domain forms a disulfide bond with a cysteine in the catalytic domain after the cleavage. This leads to the covalent attachment of the cleaved clip domain to the active SP after clip-SP activation (Kanost and Jiang, 2015; Veillard et al., 2015). After this activation via the cleavage of the *N*-terminal clip-domain, a commonly conserved consensus sequence (I/V-V-G-G) in the SP active center is liberated and leads to a conformational change, resulting in the functional active catalytic domain (Hedstrom, 2002).

Additional to the catalytically active clip-SPs, there is also a group of pseudoproteases (clip-SPHs) which also are involved in immunological processes. Those SPHs have a protease-like domain which lacks the catalytic triad required for proteolysis (Kanost and Jiang, 2015). Like clip-SPs, clip-SPHs also require cleavage to become functional. They can then for example act as co-factors which increase the efficiency of PPO activation in *Manduca sexta* or *Tenebrio molitor* (Gupta et al., 2005; Kan et al., 2008).

6.2.4.1 Serine proteases regulating PPOs in *Drosophila*

The clip-SPs that directly cleave the PPOs are also called PPO-activating enzymes or -factors (PPAEs, PPAFs). In *D. melanogaster* three PPAFs have been identified to regulate the melanization reaction in the hemolymph: MP1, Sp7 (formerly MP2) and Hayan (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Nam et al., 2012; Tang et al., 2006). The function of Sp7 was first investigated via *RNAi* experiments and a hypomorph mutant. Sp7 was found to be implicated in the activation of POs because of a reduced amount of melanin deposited at wound sites in *Sp7* deficient *Drosophila* flies (Castillejo-López and Häcker, 2005). Later, MP1 was identified through an *RNAi* screen for the suppression of a melanization phenotype. The loss of Spn27A results in the constitutive activation of melanization, which is prevented by the simultaneous knock-down of MP1 (Tang et al., 2006). The silencing of *MP1* resulted in a similar phenotype also observed after silencing *Sp7*, the reduced deposition of melanin around wound sites after injury. Additionally, Tang and colleagues demonstrated that the ubiquitous overexpression of either *MP1* or *Sp7* resulted in the formation of melanotic bodies in larvae and adults in the absence of wounding (Tang et al., 2006). The knockdown of *Sp7* also resulted in a mild susceptibility against the

entomopathogenic fungus *Beauvaria bassiana*, but not against the bacteria *Erwinia carotovora* (Gram-negative), *Enterococcus faecalis* (Gram-positive) or the yeast *Candida albicans*.

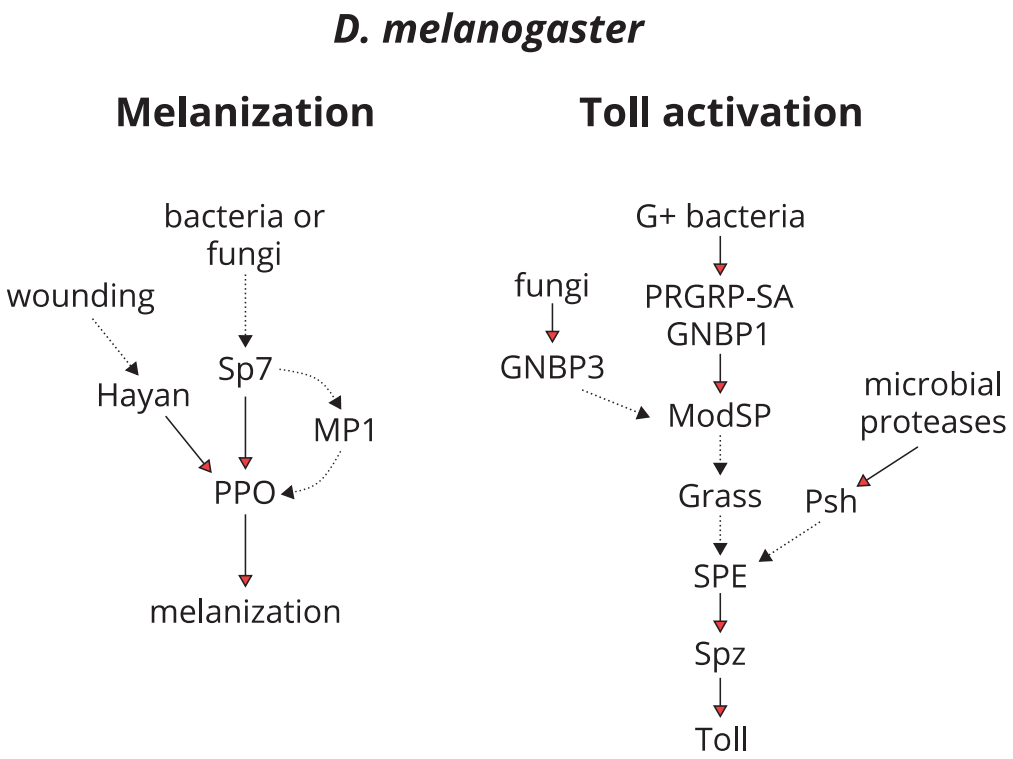

Figure 8: Schematic overview of the clip-SP cascades activating melanization or the Toll pathway in *D. melanogaster*. Two separate clip-SP cascades are proposed to independently activate either the melanization reaction (**left**), or the extracellular Toll pathway (**right**) after immune challenge. A detailed description is given in the text, figure adapted from (Kanost and Jiang, 2015). Dashed arrows represent putative steps with genetic experimental evidence, red arrowheads indicate direct experimental evidence.

No impact on the survival against those pathogens was found during the knockdown of *MP1*. Tang and colleagues also suggested that Sp7 might act upstream of MP1, as the formation of melanotic bodies via the overexpression of Sp7 was blocked by the simultaneous silencing of *MP1*, but not vice versa. With the use of recombinant protein expression, it was later biochemically proven that Sp7 can directly cleave PPO1 in *D. melanogaster* (An et al., 2013). Only recently, a third clip-SP involved in the *D. melanogaster* melanization reaction named Hayan was discovered. Hayan was identified during an RNAi-screen for clip-SPs exhibiting an impaired survival against wounding with a needle (Nam et al., 2012). Nam and colleagues demonstrated that the knockdown of *Hayan*, but not *Sp7* or *MP1*, leads to a strong susceptibility of flies against wounding. The use of a *Hayan* null mutation in this study, subsequently lead to the discovery that *Hayan* mutants fail to melanize the

wound area after the perforation of the cuticle with a needle. Also, *Hayan* mutant flies do not activate PPO1 *in vivo*, and no cleavage of PPO1 is observed in *in vitro* experiments with recombinant Hayan. Additionally, the overexpression of Hayan led to the formation of melanotic bodies, similar to the overexpression of MP1 and Sp7 noted above. Interestingly, Nam and colleagues also unveiled a neuro-protective role of Hayan. They show that after wounding flies, ROS are produced by the Hayan- and PO-dependent melanization pathway. Those ROS then diffuse to neuronal cells and act as a second messenger system to induce a JNK dependent cytoprotective program in neuronal tissues, which contributes to improved fly survival after wounding (Nam et al., 2012).

There are several reports that the activation of the PPAFs might be regulated by components of the extracellular Toll pathway. Matskevich and colleagues demonstrated that the PRRs for fungi and microbial proteases, GNBP3 and Psh respectively, are involved in the melanization reaction (Matskevich et al., 2010). First, they discovered that hemolymph of *GNBP3* mutant larvae fails to melanize spores of the entomopathogenic fungi *B. bassiana*. They further report the presence of multi-protein complexes in the hemolymph, containing GNBP3 + PPO and present evidence of the direct protein-protein interactions of both. Finally, Matskevich et al. determined that flies double-mutant for GNBP3 and Psh show no PO activity after fungal injection, while single mutants are not or only weakly affected. Additionally, a reduced cleavage of PPO1 was shown for mutants of the PRRs for Gram-positive bacteria, PGRP-SA and GNBP1, indicating that they play a role in activating PPOs. Interestingly, no defect of PPO cleavage was observed in flies mutant for Spz, indicating that only extracellular components of Toll upstream of Spz are involved in the activation of the melanization reaction (Matskevich et al., 2010). Toll is also involved in the transcriptional regulation of the melanization reaction, as several clip-SPs, -SPHs and serpins are upregulated after microbial infection in a Toll dependent manner (De Gregorio, 2002).

The painted picture of the regulation of melanization through MP1, Sp7 and Hayan as well as the Toll PRRs through those publications is however not complete. While there is direct evidence that both Sp7 and Hayan can activate PPO1 (An et al., 2013; Nam et al., 2012), there is currently no data available if they can also act on PPO2 or PPO3, and whether MP1 has any direct interactions with one of the *Drosophila* PPOs. It is also unclear if these three potential PPAFs activate the melanization reaction in a different manner, and if not, why there is this level of redundancy. Also,

there is no final evidence on how the Toll PRRs act on the PPAFs. An overview of the current model of the melanization pathway in *D. melanogaster* is depicted on the left side in **Figure 8**.

6.2.4.2 Serine proteases regulating Toll in Drosophila

The activation of the Toll pathway in the fat body after exposure to Gram-positive bacteria results in the induction of AMPs (see section 6.1.3.3). The following paragraph will address the role of SPs in the regulation of Toll activation during the immune response of *D. melanogaster*. To date, four SPs have been identified to play a role in Toll activation. In the PRR branch, those are ModSP, Grass and SPE (Buchon et al., 2009; Gobert, 2003; Gottar et al., 2006; Jang et al., 2006). Additionally, Psh can sense aberrant hemolymph protease activity after infection with microbes, which often secrete proteases as virulence factors (Chamy et al., 2008; Issa et al., 2018). ModSP was identified via homology to a modular SP in the beetle *Tenebrio molitor* (*Tm*-MSP), which is activated after the binding of PGN to the PGRPG-SA/GNBP1 PRR complex (Buchon et al., 2009; Kim et al., 2008). Flies mutant for ModSP fail to activate the Toll pathway after infection with Gram-positive bacteria or yeast, but not after injection of bacterial proteases, indicating that its role is strictly related to the PRR branch of Toll. Via epistasis experiments it was validated that ModSP acts downstream of PGRP-SA but upstream of Grass. Interestingly, ModSP does not seem to need cleavage from an upstream protease but is autoactivated after over-expression *in vitro* and *in vivo*, which points to a different mode of ModSP activity regulation. Buchon and colleagues speculate that an interaction with PRRs, like GNBP1 and GNBP3, might regulate ModSP activity *in vivo*. The close proximity of ModSP to the PRRs will auto-activate ModSP (Buchon et al., 2009). Grass was identified during a large-scale RNAi screen for SPs preventing Toll activity (Kambris et al., 2006). Interestingly, Grass cannot be cleaved by ModSP in *in vitro* experiments with recombinant proteins, suggesting the presence of another SP or a co-factor in between (Buchon et al., 2009). SPE, which terminally activates Spz, was identified through homology to Easter, a clip-SP cleaving Spz during development, and SPE mutants were shown to prevent Toll activation after immune challenge (Chasan and Anderson, 1989; Jang et al., 2006; Mulinari et al., 2006). Current models propose that Grass directly acts on SPE, although no direct evidence has yet been presented. The presence of one or more SPs in between can therefore not be excluded yet.

The second SP pathway to activate Toll is called the Persephone pathway. This activation is independent of the PRRs GNBP3, GNBP1, PGRP-SA, as well as the SPs ModSP and Grass (Buchon et al., 2009; Chamy et al., 2008; Matskevich et al., 2010). Psh was initially discovered by screening for suppressors of the necrotic-phenotype. The loss of the serpin Necrotic results in the constitutive activation of Toll in the absence of infection, and was used in screens to identify new components which play a role in Toll activation (Levashina et al., 1999; Ligoxygakis et al., 2002). A mutation in Psh suppressed both the Toll activation and the ectopic melanization caused by *necrotic*, demonstrating that Psh is likely a target of Necrotic. Subsequently, it was illustrated that Psh is not functioning downstream of the PRRs responsible for fungal or Gram-positive bacteria recognition, but rather activated by other mechanisms (Gottar et al., 2006). Finally, Psh was demonstrated to be sensitive to bacterial and fungal proteolytic activities in the hemolymph, and was described as a separate entry point into the extracellular Toll cascade at the position upstream of SPE (Chamy et al., 2008). Verification came when Psh was shown to respond to microbial proteases. A recent study identified a protease bait region in the *N*-terminal region of Psh, which is susceptible to a variety of microbial proteases and cleavage results in the activation of Psh and therefore SPE (Issa et al., 2018). An overview of the current model of the extracellular SPs involved in the activation of the immune Toll pathway in *D. melanogaster* is given on the right side of **Figure 8**.

In addition to the four SPs presented above, a role for three more SPs in the activation of Toll was described. The same study that used an RNAi screen to identify Grass also proposed a role for the clip-SP Spirit and the clip-SPHs Sphinx and Spheroide in the activation of SPE (Kambris et al., 2006). Also, the presence of a second Spz cleaving SP next to SPE was proposed, after the observation that SPE mutant *D. melanogaster* can activate Toll after *B. subtilis* infection (Yamamoto-Hino and Goto, 2016). These findings await confirmation and proper placement into the cascade.

6.2.5 Negative regulation of the melanization reaction

Due to the formation of potentially cytotoxic by-products during melanogenesis, the activity of POs must be tightly regulated to prevent damage to the host itself. Additionally, the nature of SP cascades leads to an amplification of an initially small signal, this amplification step also has to be controlled to e.g. prevent an over activation of the immune system. One mode of regulation is the inhibition of SPs through serpins. Serpins are typically ~400 amino acids long and expose a

reactive-center loop (RCL) C-terminally, which acts as a bait for an SP. When the RCL gets cleaved by the target SP, both the SP and the serpin get connected via a resulting covalent ester linkage (Ellisdon et al., 2014). Because of this irreversible bond, serpins are called suicide substrate inhibitors, as they "sacrifice" themselves for the inhibition. After the inhibition process, the serpin-SPs complex is removed from the hemolymph and degraded (Reichhart et al., 2011; Soukup et al., 2009; Veillard et al., 2015). Five *D. melanogaster* serpins, Spn27A, Spn28D, Spn77Ba, Spn5 and Necrotic (Spn43Ac), have been implicated in regulating the melanization reaction (Ahmad et al., 2009; De Gregorio et al., 2002; Ligoxygakis, 2002; Scherfer et al., 2008). A loss of function mutation in each one of them results in the formation of melanotic bodies, excessive melanization and usually lethality. Serpin mutants resulting in constitutive melanization are regularly used for genetics screens to identify new candidate genes participating in melanization, via attempting to suppress one of those phenotypes. Except for Psh as a target for Necrotic, we do not know the direct targets of these serpins.

Interestingly, serpins are not only used to regulate SP cascades from the host side. They are also produced by e.g. parasitoid wasps or blood feeding insects, like ticks or mosquitos, to suppress or modulate immune mechanisms (Meekins et al., 2017). A natural parasite of *Drosophila*, the parasitoid wasp *L. boulardi*, produces a venom which is injected in the body cavity of the fly larva during infestation. This venom contains a serpin, LbSPNy, which inhibits the melanization reaction at an unknown step (Colinet et al., 2009).

6.2.6 The connection between the melanization reaction and Toll

As described in 6.2.4.1, there is evidence of a connection between the SPs regulating the melanization reaction and the SPs activating the Toll pathway in *D. melanogaster*. Evidence suggests that this connection is caused by the utilization of the same PRRs, GNBP3, PGRP-SA, GNBP1 and the sensor for microbial protease activity Psh, for both Toll and the melanization activation (De Gregorio, 2002; Matskevich et al., 2010). Nevertheless, there is no formal proof that the SPs regulating melanization can also activate the Toll pathway in *Drosophila* and vice versa. This stands in contrast to other insects, where there is strong evidence that the SP cascades regulating these two processes are either identical, or share important components. As an

example, a brief summary of the state of knowledge on the activation of these cascades in the mealworm *T. molitor* will be presented.

The melanization cascade in *T. molitor* can be activated with peptidoglycan from Gram-positive and Gram-negative bacteria, as well as with β-1,3-glucan from fungi (Nakhleh et al., 2017; Park et al., 2006). By using affinity purification experiments for PGN, the PRRs PRGP-SA and GNBP1 were identified as upstream receptors which initiate the PO cascade (Park et al., 2006). A modular serine protease (MSP, a homolog of ModSP from *D. melanogaster*) can form a complex with these PRRs, and initiate the downstream SP cascade (Buchon et al., 2009; Kan et al., 2008; Park et al., 2006).

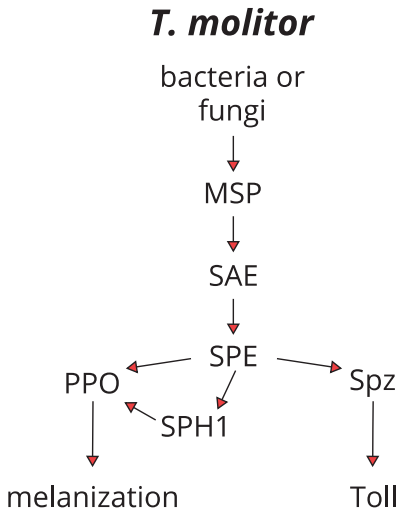

Figure 9: Schematic overview of the single clip-SP cascade activating melanization and Toll in *T. molitor.* PRR receptors recognize Gram-positive bacteria or fungi and activate downstream SPs. SPE as most downstream SP can activate both the melanization reaction or Toll. A detailed description is given in the text, figure adapted from (Kanost and Jiang, 2015).

After the complex formation of MSP with the PRRs, MSP undergoes auto-proteolysis and activates a downstream SP called SPE-AE (spätzle-processing enzyme activating enzyme) (Kim et al., 2008). The process of autoactivation is also found for ModSP from *D. melanogaster* (Buchon et al., 2009). Finally, SAE can activate the Spätzle processing enzyme (SPE), which can cleave Spz and activate Toll. Interestingly, SPE can also activate the melanization reaction by directly cleaving PPO and

additionally an SPH called SPH1, which also promotes PO activity (Kan et al., 2008; Kim et al., 2008). As a serine protease homolog, SPH1 can bind to cell wall components of microbes and is proposed to guide POs to microbial surfaces to prevent its dispersal throughout the hemolymph (Kan et al., 2008; Zhang et al., 2003). Taken together, these reports clearly demonstrate the presence of a common pathway for the melanization reaction and the activation of Toll in *T. molitor* (see **Figure 9**), contrasting the proposal of two separate pathways in *D. melanogaster*. It is important to mention that the differences in the experimental approaches, the genetic approaches in *Drosophila* versus the biochemical approaches used for other insects, could explain some observed difference in the models of these SP cascades.

6.2.7 Biological function and importance of the melanization reaction

While the melanization reaction is rapid and spectacularly visible compared to other insect immune functions, its precise relevance in insects has been disputed. This paragraph will focus on relevance of the melanization reaction as an immune response. Additionally, tangible examples will be given in the following section, where the melanization has an impact on economic revenues from agricultural industries.

6.2.7.1 Melanogenesis produces cytotoxic compounds

During melanogenesis, radical semiquinones are produced (see 6.2.1). Two semiquinones can undergo dismutation during a redox reaction, which can lead to the formation of dopaquinone and H_2O_2 (hydrogen peroxide) (Vavricka et al., 2010). Additionally, the oxidation of L-DOPA or Dopamine by PO can generate superoxide ($\cdot O_2^-$) and hydroxyl radicals (·OH). Hydrogen peroxide can further react with superoxide to form additional hydroxyl radicals. Hydroxyl radicals are highly cytotoxic by inducing lipid peroxidation, DNA damage, protein cross linking and more (Balasubramanian et al., 1998; Gutteridge, 1984; Nappi et al., 2009). Additionally, 5,6-dyhydroxyindole (DHI) is produced during melanogenesis (see 6.2.1). DHI was found to have antibacterial, antiviral, antifungal, as well as antiparasitic effects (Nappi and Christensen, 2005; Zhao et al., 2011). The importance of the PPO-mediated melanization reaction as an immune mechanism is illustrated without doubt in *D. melanogaster* and other insects (Binggeli et al., 2014; Eleftherianos et al., 2007; Lu et al., 2008). However, the final mechanisms or molecules which lead

to the clearance of those septic infections are not fully understood yet. The cytotoxic molecules produced during melanogenesis presented here appear to be appealing candidates contributing to the killing of invading microbes.

6.2.7.2 Melanization is critical to fight bacterial and fungal infections

Until recently it was debated whether the melanization reaction has a clear impact on *Drosophila* immunity. Due to the lack of PPO mutants, the studies addressing this question relied on the use of mutations or RNAi knock-downs of SPs involved in the activation of the melanization reaction. For instance, Leclerc and colleagues generated a mutant for *Sp7*, and were unable to detect activated PPOs in the hemolymph of these *Sp7* deficient flies (Leclerc et al., 2006). They did not observe any survival defects in Sp7 mutant flies after infection with *E. faecalis* (Gram-positive), *Agrobacterium tumefaciens* (Gram-negative) or the fungus *B. bassiana*, indicating that PO activity after microbial challenge in the hemolymph is dispensable. Interestingly, Leclerc et al. also used *black cells* (*Bc*) flies, a mutation that was later shown to be a gain of function mutation in PPO1, leading to complete loss of melanin production in the hemolymph (Neyen et al., 2014a). Surprisingly, *Bc* flies showed impaired survival capabilities which contrasts their results for *Sp7* deficient flies, indicating a more complex role for POs. Clear evidence that PO activity is critical to survive infections with microbes was given recently by Binggeli et al., 2014. The generation of null-mutants for *Drosophila PPO1* and *PPO2* revealed that a single mutation in PPO1, and even more the simultaneous loss of both PPOs, leads to a severe susceptibility of mutant flies to a variety of microbes, especially Gram-positive bacteria and fungi. This clearly established that the melanization reaction is critical for *D. melanogaster* to survive septic infections.

6.2.7.3 The melanization reaction is involved in encapsulation

Parasitoid wasps are a constant threat to *Drosophila*. This is illustrated by the fact that more than 50% of natural *Drosophila* populations show parasitization by wasps (Fleury et al., 2009). Parasitoid wasps lay their eggs inside other insects, where their offspring develop by consuming the host. *Drosophila* larvae respond to this infestation with the cellular encapsulation reaction (see 6.1.2.3). In this process, the wasp egg first needs to be recognized as foreign, and then subsequently hemocytes can form a capsule around the egg to contain and eventually kill it. This

capsule is subsequently melanized. Cytotoxic by-products of melanogenesis are proposed to play a role in killing the wasp embryo, while melanin itself has been propose to harden the capsule and to act as a ROS/RNS sink to protect the host (Carton et al., 2008; Keebaugh and Schlenke, 2014; Nappi and Christensen, 2005; Russo et al., 1996; Salt, 1970). The melanization of the capsule is carried out by POs (Binggeli et al., 2014; Nappi et al., 2009). The *D. melanogaster* genome harbors three PPO genes. Interestingly, Binggeli and colleagues found that flies deficient for two out of the three genes, *PPO1* and *PPO2*, lose the capability to melanize the capsule after infestation (Binggeli et al., 2014). This stands in contrast to previous reports which propose a leading role of the third PPO, PPO3, after exposure to wasps (Nam et al., 2008). While PPO1 and PPO2 are produced in crystal cells, the expression pattern of *PPO3* is unclear. While some reports propose that *PPO3* is expressed also in crystal cells (Ferjoux et al., 2007; Waltzer et al., 2003), others suggest an expression mainly in lamellocytes (Irving et al., 2005; Nam et al., 2008). The latter is supported by the observation that *PPO3* expression is upregulated after infestation with the wasp *L. boulardi*, in a similar temporal manner like lamellocytes differentiate (Keebaugh and Schlenke, 2014; Wertheim et al., 2005). This indicates a role of PPO3 in the defense against parasitoid wasps and the production of PPO3 by lamellocytes. While the clear role of the single PPOs in this defense mechanism is not revealed yet, the importance of the melanization reaction against parasitoids is further underlined by other findings. The overexpression of a negative regulator of melanization, *spn27A,* leads to a reduced efficiency of wasp egg encapsulation and increased wasp success (Nappi et al., 2005). A similar observation is made in flies lacking Dopadecarboxylase (Ddc), which is involved in the melanization reaction to produce the cytotoxic 5,6-Dihydroxyindole (see **Figure 6** and Nappi et al., 1992). Additionally, several reports indicate that parasitoid wasps have evolved mechanisms to suppress the melanization reaction in their hosts. *L. boulardi* for instance injects a venom containing a serpin that suppresses melanogenesis (Colinet et al., 2009). Another example is the venom of the wasp *Microplitis demolitor* which infests *M. sexta*, that contains protease inhibitors specifically targeting the PPAFs that activate the melanization cascade (Beck and Strand, 2007; Lu et al., 2008). Taken together, these results illustrate the importance of the melanization reaction in the encapsulation process.

6.2.7.4 Wound healing and the melanization reaction

Insects close wounds by the quick formation of a clot to prevent infection, loss of hemolymph and promote wound healing (Theopold et al., 2004, 2014). In *Drosophila* a soft clot is formed first via the aggregation of various clot proteins (e.g. Fondue and Hemolectin), which are then crosslinked by transglutaminase (Karlsson et al., 2004; Lindgren et al., 2008; Scherfer et al., 2004). After the soft clot is formed, POs further participate in the crosslinking of proteins and also harden the clot, likely through melanogenesis (Theopold et al., 2014). To which extent the formation or maturation of the clot is important to survive wounding is debated. *Drosophila* lacking a single clotting factor do not show any (Bidla et al., 2005; Lesch et al., 2007; Scherfer et al., 2004) or only subtly (Bajzek et al., 2012; Chang et al., 2012) impaired survival after wounding. Interestingly, flies lacking components of the melanization cascade seem to be stronger affected in their survival after wounding. Flies lacking both PPO1 and PPO2 show reduced survival after receiving a wound (Binggeli et al., 2014). Similar results are found in flies carrying the black cells phenotype, a mutation in PPO1 leading to the full loss of melanization due to non-functional crystal cell (Neyen et al., 2014a; Rämet et al., 2002). Additionally, the loss of the SP Hayan also leads to the impaired capacity to survive a wound. This effect is explained with the loss of ROS produced by PO, which otherwise activate the JNK pathway leading to a systemic wound response (Nam et al., 2012). It therefore seems likely that the protein crosslinking or the hardening of the clot mediated by PO participates in the survival of *Drosophila* to injury. Taken together, these reports indicate a critical role of the melanization reaction in the process of wound healing.

6.2.8 The melanization reaction and its ecological impact

The white spot syndrome virus (WSSV) is a shrimp pathogen which causes high mortality, and poses a serious threat to commercial shrimp production in aquaculture worldwide (Escobedo-Bonilla et al., 2008; Shekhar and Ponniah, 2015). The accumulated economic losses caused by several highly infective viruses, including WSSV, are estimated to reach US $1 billion since the early 1990s (Flegel et al., 2008). Recently, the melanization reaction was implicated in the defense reaction of the black tiger shrimp *Penaeus monodon* against WSSV. Gene silencing of *P. monodon PPO*s resulted in a higher mortality of infected individuals (Sutthangkul et al., 2015). It was further discovered, that WSSV produces a protein (WSSV453), which binds to the PPO activating SP (*Pm*PPAE2) and prevents its activation, which results in reduced PO activity (Sutthangkul et al., 2017). Reports in other Crustacea indicate an active downregulation of PO genes after infection

with WSSV, or a reduction in hemocyte derived melanization, although the mechanisms are still unknown (Ai et al., 2008; Jiravanichpaisal et al., 2006).

To date, no treatment of WSSV infections are available. The prevention of the infection of aquacultures with WSSV relies on the precautionary use of disinfectants. Understanding the role of the melanization reaction in the defense against WSSV could not only help to combat the economic losses, but also might reveal a specific antiviral mechanism of PO activity. It could also provide the opportunity to identify shrimp populations with increased melanization ability, for cultures with better resistance against WSSV.

Drosophila suzukii, the spotted-wing Drosophila (SWD) native to Asia, is an invasive insect pest in Europe and North America, that attacks a wide variety of soft-skinned fruit crops (Asplen et al., 2015). Instead of living, developing and feeding in already rotten fruit, *D. suzukii* attacks fresh, intact fruits and therefore poses a serious economic threat to agriculture (Walsh et al., 2011). The control of SWD currently relies on the heavy use of chemical insecticides, which is costly both in terms of risks to the environment (e.g. unspecific effects on pollinators) and financial expenses. With the additional risk of developing a resistance against repetitively used insecticides, there is a urgent need of more effective methods to manage *D. suzukii* (Schetelig et al., 2018). Currently, a variety of alternative management approaches are being explored, where some of the most promising strategies are the use of natural pathogens (e.g. viruses) or enemies, like parasitoid wasps (Haye et al., 2016). As described in 6.2.7.3, the melanization reaction contributes strongly to the encapsulation process as a defense mechanism against parasitoid wasps. For now, only few species of parasitoid wasps have been evaluated as control agents against SWD, and even fewer of them show the ability to use SWD efficiently as a host (Schetelig et al., 2018). A better understanding of the process of melanotic encapsulation, and especially the interactions between serpins and SPs, can aid in the identification and selection of adequate wasp species to control SWD.

6.3 OBJECTIVES OF THE PRESENT PHD THESIS

The goal of this PhD thesis was to provide a better characterization of the melanization reaction in *D. melanogaster* on two different levels: i) on the effector level of the PPOs and ii) on the level of their regulation through SPs.

While a recent study ascribed clear roles to two PPOs (PPO1 and PPO2) out of the three PPOs in *D. melanogaster*, the role of the third PPO (PPO3) remains unclear (Binggeli et al., 2014). Here, we generated a loss-of-function mutation of *PPO3* and analyzed its function using either a single mutant background, or analyzing the effect of combined mutations with PPO1 and PPO2. Additionally, we use bioinformatic tools to gain insights on the radiation and the evolution of the PPO genes, pointing to the existence of gene duplications and sub-functionalization. The results from this first part of my PhD resulted in a publication in BMC Biology (Dudzic et al., 2015).

The second objective of my PhD addresses the regulation of the melanization reaction through SPs. In this part we generated new loss-of-function mutants for three SPs involved in *D. melanogaster* melanization: *MP1*, *Sp7* and *Hayan*. The use of these mutants, alone and in combination, allowed us to readdress their roles in the melanization cascade and Toll pathway activation. The use of combined mutations with other SPs allowed us to identify phenotypes, otherwise hidden by a single mutant approach. Finally, we also use bioinformatic tools to gain insights in the evolution of these SPs, revealing a process of neo- or sub-functionalization of genes. Many of our results using null-mutations differ from previous publications, which used mainly RNAi experiments and hypomorph mutants. The results from this second part are published as a non-peer-reviewed pre-print and are currently under review in Cell reports (bio-Rxiv: Dudzic et al., 2018).

7 DROSOPHILA INNATE IMMUNITY: REGIONAL AND FUNCTIONAL SPECIALIZATION OF PROPHENOLOXIDASES

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Drosophila innate immunity: Regional and functional specialization of prophenoloxidases

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Abstract

Background

The diversification of immune systems during evolution involves the expansion of particular gene families in given phyla. A better understanding of the metazoan immune system requires an analysis of the logic underlying such immune gene amplification. This analysis is now within reach due to the ease with which we can generate multiple mutations in an organism. In this paper, we analyze the contribution of the three *Drosophila* prophenoloxidases (PPO) to host defense by generating single, double and triple mutants. PPOs are enzymes that catalyze the production of melanin at the site of infection and around parasites. They are the rate-limiting enzymes that contribute to the melanization reaction, a major immune mechanism of Arthropods. The number of PPO encoding genes is variable among insects, ranging from one in the bee to ten in the mosquito.

Results

By analyzing mutations alone and in combination, we ascribe a specific function to each of the three PPOs of *Drosophila*. Our study confirms that two PPOs produced by crystal cells, PPO1 and PPO2, contribute to the bulk of melanization in the hemolymph, upon septic or clean injury. In contrast, PPO3, a PPO restricted to the *D. melanogaster* group, is expressed in lamellocytes and contributes to melanization during the encapsulation process. Interestingly, another overlapping set of PPOs, PPO2 and PPO3, achieve melanization of the capsule upon parasitoid wasp infection.

Conclusions

The use of single or combined mutations allowed us to show that each PPO mutant has a specific phenotype, and that knocking out two of three genes is required to fully abolish a particular function. Thus, *Drosophila* PPOs have partially overlapping functions to optimize melanization in at least two conditions, following injury or during encapsulation. Since PPO3 is restricted to the *D. melanogaster* group, this suggests that production of PPO by lamellocytes emerged as recent defense mechanism against parasitoid wasps. We conclude that differences in spatial localization, immediate or late

availability, and mode of activation underlie the functional diversification of the three *Drosophila* PPOs, with each of them having non-redundant but overlapping functions.

Keywords: *Drosophila*, Prophenoloxidase, Melanization, Gene family, Immunity, Duplication

Background

The constant interactions of infectious microbes with their hosts explain the emergence of complex immune systems. Comparative immunology provides one of the best approaches to understand the logic of metazoan host defense mechanisms and their diversification throughout evolution. This approach reveals that despite the apparently extreme diversity of immune reactions, similar mechanisms are used across the animal kingdom to cope with microbes. Phagocytosis, epithelial production of antimicrobial peptides, sequestration of iron or mucus barriers are found in many organisms, indicating either an ancient origin or their recurrent emergence by convergent evolution. Other immune modules are specific to a limited number of organisms. Emergence, loss, and diversification of these immune modules are thought to reflect the evolutionary trajectory of Metazoan lineages facing various selective pressures from pathogens. The process of immune diversification often involved the expansion of particular gene families. For instance, the number of genes encoding Toll-like receptors (TLRs), peptidoglycan recognition proteins (PGRPs) and C-type lectins markedly varies among phyla [1]. A better understanding of the metazoan immune system requires an analysis of the rules underlying such immune gene amplification. The recently developed CRISPR/Cas9 genome editing approach offers a new strategy for generating mutations in a quasi-systematic manner. This allows for the first time to tackle the question of gene family diversification, by generating mutations in single and multiple genes belonging to the same family. We previously reported the functional analysis of two of the three *Drosophila* prophenoloxidases, PPO1 and PPO2, using single and double mutant analysis [2]. We subsequently realized that the *PPO1, PPO2* double mutant stock used in our previous study carries a mutation in the *PPO3* gene. Here, we investigated the function of *PPO3*, and performed a single, double and triple mutant analysis of the three *Drosophila* prophenoloxidases. Our study extends our previous results and attributes

specific and complementary functions to each of the three PPOs, providing an insight into how this protein family has evolved in *Drosophila*.

Melanization is a major immune module found in Arthropods but not in Vertebrates [3, 4]. It involves the rapid synthesis of a black pigment named melanin, at the site of infection or injury. A key enzyme in melanin biosynthesis is phenoloxidase (PO), which catalyzes the oxidation of phenols to quinones, which subsequently polymerize into melanin. PO is usually synthesized as a zymogen called prophenoloxidase (PPO), which is cleaved to generate the active PO enzyme upon activation of a proteolytic cascade. Several roles have been ascribed to the melanization reaction in insects [5-8]. PO activity contributes to wound healing by forming a scab at the epithelial injury site. By-products of PO activity are reactive oxygen species (ROS), which are thought to contribute to the killing of microbes and pathogens. Finally, melanization participates in the encapsulation reaction against parasites. Deposition of melanin on the parasite forms a physical barrier, allowing the localized and confined production of toxic compounds while ensuring the protection of the host. Melanization is clearly an Arthropod specific mechanism, but its functions in providing a barrier and generating microbicidal ROS are usually carried out by other mechanisms in other species.

The *Drosophila melanogaster* genome contains three *PPO* genes all on the second chromosome [2]. PPO1 and PPO2 are produced in specialized hemocytes (blood cell) called crystal cells. Crystal cells represent 5% of the hemocyte population in larvae [9, 10]. Upon injury they rupture and release PPOs into the hemolymph (the insect blood), where they are activated by a cascade of serine proteases [11]. Using null mutations in *PPO1* and *PPO2*, we recently showed that PPO1 and PPO2 are responsible for all the PO activity in the hemolymph [2]. While PPO1 is involved in the rapid early delivery of PO activity, PPO2 present in the crystals of crystal cells provides a storage form, which can be deployed in a later phase [2]. This and other studies also revealed an important role for PPO1 and PPO2 in the survival to infection with Gram-positive bacteria and fungi, underlining the importance of melanization in insect host defense [4, 12-14]. However, previous work left open the question of the function of the third PPO, PPO3, which could not be addressed because no mutant was available. The expression pattern of *PPO3* was not clear either. Some reports suggested that *PPO3* is expressed in crystal cells [15, 16], while others proposed it is expressed in lamellocytes

[17, 18]. Lamellocytes are a type of larval hemocytes, which are induced upon injury or wasp infection and play a key role in the encapsulation of foreign bodies and parasites [19]. Interestingly, while PPO1 and PPO2 require proteolytic cleavage to be activated, PPO3 is thought to be produced in an active form, although a putative cleavage site is present [18, 20].

The starting point of the present study came with the serendipitous observation that the *PPO1$^{\Delta}$,PPO2$^{\Delta}$* double mutant that we previously used to analyze the role of PPO1 and PPO2 [2], also carries a cryptic null deletion in *PPO3*. This raised the possibility that the phenotype initially attributed to PPO1 and/or PPO2 could be caused by the absence of PPO3. To clarify this question, we separated the *PPO3* mutation from the *PPO1$^{\Delta}$* and *PPO2$^{\Delta}$* mutations and additionally generated an independent *PPO3* mutant using the CRISPR/Cas9 approach [21]. Using single, double and triple mutants of the three *PPO* genes, we confirm that PPO1 and PPO2 are the only source of hemolymphatic PO upon septic injury. Our study also reveals a role for PPO3 in the encapsulation process, in association with PPO2.

Results

Identification of a cryptic *PPO3* deletion in the *PPO1$^{\Delta}$*, *PPO2$^{\Delta}$* double mutant stock

By testing primers for the *PPO3* gene, we serendipitously discovered the presence of a deletion of 336 bp, removing 112 (residues 105-217) of the 683 amino-acids of PPO3 protein (**Fig. 1A**). This deletion is referred to as *PPO3^{1},* and we use parentheses around this mutant to refer to previous results where this mutant was present in a cryptic state. As the deletion does not change the reading frame, a residual protein is expected to be produced, lacking part of a conserved copper-binding domain found in all PPOs (**Fig. 1B**). The presence of this cryptic mutation raised the possibility that some of the phenotypes observed in the *PPO1$^{\Delta}$,2$^{\Delta}$,(3^{1})* flies could be due to the absence of PPO3. To clarify the function of the three PPOs, we separated *PPO3^{1}* from *PPO1$^{\Delta}$* and *PPO2$^{\Delta}$* by meiotic recombination. We also induced a null mutation in *PPO3* by CRISPR/Cas9, referred to as *PPO3^{SK3}*. *PPO3^{SK3}* is caused by a frameshift mutation in the first exon of the gene (**Fig. 1A**). Both *PPO3^{1}* and *PPO3^{SK3}*

mutations, which were generated in two distinct genetic backgrounds, show the same phenotype in all the experiments described below, indicating that *PPO3*^{1} is also a null mutation. As expected, *PPO3* mutants are perfectly viable and do not exhibit any overt developmental or pigmentation defect. **Figure 1C** shows that *PPO1*$^{\Delta}$*,2*$^{\Delta}$ and *PPO1*$^{\Delta}$*,2*$^{\Delta}$*,3*^{1} but not *PPO3*^{1} flies have a reduced life expectancy, confirming that the simultaneous presence of PPO1 and PPO2 is required for optimal fly longevity [2].

PO1 and PO2 are the sole POs contributing to hemolymph injury-mediated melanization in larvae and adults

Injury with a needle to wild-type larvae or adults induces a melanization spot at the wound site, the extent of which is usually proportional to the injury size. This blackening reaction results from *de novo* melanin synthesis catalyzed by PO and is further enhanced by the presence of microbial products [22]. Our previous study attributed all hemolymphatic PO activity to PPO1 and PPO2 due to the absence of melanization in the *PPO1*$^{\Delta}$*,2*$^{\Delta}$*,(3*^{1}*)* stocks, while single *PPO1* or *PPO2* mutants only showed reduced or almost normal melanization, respectively. Consistent with this finding, no melanization spot on the cuticle of injured *PPO1*$^{\Delta}$*,2*$^{\Delta}$ *and PPO1*$^{\Delta}$*,2*$^{\Delta}$*,3*^{1} mutants was observed (**Fig. 2A and 2B**). In contrast, *PPO3* mutants show a wild-type level of melanization in injured larvae and adults. We next measured PO activity via L-DOPA assay in hemolymph samples extracted from larvae. We found no significant difference in PO activity between *PPO3*^{1} and wild-type hemolymph samples. In contrast, hemolymph samples of *PPO1,2* deficient larvae show no PO activity indicating that PPO3 alone is not sufficient to produce PO activity in hemolymph (**Fig S1**). This confirms that PPO1 and PPO2 together produce all injury-mediated melanization in larvae and adults [2]. Survival analyses had shown that PO is required for resistance to microbial infection, notably to Gram-positive bacteria and fungi [2]. Survival analyses using the new fly lines now show that the strict *PPO1*$^{\Delta}$*,2*$^{\Delta}$ double mutation recapitulates all the phenotypes previously described using *PPO1*$^{\Delta}$*,2*$^{\Delta}$*,(3*^{1}*)*: *PPO1*$^{\Delta}$*,2*$^{\Delta}$ flies are more susceptible to septic injury with the Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis* and exhibit a mild susceptibility to natural infection with the fungus *Beauveria bassiana* (**Fig. 3**). In contrast, *PPO3*^{SK3} flies exhibit a wild-type level of resistance upon challenge with the same microbes. These

experiments confirm the important role of melanization in fighting infection by Gram-positive bacteria and fungi, and are consistent with our previous results indicating that PPO1 and 2 are the sole sources for hemolymphatic PO. Additional data (**Fig. 4A and B**) show that *PPO3*^{1} does not markedly affect the Toll and Imd pathways in adults as revealed by the wild-type inducibility of *Diptericin* and *Drosomycin,* their respective read-out genes, in this mutant.

PPO3 is specifically expressed in lamellocytes

Drosophila larvae have two types of hemocytes in the unchallenged state: plasmatocytes, which are macrophage-like cells, and crystal cells which produce PPO1 and contain crystals of PPO2 [2, 19, 23, 24]. A third 'inducible' cell type, the lamellocytes, are produced only upon wasp infection or injury. Lamellocytes are large flat cells that differentiate in the lymph gland or from circulating plasmatocytes and adhere to foreign objects or aberrant tissue in a process called encapsulation. Capsules are usually melanized in a secondary step. The expression pattern of *PPO3* has been a source of conflicting observations. Irving *et al*. (2005) reported its localization in lamellocytes using a microarray approach. In contrast, several studies [15, 16] report expression of *PPO3* in crystal cells. The observation that ubiquitous silencing of *PPO3* by *in vivo* RNAi suppresses melanization induced by the melanotic tumor *hop*^{Tuml} mutation, led Nam et al. (2008) to hypothesize that *PPO3* is indeed expressed in lamellocytes. To distinguish between these two possibilities, we generated a reporter gene in which the yeast transcription factor GAL4 is under the control of 1.6 kb of *PPO3* upstream sequences. This transgenic line was crossed with *uas-GFP* flies to reveal the expression profile of *PPO3* by GFP. We found no expression of *PPO3-Gal4* in plasmatocytes or crystal cells from naïve larvae (data not shown). We then analyzed the expression pattern of *PPO3-Gal4; uas-GFP* in larvae upon infestation with the parasitoid wasp *Leptopilina boulardi,* which induces a massive production of lamellocytes. **Figure 5A and 5B** show that the PPO3 reporter was not expressed in plasmatocytes or crystal cells in parasitized larvae, but was strongly expressed in all lamellocytes. High numbers of GFP positive lamellocytes were observed in circulation or around the wasp egg (**Fig. 5C and 5D**). No other tissue or

cell expressed the reporter and no expression was detected in embryos and adults, consistent with the absence of lamellocytes at these two stages.

Consistent with these observations and previous reports [25, 26], RT-qPCR analysis indicated that the level of *PPO3* transcripts is low in unchallenged wild-type larvae but markedly up-regulated in larvae collected 52h after wasp infestation, when lamellocytes have become abundant (**Fig. 5E**). While PPO1 and PPO2 are produced by crystal cells, our data clearly demonstrate that PPO3 is specifically expressed in lamellocytes. Since lamellocytes are absent in adults, this observation also explains why PPO3 does not contribute to hemolymphatic PO activity upon injury during this stage.

Both PPO2 and PPO3 contribute to capsule melanization.

A surprising result of Binggeli et al. was the observation that capsules around parasitoid wasp eggs were not melanized in $PPO1^{\Delta},2^{\Delta},(3^{1})$ larvae [2]. This led us to prematurely conclude that PPO3 was not essential for the melanization process during encapsulation. The discovery of the cryptic $PPO3^{1}$ mutation in the original stock and the specific expression of *PPO3* in lamellocytes prompted us to further analyze the role of PPO3 in the encapsulation process. Wild-type, $PPO3^{1}$, $PPO1^{\Delta},2^{\Delta}$ and $PPO1^{\Delta},2^{\Delta},3^{1}$ second instar larvae were infested by *Leptopilina boulardi*, and the presence of a melanized wasp egg was subsequently analyzed. **Figure 6** shows the presence of melanized *L. boulardi* eggs in wild-type, $PPO1^{\Delta},2^{\Delta}$ double mutant and $PPO3^{1}$ single mutant third instar larvae. In contrast, no melanized capsules were ever observed in the infested triple $PPO1^{\Delta},2^{\Delta},3^{1}$ mutants (the presence of intact wasp eggs was checked by dissecting the larvae). In contradiction with our previous conclusion, this result indicates that PPO3 does play a role in encapsulation that can be masked by the presence of PPO1 and/or PPO2. We then generated $PPO1^{\Delta},3^{1}$ and $PPO2^{\Delta},3^{1}$ double mutants to determine which of PPO1 or PPO2 contributes to capsule melanization together with PPO3. The result was unambiguous: we never found melanized capsules in *L. boulardi* infested $PPO2^{\Delta},3^{1}$ larvae while $PPO1^{\Delta},3^{1}$ larvae were phenotypically indistinguishable from $PPO3^{1}$ (**Fig. 6**). Thus, two PPOs from different origins, PPO2 from crystal cells and PPO3 from lamellocytes, contribute to melanization that accompanies encapsulation.

Heating larvae at 65°C for 10 min induces the spontaneous activation of PPO [9]. As a consequence of this treatment, the population of sessile crystal cells that are attached underneath the integument can easily be visualized through the cuticle as

black dots [27]. We used this method to investigate the respective contribution of the three PPOs to the melanization reaction. Consistent with the notion that black dots after heating are caused by the presence of PPO2 in crystal cells, *PPO1*$^{\Delta}$ and *PPO3*^{1} mutant larvae had melanized dots corresponding to the sessile crystal cells, but *PPO2*$^{\Delta}$ mutants did not (**Fig. 7A**). We then subjected third instar larvae previously infested by *L. boulardi* to the heat treatment. In addition to the black dots corresponding to the spontaneous activation of PPO2 in crystal cells, wild-type larvae infested with *L. boulardi* show large black spots that were not observed in unchallenged larvae (**Fig. 7B**). The same treatment (infestation followed by heating) applied to the various PPO mutants suggests that the large black spots are due to PPO3 activity in lamellocytes, as they are totally absent in *PPO3*^{1} larvae. Our conclusions were further strengthened by the observation that silencing *PPO3* by RNAi in lamellocytes alone using the *PPO3-Gal4* driver phenocopied the *PPO3*^{1} phenotype. Furthermore, a *knot* mutant (also called *collier*), which cannot produce lamellocytes [28] shows a melanization pattern similar to the *PPO3*^{1} mutants following wasp infestation.

Melanization linked to encapsulation is not dependent on Hayan or Sp7

It has been proposed that PPO3 is produced in its active form, while PPO1 and PPO2 are synthesized as zymogens, which are cleaved by serine protease activity to generate the active form [18, 20]. This cleavage is thought to be mediated by a clip-domain serine protease (SP) named Hayan [22]. Hayan also exists as a zymogen that is itself stimulated through a stepwise process involving other serine proteases. One upstream clip-domain SP is SP7 (also called MP2), which is specifically expressed in crystal cells [12-14, 29]. Indeed, while a null mutation in *Hayan* totally abolishes hemolymphatic PO activity in adults [22], a partial *Sp7* loss-of-function, *Sp7*^{PAE1}, slightly reduces it [14]. To date, the roles of Hayan and Sp7 in the encapsulation reaction in larvae have not been fully clarified.

To address the role of SP cleavage in larval and adult melanization, we first generated by CRIPR/Cas9 two null mutations in *Hayan* and *Sp7* and analyzed their contribution to the melanization reaction using the same techniques as described above. We confirmed that both *Sp7*^{SK6} and *Hayan*^{SK3} mutations affect melanization at an injury site, with slight differences according to the stage of the animal. As expected, the newly generated null *Sp7* mutant, *Sp7*^{SK6}, induces a much stronger

phenotype than the previously described hypomorphic *SP7^{PAE1}* allele: *Sp7^{SK6}* mutant adults show a very weak melanized spot at the injury site compared to *SP7^{PAE1}* (**Fig 8A**). As expected [22], the *Hayan^{SK3}* mutation totally abolishes melanization at the injury site in adult flies. Interestingly, injured *Hayan^{SK3}* and *Sp7^{SK6}* larvae had opposite phenotypes compared to mutant adults. Whereas *Sp7$^{\Delta}$* larvae do not develop any melanization at the injury site, *Hayan^{SK3}* larvae still display a very weak blackening reaction (**Fig 8B**). However, both *Sp7^{SK6}* and *Hayan^{SK3}* larvae showed a wild-type pattern of melanized black dots upon heating at 65°C (**Fig 8C**). This result indicates that heat treatment can induce spontaneous activation of PPO in crystal cells in the absence of upstream serine proteases. Finally, we observed that both *Sp7^{SK6}* and *Hayan^{SK3}* mutant larvae can produce melanized capsules around *L. boulardi* eggs (**Fig 8D**).This indicates that Hayan and Sp7 are not mandatory for the melanization around the capsule, although a minor contribution of these serine proteases cannot be excluded. In summary, the loss of *Hayan* or *Sp7* both mimic the *PPO1$^{\Delta}$,2$^{\Delta}$* phenotype, pointing to a major role of these SPs following septic injury. The formation of wild-type melanized capsules in both SP mutants is consistent with the observation that PPO3 does not need cleavage to become active.

PPO3 arose by gene duplication from PPO2 during the melanogaster group radiation

To place the functional differences among *Drosophila PPO* genes in an evolutionary context, we reconstructed the phylogeny of the PPO gene family using genome sequences from 23 species of *Drosophila* [30, 31]. We confirmed previous observations by Salazar-Jaramillo et al (2014) [26] that *PPO1* and *PPO2* genes are conserved across all *Drosophila* genomes sequenced, whereas *PPO3* is restricted to the melanogaster group (**Fig S2**). By analyzing a larger panel of species in the *D. melanogaster* group than in previous work [26], we found that a canonical *D. melanogaster*-like *PPO3* gene can only be found in the *D. melanogaster* subgroup (containing *D. melanogaster, D. simulans, D. sechellia, D. erecta, D. yakuba*) and *D. eugracilis,* but not in other members of the melanogaster group or species outside this group. Evidence for a partial *PPO3* gene can be found in *D. takahashii* (**Fig S2**), which may indicate that a *PPO3*-like gene was present in the ancestor of these species. All *Drosophila* species with canonical (or partial) *PPO3* genes are able to

melanotically encapsulate parasitoid wasps and to produce lamellocytes [26, 32, 33]. However, additional species in the melanogaster group (i.e. *D. ficusphila*) are also able to cellularly encapsulate wasps and produce lamellocytes [34], suggesting that the acquisition or retention of *PPO3*-based melanization in lamellocytes occurred after the cellular basis for encapsulating parasitoid wasps evolved.

Phylogenetic evidence of all *PPO* genes in *Drosophila* shows the *PPO3* clade clusters more closely with *PPO2* than with *PPO1* (**Fig S3**) (see also [8]), consistent with the functional overlap between *PPO2* and *PPO3* reported here. Moreover, both *PPO3* and *PPO2* have a four-exon gene structure, whereas *PPO1* has six exons. The *PPO* gene tree also shows that all *PPO3* genes cluster together outside the *PPO2* clade, which is consistent with two hypotheses for the timing of the *PPO3* duplication event: (i) the *PPO3* gene arose prior to the diversification of the *Drosophila* and *Sophophora* subgenera and was subsequently lost in multiple lineages, or (ii) the *PPO3* arose in the melanogaster group and underwent a transient period of accelerated sequence evolution shortly after duplication. Assuming the species tree in Ometto et al., (2013) [35], an ancient origin is unlikely because it requires on gain and at least seven independent losses on different *Drosophila* lineages, while simultaneously invoking selective maintenance of an ancestral *PPO3* prior to the evolution of the lamellocyte cell type on the lineage leading to the melanogaster group. A recent origin is more parsimonious and only requires a single evolutionary event followed by an accelerated rate of evolution in one of the genes after duplication (in this case *PPO3*), a pattern that has been observed for duplicate genes in yeast and mammals [36]. Thus, we conclude a *PPO2*-like ancestral gene was likely the source for the *PPO3* gene duplication event, and that this event most likely occurred recently during the radiation of the melanogaster group species.

The maximal genomic extent of the *PPO3* duplication (inferred by alignment with *D. ananassae*, the closest outgroup species that lacks this locus with a high quality genome assembly) does not fully contain the sequence in our *PPO3-Gal4* reporter construct that is needed to give lamellocyte expression (**Fig S2**). In fact, the region of our reporter that extends the regulatory region reported by Ferjoux et al (2007) [16] and likely contains lamellocyte specific regulatory elements lies within the neighboring gene CG9890, which is conserved in *D. ananassae*. Thus, it is likely that regulatory sequences responsible for lamellocyte expression were not a part of the

ancestral locus that gave rise to *PPO3*, but rather arose during or after the *PPO3* duplication event.

After the gain of *PPO3*, *D. sechellia* subsequently lost the ability to produce lamellocytes and melanotically encapsulate parasitoids [26, 32, 33]. Salazar-Jaramarillo et al (2014) [26] found an accelerated rate of evolution in the *D. sechellia PPO3* gene (Dsec\GM15980), which they proposed reflected relaxation of selective constraints on *PPO3* associated with the loss of the melanotic encapsulation phenotype. We found no evidence for an increased rate of evolution on the *D. sechellia PPO3* gene in our data, and tracked this discrepancy to a gene model error in FlyBase that fuses incomplete versions of *PPO3* and a neighboring gene (CG44252) in *D. sechellia* that was used by Salazar-Jaramarillo et al (2014). We did, however, find evidence for an inactivating mutation in the *D. sechellia PPO3* gene at amino acid position 48, which converts the terminal Glutamine residue of the propeptide region to a stop codon, and is predicted to generate a truncated version of the *PPO3* protein. This loss of function coding sequence mutation together with loss of *PPO3* expression in *D. sechellia* [26] supports the general model that PPO3 functions specifically in lamellocytes, and that the proper cellular context for its function in melanotic encapsulation is required to maintain selective constraint on this locus.

Discussion

Gene duplication is recognized as an important process in evolution. About 40% of the 13,601 *Drosophila* genes are duplicates of other genes [37]. Duplication is often associated with sub-functionalization in which each of the daughter genes adopts part of the function of the parental gene or neo-functionalization in which duplication is at the origin of a novel function [37]. Gene duplication can lead to the formation of a large gene family. Both animals and plants harbor large families of genes devoted to immune defense. Immune genes encoding effector or recognition molecules often exist in multiple copies, while genes encoding signaling pathway components are typically single copy [38, 39]. It is expected that a large repertoire of immune molecules confer broader recognition and effector capacity as well as more regulatory flexibility in the use of this repertoire. Thus the higher number of genes encoding PGRPs or antimicrobial peptides in the fruit fly *Drosophila* compared to the

Pea aphid can easily be understood by their respective environments: aphids feed on sap, a rather sterile diet while *Drosophila* feed on rotting fruits swarming with microbes [40]. Beside these general statements, the '*raison d'être*' of the size of immune gene family remains speculative.

Phenoloxidases are rate-limiting enzymes which determine the extent of melanization, and as such can be considered as effector immune molecules [8]. The number of *PPO* genes is variable among insects, ranging from one in the bee *Apis mellifera* to ten in the mosquito *Aedes aegypti* [8]. In this work and in Binggeli *et al*. (2014) we have generated loss-of-function mutations in the three *PPO* genes of *Drosophila* and analyzed the contribution of each of them to immune reactions. Our gene deletion and double mutant analysis allowed us to ascribe specific functions to each of the three PPOs. Our study confirms that two crystal cell PPOs, PPO1 and PPO2, contribute to the bulk of melanization induced by injury, with PPO1 immediately available and PPO2 being deployed later [2]. Melanization is one of the most rapid immune responses, as the dark spot is visible as soon as 10 minutes after injury. This suggests a key role of this mechanism in the early steps of wound healing and pathogen control. This could explain why PPOs are rather stored as ready-to-use proteins and not regulated at the transcriptional level. The existence of crystal cells, whose sole reported function so far is melanization, provides *Drosophila* with an efficient way to store and quickly release this key enzyme. Recent studies have shown that crystal cells derive either from progenitor blood cells or can differentiate from plasmatocytes [15, 27, 41, 42]. Thus, *Drosophila* can indirectly modulate the amount of PPO1 and PPO2 by regulating crystal cell differentiation. The observation that the *PPO1* deletion does not affect crystals in crystal cells led us to propose that PPO2 is the main source of crystalline PPO and that PPO1 is either localized in the cytoplasm of crystal cells or continuously secreted into the hemolymph [2]. The extreme fragility of crystal cells did not allow us to clarify this point.

Our work here demonstrates that PPO3 is restricted to lamellocytes and contributes to the encapsulation of the wasp egg. This explains why the *PPO3* transcripts are present at barely detectable levels in unchallenged larvae, which contain few or no lamellocytes. An intriguing observation is that two PPOs, PPO2 and PPO3, contribute to the melanization of the capsule formed around a wasp egg. This means that it is achieved by phenoloxidases derived from two hemocyte types: crystal

cells and lamellocytes. Lamellocytes are large flat cells with adhesive properties that can bind to wasp eggs and form multilayers. Our study suggests that crystal cells could also be guided to the capsule where they would release PPO2 crystals close to the encapsulated egg. It is still unknown whether crystal cells have the ability to stick to non-self elements or if they need the assistance of other hemocytes to reach their target as suggested by [27]. The absence of any marked role of PPO1 in encapsulation is puzzling. We speculate that localization of PPO1 in the hemolymph, which remains to be assessed, could explain why PPO1 is not involved in encapsulation. PPO in circulation would not have the ability to be directed to a foreign element such as a wasp egg, but could be immediately activated by the presence of oxygen at a wound site.

Combining the various mutations allowed us to show that, while each single *PPO* mutant has a specific phenotype, knocking out two genes out of three is required to fully abolish a specific function. Thus, *Drosophila* PPOs have partially overlapping functions to optimize melanization in at least two stress conditions, injury or parasitization (**Fig. 9**). Future studies should investigate the role of these PPOs in the melanization of organs such as the gut, salivary gland or trachea, which is sometimes observed upon oral infection or tumorous-like state [43, 44]. The selective pressure exerted by parasitoid wasps on *D. melanogaster* has probably led to the development of dedicated cellular immune defenses, with the emergence of inducible adhesive cells with PPO3 (lamellocytes), storage cells (crystals cells with PPO2) and hematopoietic organs (the lymph gland and sessile islets). All these elements are activated upon parasitization, to collectively contribute to capsule formation by mechanisms which are still poorly understood [45].

Most *Drosophila* species possess two *PPO* genes, *PPO1* and *PPO2*, except for some species from the *D. melanogaster* group (*D. melanogaster, D. simulans, D. sechellia, D. erecta, D. yakuba* and *D. eugracilis*) which have three (**Fig. S2**). Since the *PPO3* gene sequence, structure and function is more similar to that of *PPO2* than of *PPO1*, we propose that a duplication of an ancestral *PPO2*-like gene gave rise to an ancestral *PPO3*-like gene in the common ancestor of the lineage leading to *D. eugracilis* and the *D. melanogaster* subgroup. This was followed by a functional diversification with *PPO2* maintaining expression in crystal cells and *PPO3* becoming expressed only in lamellocytes. This scenario is consistent with a period of

accelerated evolution on the *PPO3* lineage after duplication (**Fig. S3**). Future studies in other *Drosophila* species might shed light whether this gene duplication event led to sub-functionalization (the ancestral gene was expressed in both crystal cell and lamellocyte lineages) or neo-functionalization (the ancestral gene was only expressed in crystal cells and the duplicate gene acquired a new expression pattern in lamellocytes). The observation that the existence of a third PPO is restricted to the *D. melanogaster* group suggests that massive production of PO emerged as a crucial mechanism to reinforce the defense against parasitoids in this lineage. The same selective pressure has probably led to other anti-parasites defense. Recent studies have shown that wasp encapsulation in *D. ananassae* (a species outside the *D. melanogaster* subgroup) is mediated by another mechanism involving multinucleated giant hemocytes, that are formed by the aggregation of hemocytes [46]. The capsule around the wasp is not melanized in this species [26, 33, 46]. The melanization of the capsule points to a critical role of melanization against parasites. This is also supported by studies showing that virulent wasps have suppressive mechanism against melanization such as the injection of serpins [47]. We performed survival analysis with *PPO* mutants using a highly virulent wasp species, *L. boulardi* and a weakly virulent one, *L. clavipes*. The results did not reveal any striking effect although there was a trend for a lower resistance of larvae to *L. clavipes* infestation in the absence of PPO (**Fig. S5**). Consistent with this, we noticed that it is easier to cultivate the wasp *Asobora tabida* using *PPO1,2,3* flies compared to wild-type. Taking possible effects of the genetic backgrounds in consideration, the results of our survival analysis in the laboratory should be taken with caution. It is possible that the optimum level of melanization for successful encapsulation is an ongoing arm-race process requiring a very tight balance, as melanization is also toxic for flies. This would explain why *PPO3* mutants tend to be more susceptible than *PPO1,2,3* deficient flies upon infection with *L. clavipes* (**Fig. S5**). Thus, the relevance of PPO in wasp encapsulation requires further analysis that should take in account the influence of the genetic background and the use of a more natural setting of infection.

The mechanisms of activation of PPO in *Drosophila* are not yet fully understood. Previous studies suggested that PPO1 and PPO2 require cleavage by serine proteases while PPO3 would be constitutively active. Indeed over-expression of PPO3 is sufficient to induce melanization of tissues as diverse as the eye or the

salivary gland [18]. Here, we show that mutations in *Hayan* or *SP7* strongly reduce melanization due to septic injury but do not affect melanization around a wasp capsule. This suggests that either PPO3 does not require proteolytic cleavage as suggested by molecular modeling and *in vitro* testing of PPO3 mutants [20] or that its maturation requires distinct serine proteases. Interestingly in this context, a heat-treatment of wasp infected larvae produced melanized cell aggregates, which corresponds to PPO3 activity in lamellocytes. This indicates that PPO3 is present in lamellocytes in an inactive form that can be activated by heat. Thus, PPO3 either needs an additional step to be activated, or is produced together with an inhibitor, or is inactive due to the absence of its substrate. The mechanism by which PPO3 is activated only in lamellocytes around a capsule remains to be deciphered.

This analysis started by the serendipitous discovery of a *PPO3* deletion in the *PPO1,PPO2* double mutant fly stock. Since this deletion was also found in a *white* control stock, we assume that this mutation was introduced when backcrossing *PPO1*. Since fly stocks in the laboratory are cultivated in the absence of parasitoid pressure, it cannot be excluded that this gene quickly pseudogenizes as it has no role beyond encapsulation. We were fortunate that the presence of this mutation does not affect the main conclusion of Binggeli et al. (2014) [2] stating that PPO1 and PPO2 are responsible for all hemolymphatic PO activity. The absence of melanization around the wasp egg in $PPO1^{\Delta},2^{\Delta},(3^{1})$ larvae led us to miss the important contribution of PPO3 to encapsulation. This underlines that one of the main issues in generating an extensive characterization of the *Drosophila* immune system is that of the genetic background and namely what we refer to as ‘wild-type’. Fly geneticists have usually a number of ways to assess the effect of the genetic background by analyzing the phenotype caused by mutations in different contexts. This can be done by placing the mutation over a deficiency or by extensive backcrossing to generate isogenic stocks as recently described in [48]. An important task should be to analyze how the studied phenotype varies in diverse backgrounds. In this study the phenotypes of each of the *PPO* single mutations were clear enough and in accordance with their expression patterns so that we could be confident about their respective functions. The striking phenotypes of $PPO1^{\Delta},2^{\Delta}$ and $PPO2^{\Delta},3^{1}$ double mutants which fail to melanize upon injury and wasp infection, respectively, reinforced our conclusions, which would have been more difficult to draw from only single

mutant analysis. Thus, generation of multiple mutation in combination is an adequate approach to better assess the function of gene families, notably those involved in effector mechanisms. Similar studies on other immune gene families should provide insights into the organization of the *Drosophila* immune system and that of other organisms.

Conclusions

We conclude that differences in spatial localization, immediate or late availability, and mode of activation underlie the functional diversification of the three *Drosophila* PPOs, with each of them having non-redundant but overlapping functions.

Methods

Insects Stocks and Mutant Generation

Unless indicated otherwise, w^{1118} or $y^{1}w^{1118}$ flies were used as wild-type controls. The $PPO1^{\Delta}$, $PPO2^{\Delta}$ and $PPO1^{\Delta},2^{\Delta},(PPO3^{1})$, $Relish^{E20}$ (Rel^{E20}), $spätzle^{rm7}$ (spz^{rm7}) and $SP7^{PAE1}$ lines are described previously [2, 10, 14, 49]. kn^{col-1} lamellocyte deficient larvae were obtained by using the *w*; kn^{col-1};P{col5-cDNA.C}* fly line that carries the lethal mutation *knot* and a *knot* transgene with a restricted expression pattern rescuing this lethality [28]. *w*; P{10XUAS-mCD8::GFP}attP2* (*UAS-GFP*) lines was obtained from the Bloomington Stock Center. The parasitoid wasp *L. boulardi* (kindly provided by M. Crozatier) was reared on $PPO1^{\Delta},2^{\Delta},3^{1}$ triple mutant fly stocks at room temperature. After emergence wasps were kept at room temperature and provided with honey until use for experiments. The $PPO3^{SK3}$, $Hayan^{SK3}$, and $Sp7^{SK6}$, mutant lines were generated by CRISPR/Cas9 as described in [21] (**Fig S4**). The *uas-PPO3-IR* (ch II) was obtained from VDRC (50737). The *PPO3-Gal4* line was generated by cloning a 1.6 kb sequence upstream of the *PPO3* gene (*PPO3-Gal4* forward 5'-TTGAGGGCGGTGAAGTTGTC-3', reverse 5'- GGAGGACCTTTAGCGAGCAG-3') into pBPGUw vector [50] followed by PhiC31 integrase-mediated transgenesis (BestGene Inc.). The $PPO1^{\Delta},2^{\Delta}$, $PPO1^{\Delta},3^{1}$ and $PPO2^{\Delta},3^{1}$ lines were generated by reintroducing the corresponding wild-type alleles into

the *PPO1*$^{\Delta}$*,2*$^{\Delta}$*,3*^{1} mutant by meiotic recombination. *Drosophila* stocks were maintained at 25°C on standard fly medium.

Microorganism culture and infection experiments

The bacterial strains used and their respective optical density of the pellet (O.D.) at 595 nm were: the Gram-negative bacteria *Erwinia carotovora* (*Ecc15*, O.D. 200); the DAP-type PGN containing Gram-positive bacteria *Bacillus subtilis* (*B. subtilis*, O.D 5); the Lys-type PGN containing Gram-positive bacteria *Micrococcus luteus* (*M. luteus*, O.D. 200), S*taphylococcus aureus* (*S. aureus*, O.D. 0.5) and *Enterococcus faecalis* (*E. faecalis*, O.D. 0.5). Strains were cultured in Luria Broth (LB) at 29°C (*E. carotovora*, *M. luteus*) or 37°C (other species). Spores of entomopathogenic strains *Beauveria bassiana 802* (*B. bassiana*) were grown on Malt agar plates at 29°C for approximately 3 weeks until sporulation [49]. Systemic infections (septic injury) were performed by pricking adult females in the thorax with a thin needle previously dipped into a concentrated pellet of bacteria. Natural infections were initiated by shaking anesthetized flies in a petri dish containing a sporulating culture of entomopathogenic fungi *B. bassiana*. Infected flies were subsequently maintained at 29°C (*E. carotovora*, *M. luteus, B. bassiana*) or at 25°C (all other bacteria). At least three tubes of 20 flies were used for each survival experiment and survival was scored daily. For lifespan experiments, flies were kept on normal fly medium and were flipped every two days.

Wounding experiment

'Clean' injury (CI) referred to an injury performed with a sterilized needle. A low level of bacterial contamination is still possible since the surface of the insect was not sterilized. For imaging of the melanization reaction upon pricking, the thorax of the animal was pricked (as described in infection experiments) using a sterile needle (diameter: ~5 µm). Pictures were taken 16 hours post-pricking. Third instar larvae were pricked dorsally near the posterior end, using a sterile needle (diameter: ~5 µm). Pictures of melanized larvae were taken 1 hour post-injury. Pictures were captured with a Leica DFC300FX camera and Leica Application Suite. For publication purposes, brightness and contrast were increased on some images.

Live Imaging and Immunofluorescence

For hemocyte imaging, larvae were cleaned in PBS and dissected on silane coated microscope slides (TEKDON, INC.) into a drop of 1% BSA-PBS. Hemocytes were allowed to settle for 30 minutes, then fixed with 4% PFA-PBS for 15 minutes and rinsed with PBS.

For staining of capsules from wasp infested larvae, capsules were dissected three days after infection, fixed with 4% PFA-PBS and rinsed with PBS. Samples were observed for fluorescence with an Axioplot imager Z1 and Axiocam mRM camera (Zeiss).

PO activity

Hemolymph was collected by dissecting larvae in 4 °C PBS. Protein concentration was adjusted after a Bradford test. Sample volumes were adjusted in 20 µl 5 mM $CaCl_2$ solution and after addition of 80 µl L-DOPA solution (20 mM, pH 6.6) the samples were incubated at 29 °C in the dark. Optical density at 492 nm was measured consequently. L-DOPA solution without hemolymph was used as blank. Each experiment was repeated three times.

Quantitative RT-PCR

For quantification of mRNA, whole flies or larvae were collected at indicated time points. Total RNA was isolated from 10-15 adult flies or larvae by TRIzol reagent and dissolved in RNase- free water. 1 microgram total RNA was then reverse-transcribed in 10 µl reaction volume 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 LightCycler 480 SYBR Green I master mix or on a LightCycler 2.0 (Roche) in capillaries using dsDNA dye SYBR Green I (Roche). Primers were as follows: *Diptericin* forward 5'- GCTGCGCAATCGCTTCTACT-3', reverse 5'- TGGTGGAGTGGGCTTCATG-3'; *Drosomycin* forward 5'- CGTGAGAACCTTTTCCAATATGAT-3', reverse 5'- TCCCAGGACCACCAGCAT-3'; *Rp49* forward 5'-GACGCTTCAAGGGACAGTATCTG-3', reverse 5'- AAACGCGGTTCTGCATGAG-3'; *PPO3* forward 5'-GGCGAGCTGTTCTACT-3', reverse 5'- GAGGATACGCCCTACTG-3'.

Wasp infestation and quantification of fly survival to wasp infestation

For wasp infections, 30 synchronized second instar wild-type or mutant larvae were placed on a pea-sized mound of fly food within a custom-built wasp trap in the presence of 3 female wasps for 2h (*L. boulardi*) or 72h (*L. clavipes*). For survival experiments, parasitized larvae were kept at room temperature and scored daily for flies and wasps. The difference between enclosed flies and wasps to the initial number of larvae was set as dead larvae/pupae. For imaging of lamellocytes or wasp eggs, infested larvae were dissected 72 hours after being exposed to wasps. For qRT-PCR of PPO3, total RNA of larvae was isolated 52 hours after wasp infections.

Heating of larvae to induce spontaneous melanization

Larvae were heated in PBS at 67 °C for 20 minutes to induce the spontaneous activation of PPO within hemocytes [9, 27].

PPO sequence analysis

Predicted orthologs of *PPO* genes from 23 species in the *Drosophila* genus were identified by extracting and concatenating exonic regions from the UCSC Genome Bioinformatics Database multiz27way insect alignments (http://hgdownload.cse.ucsc.edu/goldenPath/dm6/multiz27way/alignments/). Genomic alignments extracted from UCSC for each gene were cross-referenced with orthology predictions for 12 *Drosophila* genomes from OrthologDB [51] and in three cases where UCSC genomic alignments lacked a gene model for a particular species (*PPO1* from *D. mojavensis*, *PPO1* from *D. grimshawii*, and *PPO2* from *D. ananassae*), the FlyBase gene model indicated by OrthologDB was added to the alignment [30, 52].

Sequences for all three *PPO* genes were combined and aligned using Clustal Omega [53] using default settings in SeaView 4.0. [54] A preliminary phylogenetic tree was produced using the BioNJ algorithm [55] in SeaView and *PPO* sequences clustering in the incorrect gene or species clade were inspected in the UCSC Genome Browser. Three predicted *PPO* genes based on the UCSC alignments (*PPO1* for *D. miranda*, *PPO1* for *D. kikkawai,* and *PPO3* for *D. kikkawai*) clustered incorrectly in the *PPO2* clade. Inspection of global alignment nets and chains in the UCSC browser revealed these to be alignment artifacts. Likewise, systematic inspection of nets and chains for all *D. melanogaster* group species revealed that a predicted *PPO3* sequence from *D. takahashii* based on UCSC whole-genome alignment was composed of two non-syntenic

fragments, and there was no evidence for a *PPO3* sequence in the two species most closely related to *D. takahashii* (*D. biarmipes* and *D. suzukii*). Thus, these four sequences were removed from the final alignment before producing the final phylogenetic tree using RAxML version 8.0.26 [56] (Stamatakis 2014). Maximum likelihood tree searches were conducted using a general time reversible (GTR) model of nucleotide substitution with Γ rate heterogeneity, with all model parameters estimated by RAxML. Trees were inferred using a combined approach, with an initial 100 bootstrap replicates and a full ML search for the best-scoring tree, using the rapid bootstrap algorithm [57].The best-scoring ML tree was visualized and annotated in FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

The PPO3 gene overview was adapted with FancyGene [58]. The protein structure of PPO3 wild-type and PPO3^{1} was predicted by Phyre2 [59] and visualized by using VMD suite [60]. All structural illustrations were rendered in VMD.

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, and p values are indicated in figure legends. Statistical significance of PO activity was calculated with Two-way ANOVA with Tukey correction. Otherwise statistical significance was calculated using Mann-Whitney test and p values of < 0.05 = *, < 0.005 = **, and < 0.0005 = *** were considered significant.

Authors' contributions

JD and BL designed the study. JD performed the experiments. JD, CMB and BL analyzed the data and wrote the manuscript. SK and RU supplied critical reagents. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

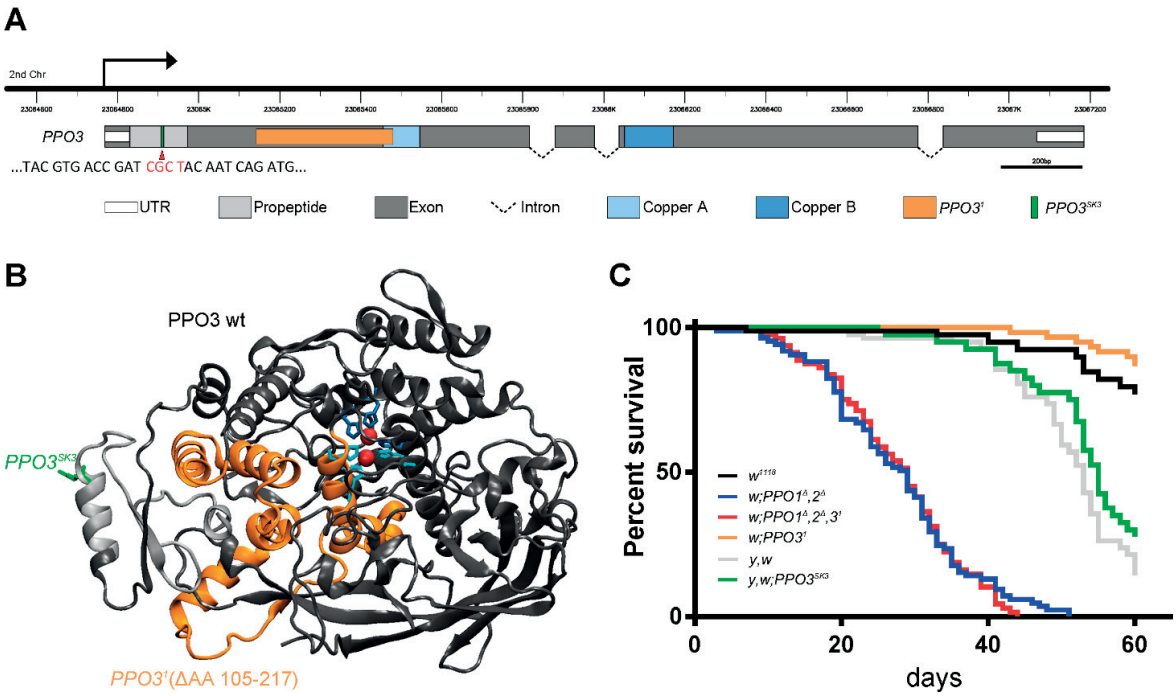


Figure 1 - Molecular characterization of two novel *PPO3* mutations. (**A**) Schematic representation of the *PPO3* gene locus and *PPO3* coding sequence. The gene map was adapted from FlyBase. The $PPO3^{1}$ mutation was present in the $PPO1^{\Delta},2^{\Delta}$ double mutant stock while the $PPO3^{SK3}$ mutation was generated by CRISPR/Cas9. Shown are the protein domains of PPO3 and a partial sequence where the deleted nucleotides are marked red. (**B**) Structural modeling shows that the $PPO3^{1}$ mutation (orange portion) deletes most of the catalytic pocket, including copper-coordinating residues of the copper binding domain A (light blue). Protein domains are colored as in **(A)**. (**C**) Lifespan analysis of unchallenged flies reveals an increase in mortality rate of $PPO1^{\Delta},2^{\Delta}$ and $PPO1^{\Delta},2^{\Delta},3^{1}$ mutant flies but not $PPO3^{1}$ mutants. Each survival curve corresponds to 3 independent experiments with 20 flies each.

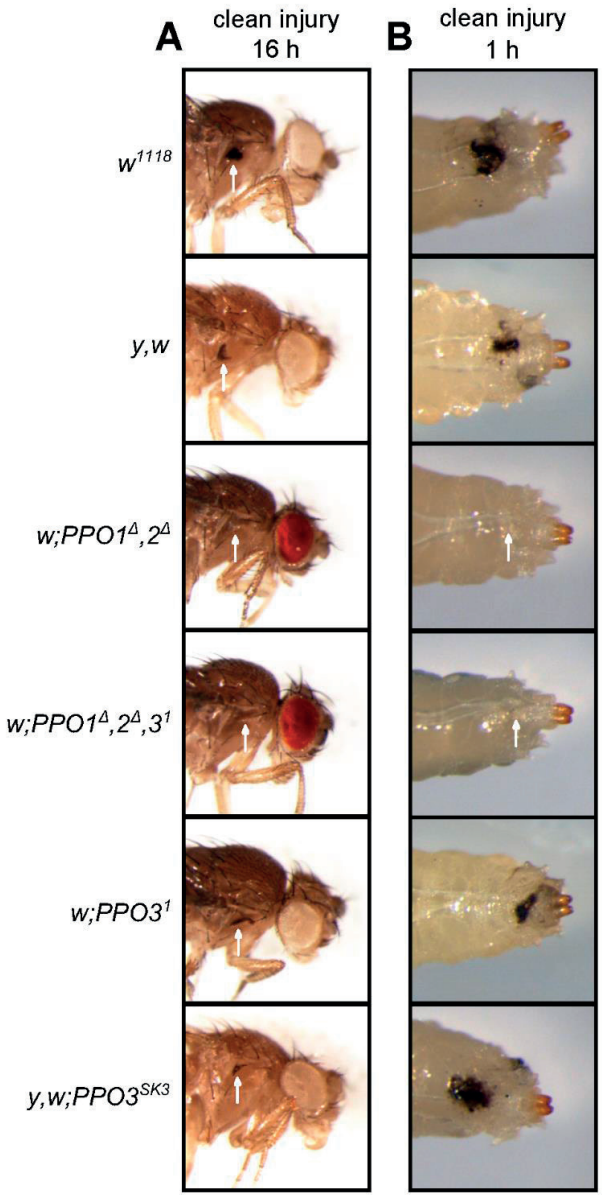


Figure 2 - Both PPO1 and PPO2 but not PPO3 contribute to injury related melanization in adults and larvae. Melanization of adults (**A**) and larvae (**B**) after clean injury is abolished only in the simultaneous absence of *PPO1* and *PPO2*. A normal melanization spot was observed in the two *PPO3* mutants. Arrows indicate the pricking site. Adults and larvae were wounded with a tungsten needle and blackening of the wound was recorded 1h later in larvae and 16h later in adults. A representative picture is shown for each genotype.

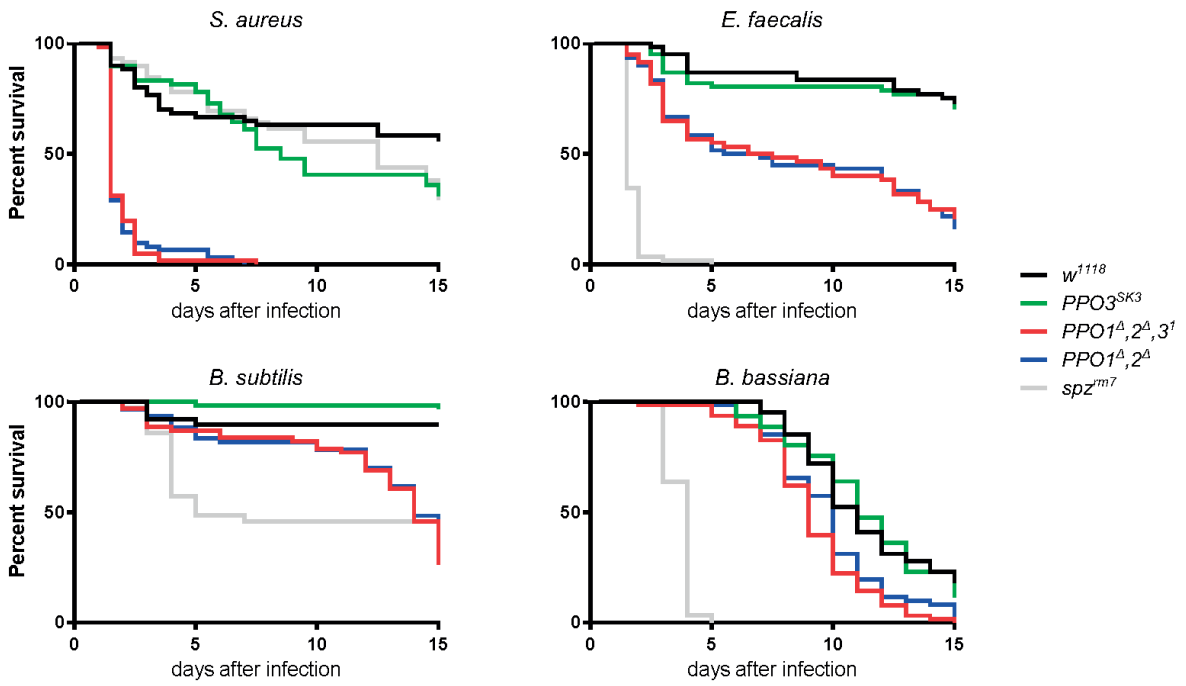


Figure 3 - Contribution of PPO1,2 and PPO3 to host defense against bacteria and fungi. Survival rate of flies following septic injury with Gram-positive Lysine-type bacteria (*S. aureus* and *E. faecalis)*, Gram-positive DAP-type bacterium (*B. subtilis)* and natural infection with the entomopathogenic fungus *B. bassiana*. Flies lacking the Toll ligand *spätzle* was used as immune deficient control. X-axis: Time post-infection in days; y-axis: Percentage of living flies. $PPO1^{\Delta},2^{\Delta}$ and $PPO1^{\Delta},2^{\Delta},3^{1}$ flies are less resistant to infections with *S. aureus* ($p < 0{,}0001$), *E. faecalis* ($p < 0{,}0001$), *B. subtilis* ($p < 0{,}0001$) and *B. bassiana* ($p < 0{,}0005$) compared to wild-type flies. Differences between $PPO3^{sk3}$ and w^{1118} do not reach statistical significance. Data were analyzed by Log-rank test.

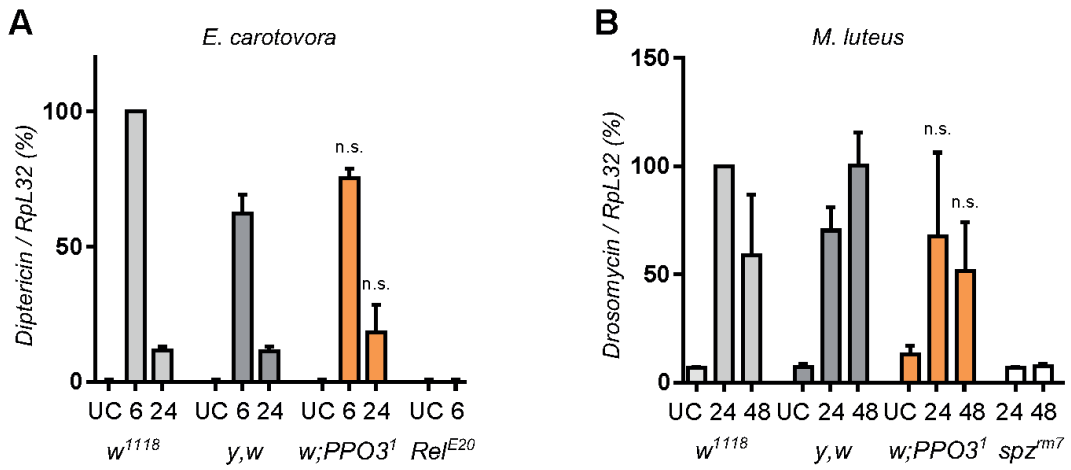


Figure 4 - PPO3 is not required for Toll and Imd pathway activities. (**A**) Expression of *Diptericin* (*Dpt*) in *PPO* mutant flies. Total RNA was extracted from animals either uninfected or collected 6h and 24h after septic injury with Gram-negative bacteria *E. carotovora*. Shown are the relative expression levels of *Dpt* in relation to *Rp49*. *PPO3*^{1} flies show normal induction of *Dpt* expression levels. The Imd pathway mutant *Relish* was used as an immune-deficient control. (**B**) Expression of *Drosomycin* (*Drs*) in *PPO3*^{1} mutant flies 24h and 48h after septic injury with the Gram-positive bacteria *M. luteus* shows that *PPO3*^{1} mutant flies have a normal induction of *Drs*. The Toll pathway mutant *spätzle*^{rm7} was used as an immune deficient control. Shown are the relative expression levels of *Drs* in relation to *Rp49*. 100% corresponds to *Dpt* or *Drs* expression level of wild-types flies 6h respectively 24h after septic injury. Data was analyzed using Mann-Whitney test, values represent the mean±s.d. of at least three independent experiments.

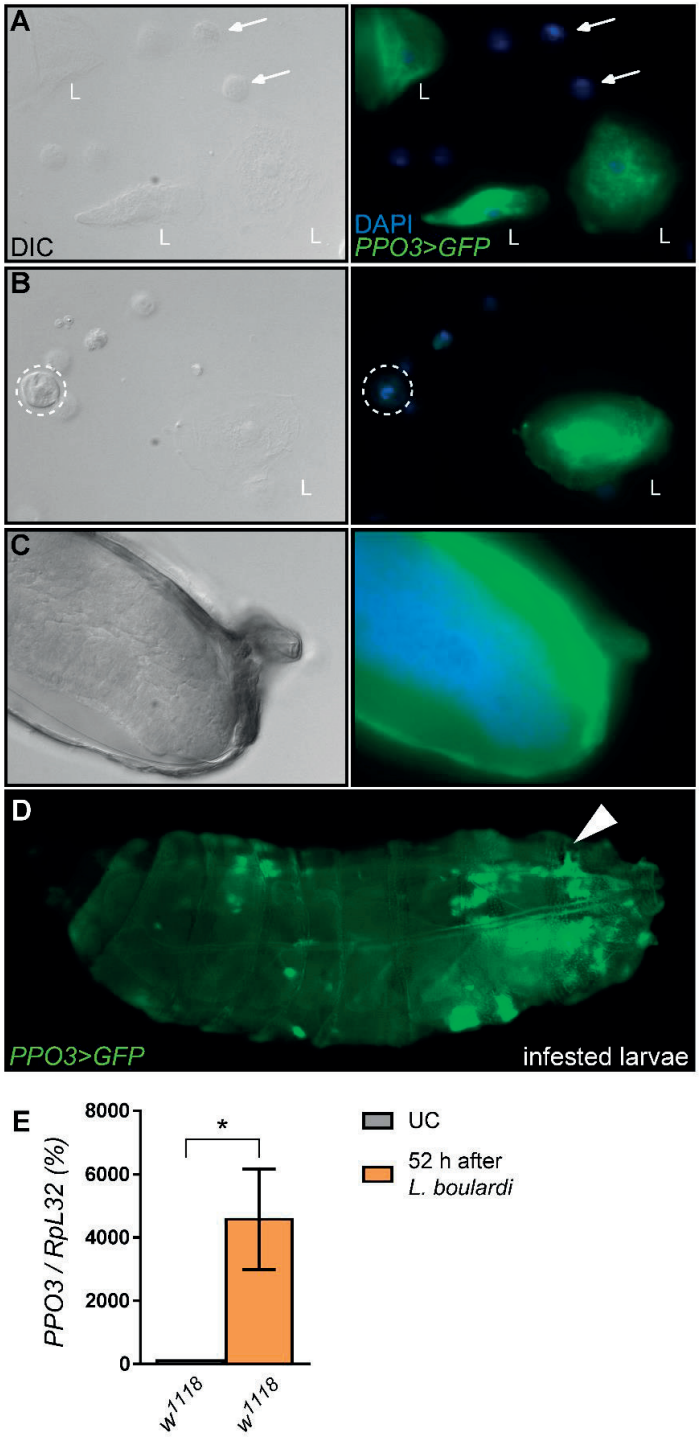


Figure 5 - PPO3 is specifically expressed in lamellocytes. Differential interference contrast (DIC) and GFP fluorescence micrographs of hemocytes from larvae infested with *L. boulardi* expressing a *PPO3-Gal4*, *UAS-GFP* construct show that PPO3 is

specifically expressed in all lamellocytes (L) either in circulation (**A, B**) or in the capsule surrounding the wasp egg (**C**). Plasmatocytes (arrows) and crystal cells (dashed line) do not express the reporter gene. GFP expression is green and DAPI staining is shown in blue. Overview of the whole infested larvae is shown in (**D**). Arrow indicates the site of the wasp larvae. Note that lamellocytes are found both around the egg and in circulation. (**E**) RT-qPCR shows that the *PPO3* gene expression is higher in larvae infested with *L. boulardi* compared to unchallenged larvae. 100% corresponds to *PPO3* expression levels of naïve larvae.

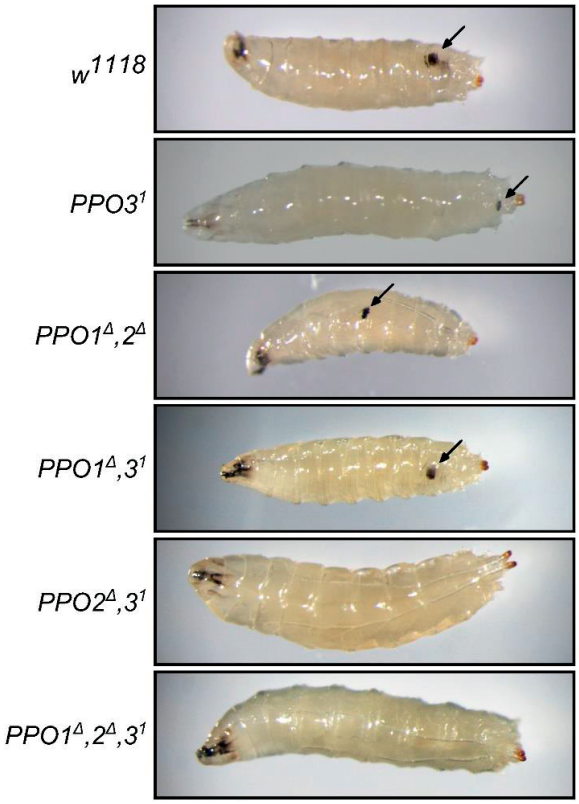


Figure 6 - Both PPO3 and PPO2 contribute to melanization around wasp capsule. Representative photos showing infested larvae containing eggs of *L. boulardi* parasitoid wasp. Larvae mutant for $PPO3^{1}$ or $PPO1^{\Delta},2^{\Delta}$ show a melanized egg. No melanized capsule was found $PPO2^{\Delta},3^{1}$ and $PPO1^{\Delta},2^{\Delta},3^{1}$ infested mutant larvae. The presence of a non-melanized egg in $PPO2^{\Delta},3^{1}$ and $PPO1^{\Delta},2^{\Delta},3^{1}$ mutant larvae was confirmed by subsequent dissection. Arrows indicate the position of the melanized wasp egg.

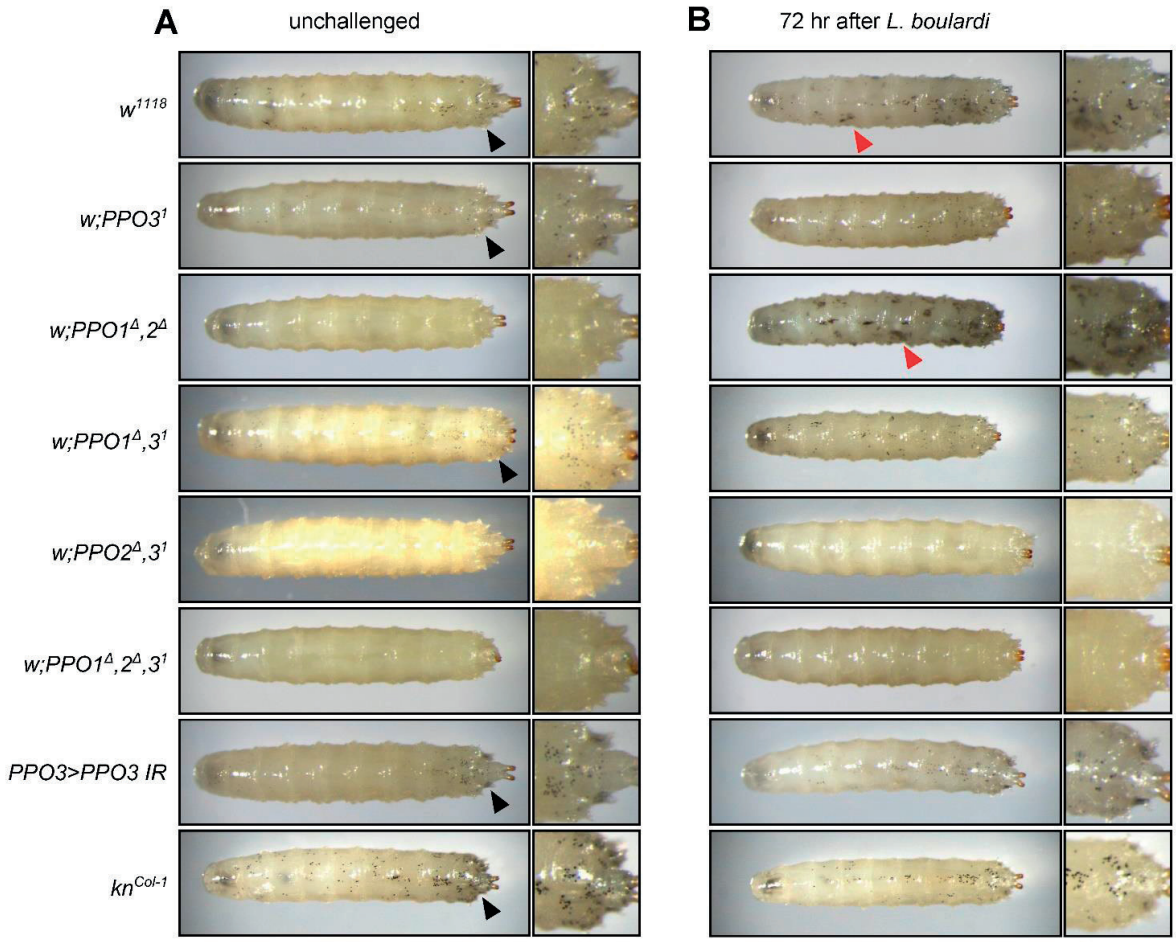


Figure 7 - Phenoloxidase activity in PPO mutant larvae upon wasp infestation as revealed by a heat-treatment. Representative photos showing unchallenged larvae (**A**) and larvae infested by *L. boulardi* parasitoid wasp after a heat treatment (**B**). Black dots on the cuticle correspond to melanized crystal cells due to PPO2 activity (Black arrows, absent in *PPO2* mutants). Large black patches corresponds to melanized lamellocyte due to PPO3 activity (Red arrows, absent in *PPO3* mutants). *Knot*$^{Col-1}$ larvae have no lamellocyte [28].

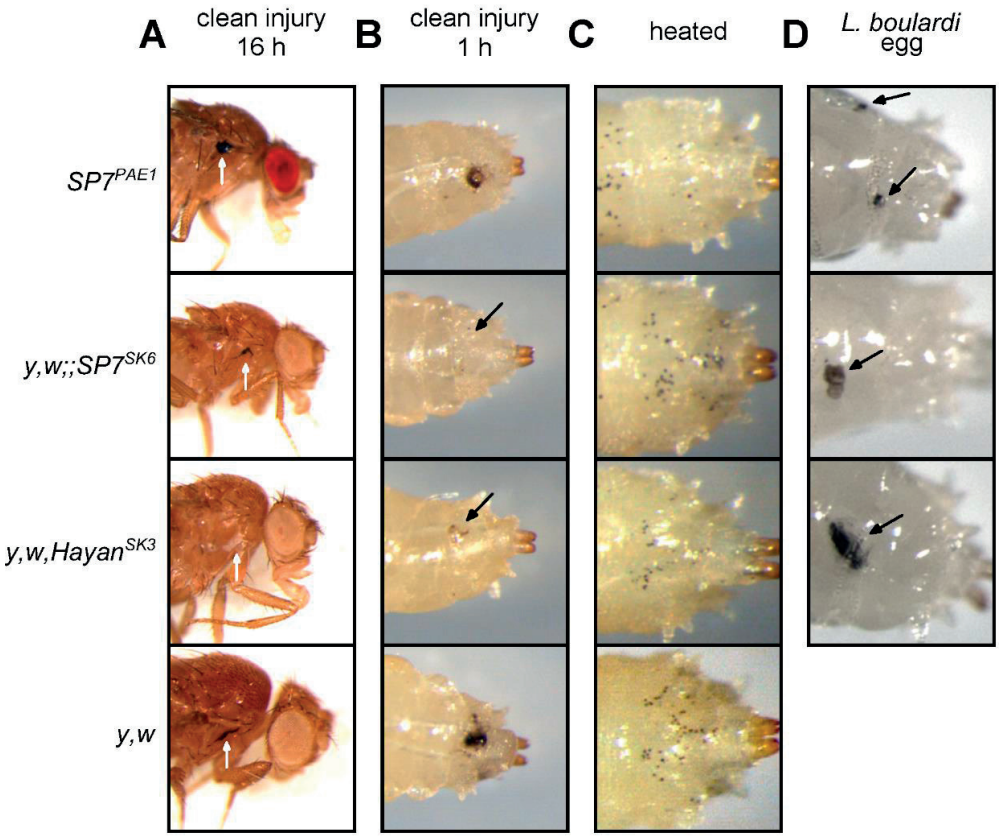


Figure 8 - Hayan and SP7 do not contribute to melanization of wasp capsule. Melanization of adults (**A**) after clean injury is abolished only in the *Hayan*^{SK3} mutant. A slightly reduced melanization spot was observed in the *Sp7*^{SK6} mutant. In contrast, after clean injury of larvae (**B**), melanization is abolished only in *Sp7*^{SK6} whereas *Hayan*^{SK3} shows residual melanization. Arrows indicate the pricking site. Adults and larvae were wounded with a tungsten needle and blackening of the wound was recorded 1h later in larvae and 16h later in adults. After heat treating larvae (**C**) black dots, corresponding to crystal cells, appeared in *Hayan*^{SK3} and *SP7*^{SK6} comparable to wild type controls. Infested larvae containing eggs of *L. boulardi* parasitoid wasp (**D**) show the presence a melanized egg in *Hayan*^{SK3} and *SP7*^{SK6}. Arrows indicate the position of the melanized wasp egg. A representative picture is shown for each genotype.

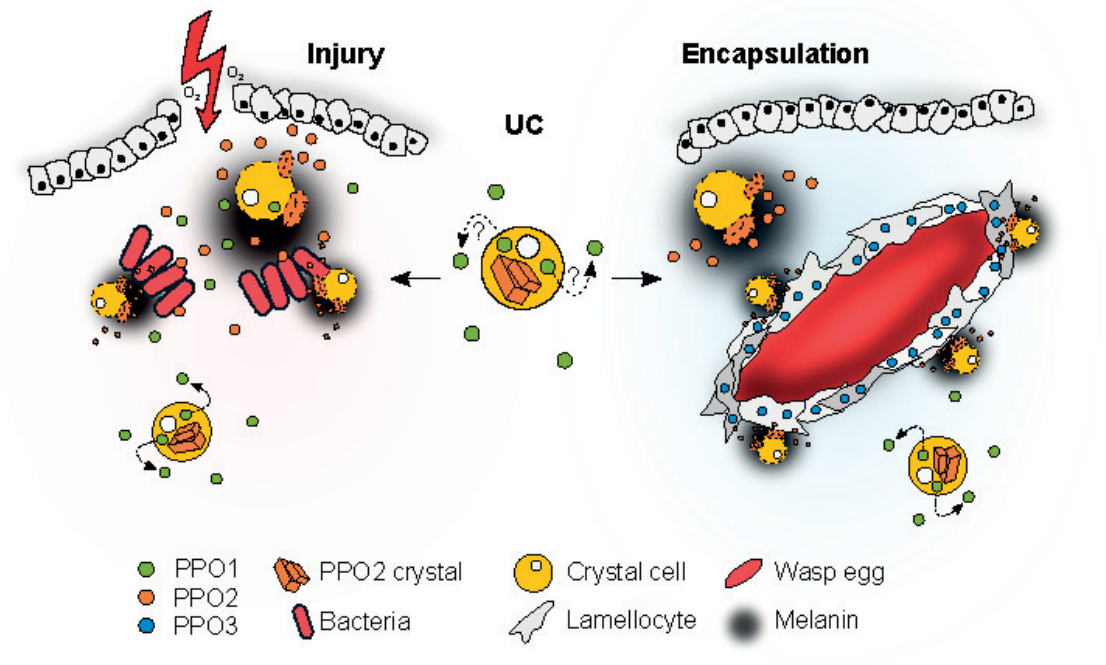
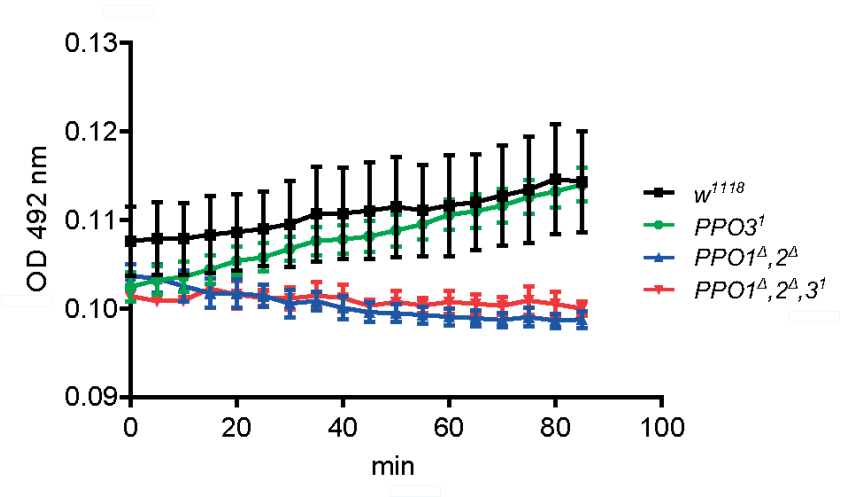


Figure 9 - Model of phenoloxidase activation after injury and wasp infestation

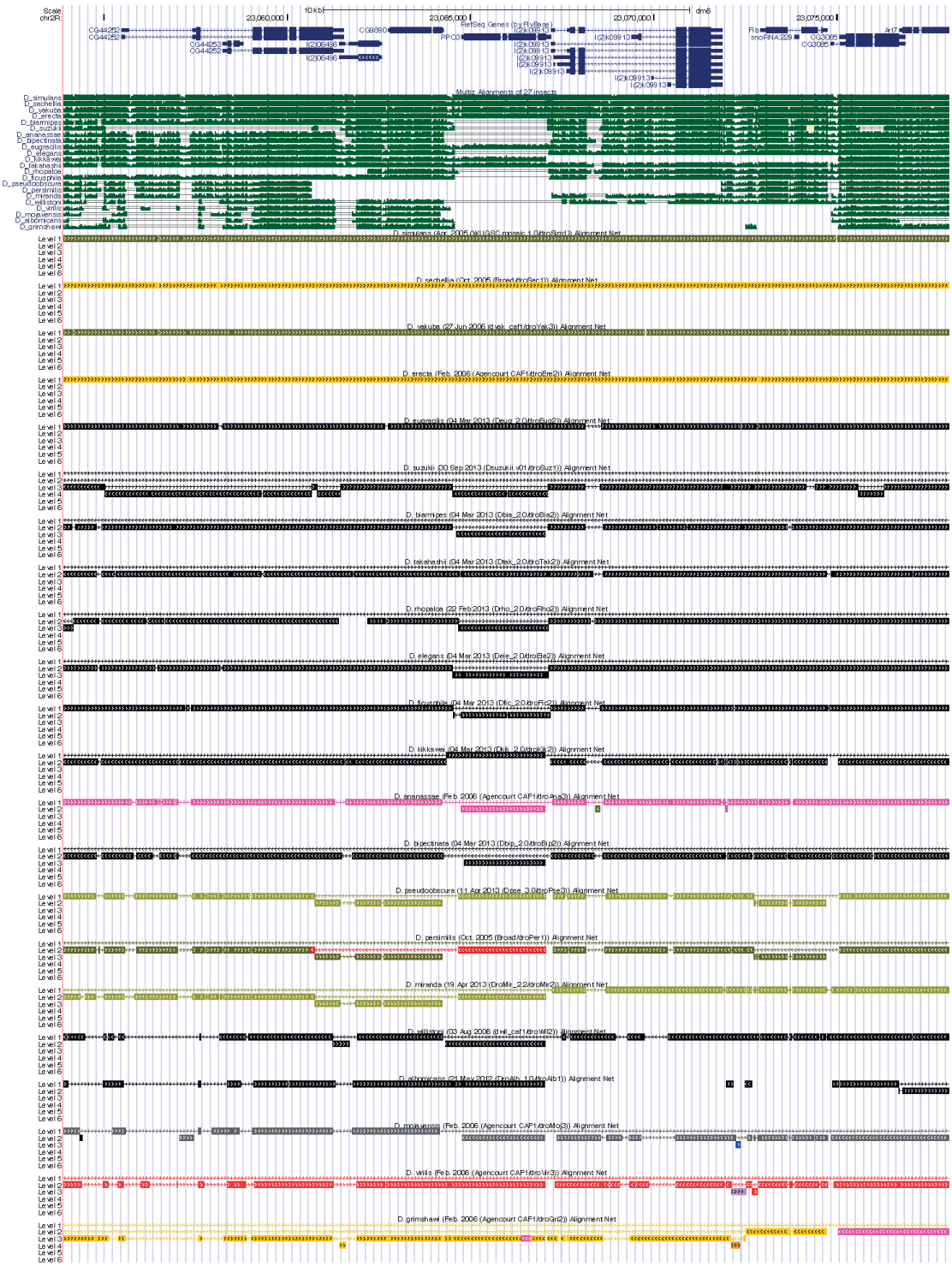
Both PPO1 and 2 are synthetized by crystal cells. In unchallenged condition PPO2 is stored in the crystal cells while the localization of PPO1 is still unknown (here shown in the hemolymph) (middle). After clean or septic injury, PPO1 and PPO2 are the only source of hemolymphatic PO activity (left). After parasitic infestation, PPO3 is produced by lamellocytes and contributes with PPO2 to the melanization around the wasp egg during encapsulation (right).

Additional files



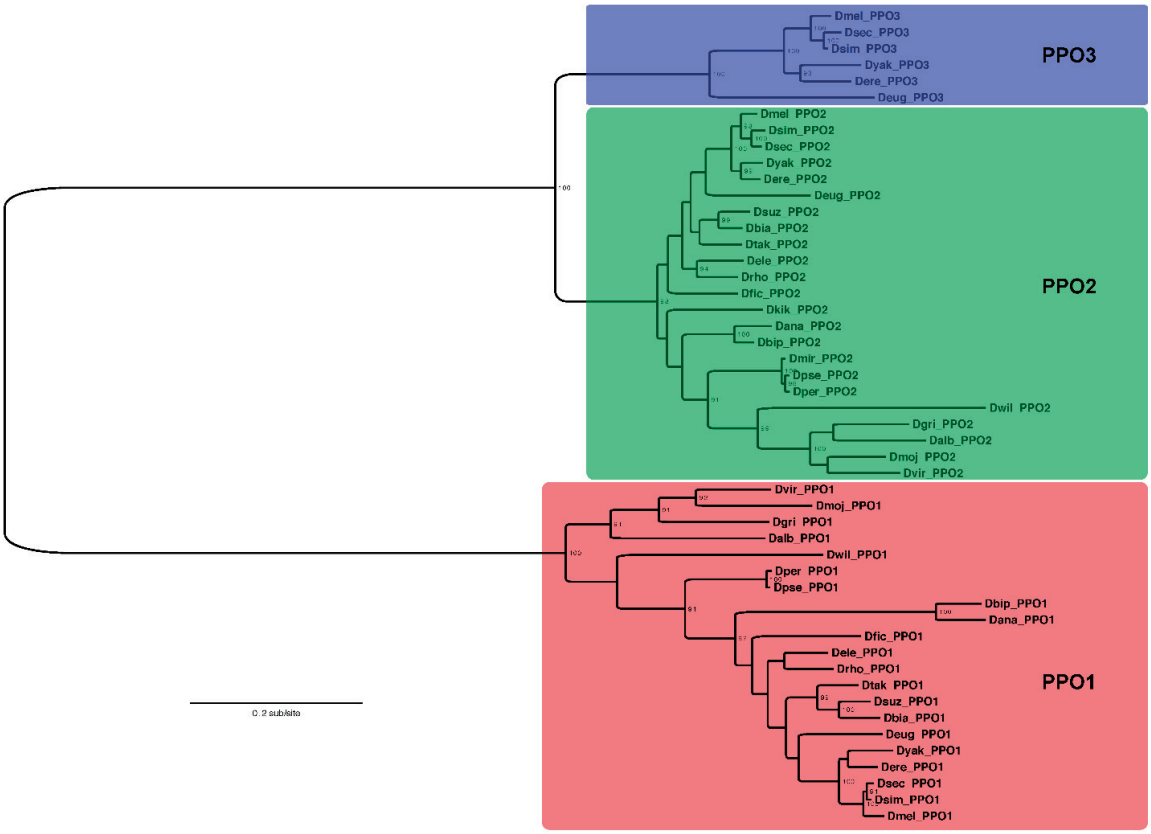
Additional file 1: Figure S1 - PPO3 does not contribute to hemolymph PO activity.

Hemolymph PO activity of unchallenged larvae was monitored by L-DOPA assay. Spontaneous PO activity is not affected in *PPO3*^{1} mutant larvae compared to wild-type. Hemolymph of wandering larvae was collected and examined for PO activity by transferring L-DOPA to dopachrome. Hemolymph of *PPO1,2,3* mutant larvae was used as negative control. Differences between *PPO3*^{1} and *w*^{1118} wild-type control do not reach statistical significance. Data were analyzed by Two-way ANOVA with Tukey correction. Values represent the mean±sem of three independent experiments.



Additional file 2: Figure S2 – Comparative genomics of the *PPO3* locus.

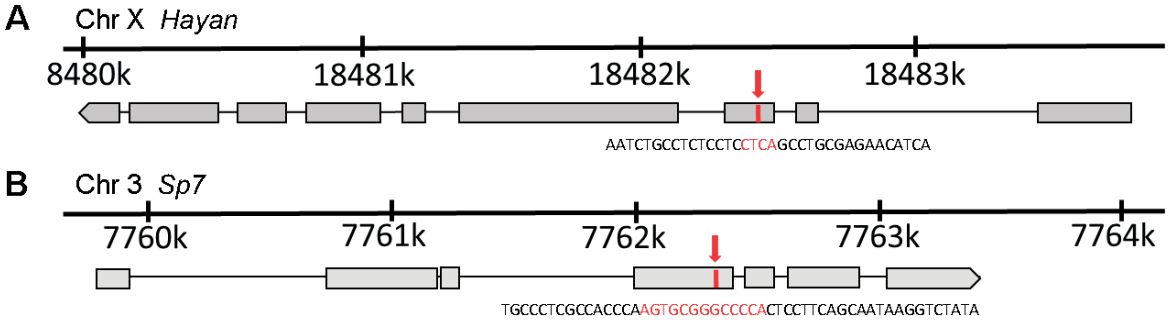
Shown are the best pairwise genome alignments for 22 *Drosophila* species versus *D. melanogaster* based on the UCSC genome browser net tracks. Species in the *D. melanogaster* group plus *D. eugracilis* have only one net that aligns contiguously the entire *PPO3* microsyntenic region. Conversely, other species (besides *D. takahashii*) have a gap in the alignment at *PPO3* that is filled by a secondary net that arises from alignment to paralogous sequences elsewhere in the genome. *D. takahashii* has only a single alignment net in the *PPO3* region, however this net is fragmented precisely in the *PPO3* gene, consistent with the possible presence of non-canonical *PPO3*-like gene sequences in this species.



Additional file 3: Figure S3 - Phylogenetic tree of PPO genes from Drosophila species.

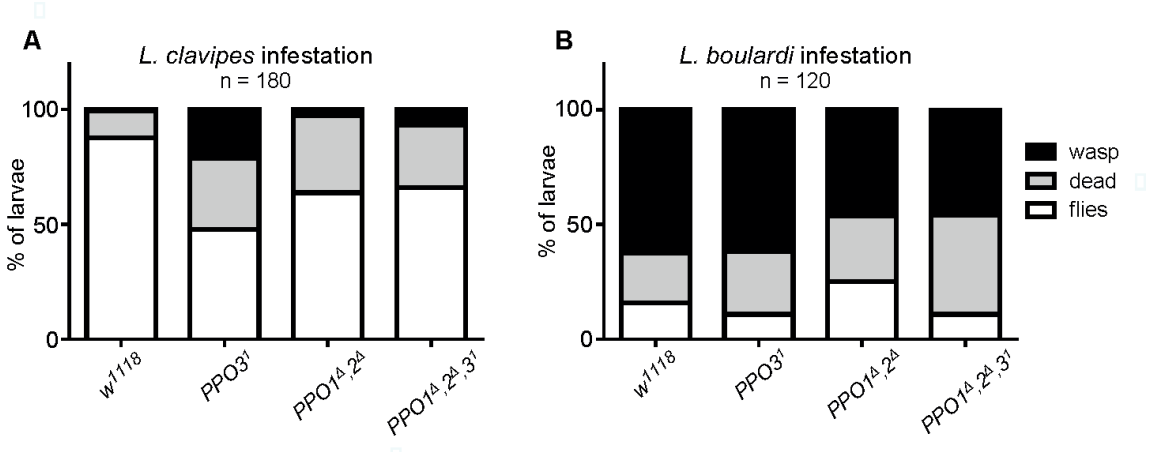
A maximum likelihood phylogenetic tree was constructed with the exonic sequences of *PPO1*, *PPO2* and *PPO3* using RAxML. Nodes with more than 90% bootstrap support are labeled. Branch lengths are in substitutions per site. The tree is midpoint rooted and based on a multiple alignment of all *PPO* genes from *D.*

melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. eugracilis, D. suzukii, D. biarmipes, D. takahashii, D. rhopaloa, D. elegans, D. ficusphila, D. kikkawai, D. bipectinata, D. ananassae, D. pseudoobscura, D. persimilis, D. miranda, D. willistoni, D. mojavensis, D. virilis, D. albomicans and *D. grimshawi*. The placement of the root in our tree is supported by a previous phylogenetic analysis of *PPO* genes across arthropods [8].



Additional file 4: Figure S4 - Schematic representation of novel CRISP/Cas9 mutants.

Overview of the Hayan (**A**) and *Sp7* (**B**) gene. Arrows indicating the position of the mutation. Partial sequence is shown, where deleted nucleotides of $Hayan^{SK3}$ respectively $Sp7^{SK6}$ are marked in red. The gene map was adapted from FlyBase.



Additional file 5: Figure S5 - Survival analysis to *L. clavipes* and *L. boulardi* wasp infestation in wild type and *PPO* mutants. Synchronized L2 larvae were exposed to gravid female *L. clavipes* (**A**) or *L. boulardi* (**B**) wasps in custom infection traps, re-

isolated and cultured at room-temperature until wasps emerged. Vials were scored daily for total number numbers of eclosed flies and wasps. *PPO3* mutant larvae show enhanced susceptibility against *L. clavipes*, whereas the loss of all PPO does not further increase this effect. The mild effect of PPO mutants on wasp encapsulation led us hypothesize that an optimal encapsulation required a precise level of phenoloxidase activity due to possible toxic effect on the host.

8 MORE THAN BLACK AND WHITE: COMPLEX RELATIONSHIPS INVOLVING SERINE PROTEASES REGULATE THE TOLL PATHWAY AND THE MELANIZATION RESPONSE IN DROSOPHILA

J.P. Dudzic, M.A. Hanson, I. Iatsenko, S. Kondo, and B. Lemaitre. More than black and white: complex relationships involving serine proteases regulate the Toll pathway and the melanization response in Drosophila. Manuscript under review in Cell Reports, 02.08.2018. Pre-published in bioRxiv.org: doi: https://doi.org/10.1101/383257.

More than black and white: complex relationships involving serine proteases regulate the Toll pathway and the melanization response in *Drosophila*

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Abstract:

The melanization response is a rapid and important defense mechanism in arthropods. Melanin is produced around wound sites and invading microorganisms by phenoloxidases (POs), which need to be activated by the sequential activation of an extracellular serine protease (SP) cascade. *Drosophila melanogaster* has been a useful genetic model for dissecting insect immune signaling, but understanding these proteolytic cascades has

been complicated by the large number of SP genes, possibly with redundant function. Taking advantage of recently-generated null and compound mutants, we re-investigated the role of SPs involved in the melanization response in *D. melanogaster* and discovered phenotypes previously concealed in single mutant analysis. We found that two of them, Hayan and Sp7, can activate the melanization response in two different manners: Hayan is required in the local blackening of wound sites, while Sp7 regulates an alternate melanization reaction responsible for the clearance of septic infections with *Staphylococcus aureus*. We present evidence that both Sp7 and Hayan regulate the Toll NF-κB pathway. Sp7 is regulated by canonical Toll signaling downstream of PGRP-SA, ModSP, and Grass, leading to control of septic infections via a Sp7-dependent melanization response. Additionally, we found that Hayan and the Toll-regulating SP Psh are the result of a recent gene duplication. Using genetic manipulations, we reveal the hidden role for Hayan, alongside Psh, in propagating Toll signaling downstream of pattern recognition receptors. Thus, we describe the existence of two pathways leading to the melanization response and reveal previously unknown dynamics in the activation of the Toll pathway.

Introduction:

Sequential activation of extracellular serine protease (SP) cascades regulates important aspects of insect innate immune reactions, notably 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*, the Toll pathway mediates resistance to Gram-positive bacteria and fungi by regulating a subset of antimicrobial peptides in the fat body. Unlike mammalian Toll-like receptors that function as pattern recognition receptors, the *Drosophila* Toll receptor does not interact directly with microbial determinants and is instead activated by a cleaved form of the secreted molecule Spätzle (Spz) (Lemaitre et al., 1996; Weber et al., 2003). The immune-regulated CLIP-domain SP Spätzle processing enzyme (SPE) 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 pattern recognition receptor (PRR) and Persephone (Psh) pathways. In the PRR pathway, PRRs involved in the sensing of Gram-positive bacteria (GNBP1 or PGRP-SA) or fungi (GNBP3) bind to their respective microbial ligands to activate an upstream SP, ModSP, which then leads to the activation of the SP Grass, leading to the maturation of SPE (Buchon et al., 2009; Chamy et al., 2008; Gobert, 2003; Gottar et al., 2006). In the Psh pathway, infectious agents are detected directly through the cleavage of the protease bait region of the SP Psh by microbial proteases, leading to Spz cleavage by SPE (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 microbicidal 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 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, while the role of the lamellocyte-derived PPO3 is confined to encapsulation (Binggeli et al., 2014; Dudzic et al., 2015; Nam et al., 2008). Thus far, 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). While a null mutation in *Hayan* totally abolishes melanization in adults, a null *Sp7* mutation results in only a slight reduction (Dudzic et al., 2015). The positions of these SPs in the melanization cascade has not been fully established, although Hayan and Sp7 have been shown to 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 the same or 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). However, a common opinion is that the SPs regulating the Toll pathway in *Drosophila* upon infection are independent of the melanization cascade and vice versa. Nevertheless,

the melanization and Toll pathways interact, as the Toll pathway regulates the expression of many genes encoding SPs and serpins involved in the melanization pathway (De Gregorio, 2002; De Gregorio et al., 2002; Ligoxygakis, 2002). Moreover, there has been disparate 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 new mode of systemic infection using a low dose of *Staphylococcus aureus* (*S. aureus*) where the survival of flies relies entirely on the melanization response, but not on expression of antimicrobial peptides or 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, while the Sp7-dependent melanization response is mediated by Toll PRR signaling, which diverges at Grass to activate either SPE or Sp7. 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 previously unappreciated role for both Hayan and Psh in Toll signaling.

Results:

Melanization is important to survive *Staphylococcus aureus* infection

While reviewing survival data from Binggeli et al. (2014), we confirmed that survival of *D. melanogaster* 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* (OD 0.5, 1-100 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 (Fig. 1A). Analysis of the bacterial load of $PPO1^{\Delta},2^{\Delta}$ flies reveals that melanization-deficient flies already have a higher bacterial burden 6 hours (h) after infection compared to their wild-type (w^{1118}) counterparts (Fig. 1B). Their inability to

control *S. aureus* growth is even more prominent 24 h after infection. Following injection of *GFP*-producing *S. aureus, PPO1$^{\Delta}$,2$^{\Delta}$* flies do not melanize the wound (Fig. 1C arrows) and exhibit a local GFP signal after 18 h, indicating *S. aureus* growth at the wound area (Fig. 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 (Fig. 1C). This contrasts with wild-type flies, which deposit melanin at the wound area and control bacterial growth over 24 h (Fig. 1D). The importance of the melanization response to resist *S. aureus* infections is further illustrated by the observation that *PPO1$^{\Delta}$,2$^{\Delta}$* flies show no defect in Toll or IMD pathway activation after *S. aureus* infection. Compared to wild-type flies, they even show a ~7x higher expression of the Toll-activity readout *Drosomycin* (*Drs*) as well as ~8x higher expression of the IMD-activity readout *Diptericin* (*Dpt*) (Fig. S1A,B), likely due to unconstrained bacterial growth. To rule out the involvement of hemocytes in the resistance to *S. aureus*, we produced hemoless flies using a plasmatocyte-specific gal4 (*hml$^{\Delta}$-Gal4*) to express the pro-apoptotic gene *bax* (Defaye et al., 2009), and additionally 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 like the wild-type (Fig. S1C). Our results contrast with previously published data showing that hemocytes are critical to survive *S. aureus* infections via phagocytosis (Defaye et al., 2009; Garg and Wu, 2014). We attribute this discrepancy to using a low dose of *S. aureus* that is controlled specifically by the melanization response but not phagocytes.

Cytotoxic by-products of the melanization reaction include reactive oxygen species (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 a time-span of 6 hours following *S. aureus* infection in wild-type flies. Interestingly, *PPO1$^{\Delta}$,2$^{\Delta}$* flies show a non-significant but consistent reduction in H_2O_2 levels compared to wild-type flies (Fig. S1D).

Taken together, these results demonstrate that the melanization response is critical to resist a low dose of *S. aureus* infection consistent with a previous study (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 are 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) that were previously described to be involved in melanization. For this, we used a newly generated CRISPR null mutant for *MP1* (*MP1^{SK6}*, this study), 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 immune phenotype characterization is further described in Fig. 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*, while *Hayan* deficient flies behave like wild-type (Fig. 2A). By injecting GFP-producing *S. aureus* in flies lacking these serine proteases, we observed that *Sp7^{SK6}* flies fail to restrict *S. aureus* growth first locally after 18 h, then systemically after 24 h (Fig. 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 (Fig. 2D, arrows).

These results were surprising since *Hayan*, but not *Sp7* mutants have reduced melanization at the wound site (hereon referred to as the “blackening” reaction) after clean injury (CI) (Fig. S2A) and *S. aureus* (Fig. 2C,D) (Dudzic et al., 2015; Nam et al., 2012) We then investigated the role of Sp7 and Hayan in the melanization of the hemolymph in both adults and larvae. Extracting hemolymph with subsequent incubation at room temperature leads to blackening, due to PO-dependent melanin production. Surprisingly, hemolymph from both adults and larvae shows that *Hayan^{SK3}* mutants fail to produce melanin, while hemolymph of *Sp7^{SK6}* mutants turns black over time (Fig. 2B for larval hemolymph, data not shown for adults). Together, these results disconnect the blackening of the hemolymph from a melanization reaction-dependent clearance of *S. aureus*, suggesting that intermediate metabolites in the melanization reaction contribute to microbial control.

Collectively, our results show that Sp7*,* but not Hayan*,* controls *S. aureus* and that the underlying resistance mechanism does not involve melanin deposition, a terminal step in the melanization cascade. This suggests that by-products of the melanization reaction, such as ROS or other metabolic intermediates, might be the active molecules controlling bacterial growth. We also reveal the complexity of the PPO cascade 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 or by the presence of microbial products. To further explore the relationship between the blackening reaction and the two SPs, we compared the blackening reaction at the wound site upon clean injury or septic injury with the avirulent Gram-positive bacterium *Micrococcus luteus*. For this, we categorized the blackening reaction of the cuticle into three levels: strong, weak and none. An example for each category is given in Fig. S3. For wild-type w^{1118} flies, after clean injury 91.8% 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, Fig. 3A grey columns), while *Hayan* mutants were almost deficient for the blackening reaction (0% strong, 38.9% weak, 61.1% none, p < 0.0001). Interestingly, some cuticle blackening is recovered in *Hayan* mutants upon septic injury with *M. luteus* (16.2% strong, 64.6% weak, 19.2% none, p < 0.0001, Fig. 3A red columns). Thus, Hayan is not solely responsible for the blackening reaction, as the presence of bacteria can induce cuticle blackening in a Hayan-independent manner.

In *Drosophila,* two PPOs, PPO1 and PPO2, produce the bulk of hemolymph PO activity as no blackening is observed in *PPO1, PPO2* deficient mutants. However, it is unclear whether these PPOs are activated by the same SP or are differentially regulated**.** To further understand how the SPs Hayan and Sp7 relate to PPO1 and PPO2, we generated double mutants and subsequently analyzed their melanization capabilities upon clean injury and *M. luteus* infection. 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, $PPO1^{\Delta}$,$Sp7^{SK6}$ and $PPO2^{\Delta}$,$Sp7^{SK6}$ retain a blackening reaction, only slightly reduced compared to $PPO1^{\Delta}$ *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 (Fig. 3B). In contrast, $Hayan^{SK3}$;$PPO1^{\Delta}$ double mutants fail to blacken, even upon septic injury with *M. luteus*, which is not the case for $Hayan^{SK3}$;$PPO2^{\Delta}$ (Fig. 3B). As Hayan is not responsible for the additional blackening we see upon septic injury (Fig. 3A red), this indicates that another SP acts on PPO1, possibly Sp7, after exposure to Gram-positive bacteria. Finally, we

observed that flies, double mutant for both *Hayan* and *Sp7* have strongly reduced but still detectable levels of the blackening reaction (Fig. 3C). This suggests the existence of a third, although minor, branch for activating PPO that leads to blackening of the wound site. A summary of these results is shown in Table 1.

Together, these results demonstrate that cuticle blackening after clean injury relies strongly on the presence of Hayan, acting through both PPO1 and PPO2. In the absence of Hayan, cuticle blackening can be partially restored by septic injury with the Gram-positive *M. luteus*, and this relies on PPO1 and on the presence of Sp7.

Extracellular components of the Toll pathway are required to activate melanization and to survive *S. aureus* infections

Previous studies have suggested a link between the melanization and Toll pathways (Ligoxygakis, 2002; Matskevich et al., 2010), the latter being critical to resist infection to Gram-positive bacteria in *D. melanogaster* (Leulier et al., 2000; Rutschmann et al., 2000). Currently, how the Toll pathway can impact melanization is not well understood. Our finding that the Gram-positive bacteria *M. luteus* activates blackening in the absence of Hayan prompted us to investigate the role of pattern recognition receptors (PRRs) in melanization. First, we monitored the survival of flies lacking upstream or downstream components of the Toll pathway using our melanization-sensitive *S. aureus* 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 (Fig. 4A). In contrast, flies lacking GNBP3, a PRR sensitive to fungal ß-glucans, resist as wild-type to *S. aureus* infection. Importantly, *spätzle* (spz^{rm7}) flies lacking intracellular Toll signaling, exhibit wild-type resistance to *S. aureus* infection (Fig. 4B). This indicates that the high susceptibility of flies lacking PGRP-SA and GNBP1 is not linked to the intracellular Toll pathway, which regulates the Toll transcriptional output. We therefore analyzed the role of the four serine proteases upstream of Spz in the Toll pathway, ModSP, Grass, SPE, and Psh, for their role in resistance to *S. aureus*. 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 (Fig. 5A). We conclude that a subset of the extracellular components functioning upstream of the Toll ligand, namely PGRPS-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 to activate the melanization pathway. This is consistent with studies in other insects that have shown that melanization is controlled by the same SPs that regulate Toll (An et al., 2009; Kan et al., 2008). Thus, ModSP and Grass could 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. Consistent with our hypothesis that ModSP contributes to Hayan-independent blackening, $Hayan^{SK3}$;;$ModSP^{1}$ mutants failed to show cuticle blackening regardless of treatment (Fig. 5B). On the contrary, $Sp7^{SK6}$, $ModSP^{1}$ mutant flies showed a similar level of blackening reaction found in *Sp7* mutants alone (Fig. 5B).

We conclude that at least two independent pathways contribute to cuticle blackening in *D. melanogaster* adults: one involving Hayan and another involving PGRP-SA, GNBP1, ModSP, Grass and Sp7.

Neither Hayan nor Sp7 alone is required to activate Toll signaling

We found that components of the Toll pathway directly regulate the melanization response. We then tested if the two SPs involved in the melanization pathway, Sp7 or Hayan, could be involved in the activation of the Toll pathway by Spätzle. Consistent with previous studies (Nam et al., 2012; Tang et al., 2006), neither *Sp7* nor *Hayan* single mutants reduce Toll pathway activation after *M. luteus* infection as monitored by the expression of *Drs* (Fig. 6A). Of note, we observed a mild overactivation of the Toll pathway in *Sp7* mutant flies upon *M. luteus* infection compared to wild-type (Fig. 6 A). Additionally, no effect on the activation of the Toll pathway could be observed in in *Sp7* nor *Hayan* mutant flies upon *Candida albicans* infection (Fig. S4A). We conclude that Hayan and Sp7 alone are not required for Toll pathway activation by bacteria or fungi.

Evidence of a close evolutionary relationship between Hayan and Psh

Hayan and the Toll-regulating SP *psh* are only 751 bp apart on the *D. melanogaster* X chromosome. This stimulated us to investigate the possible relationship between Hayan and Psh. We performed a phylogenetic analysis of all *D. melanogaster* CLIP-domain SPs listed in Veillard et al. (2015) using protein sequences from the conserved catalytic domain. We found that Hayan and psh form a closely-related monophyletic lineage amongst CLIP-domain SPs (Fig. 7 A). Interestingly, it appears that *psh* is a lineage-restricted duplication found only in Melanogaster group flies, arising from an ancestral *Hayan* gene, the latter being conserved across the genus *Drosophila* (Fig. 7B and Fig. S5A). We found that the Psh protease "bait region", a region prone to cleavage by pathogen proteases (Issa et al., 2018), is also present in Hayan (Fig. S5B). Furthermore, we recovered a striking similarity in transcript structure of *psh*-RA and *Hayan*-RA, where alternative splicing excludes the fourth exon found in *Hayan*-RB and *Hayan*-RD (Fig. 7 C). This 4th exon encodes a "*Hayan*-exclusive domain," not found in other *D. melanogaster* CLIP-domain SPs. Nevertheless, this alternative splicing is conserved in FlyBase v2018-02 annotations for *Drosophila pseudoobscura* (subgenus Sophophora) (Fig. S6A), and we independently confirmed this pattern across the genus *Drosophila* using RT-PCR (Fig. S6B). Thus, alternative splicing of *Hayan* is an evolutionarily conserved mechanism to produce *Hayan* transcripts either similar to *psh*, or exclusive to *Hayan*. To shed light on the functional regions of this *Hayan*-exclusive domain, we extracted *Hayan* sequences from diverse *Drosophila* and used FEL and SLAC to infer codons under selection (Delport et al., 2010). We found multiple sites under purifying selection ($p < .05$, Fig. S6C, Supplementary Data File 1), forming largely-conserved motifs corresponding to *D. melanogaster Hayan*-RB residues from A^{201}-P^{206}, R^{226}-P^{234}, L^{261}-V^{282}, and D^{303}-G^{312}. We did not find homologues for the *Hayan*-exclusive domain outside *Drosophila* in GenBank, UniProt, and Pfam databases. Globally, we conclude that *psh* is a recent duplication of *Hayan*, lacking the *Hayan*-exclusive domain. Furthermore, transcripts from both genes can encode highly similar proteins.

Hayan and Psh redundantly regulate Toll pathway activation by the PRR and Psh branch

The duplication producing *Hayan* and *psh* is quite recent raising the possibility that both genes share an overlapping function that would be masked in single mutant analyses. This

prompted us to analyze the combined effect of *Hayan* and *psh* mutations by generating a deficiency that removes both genes by CRISPR referred to as *Hayan-psh^{Def}*. In these experiments, we also use a newly generated null *psh^{SK1}* mutants. *Hayan-psh^{Def}* mutant flies are viable and show no overt morphological defects. Strikingly, we found that *Hayan-psh^{Def}* flies fail to activate the Toll pathway upon *M. luteus* infection in contrast to single mutants (Fig. 6 B). Similarly, *Hayan-psh^{Def}* mutants also fail to activate Toll after *C. albicans* infection and *Bacillus sp.* protease injection (Fig. S4B,C). Interestingly, while *Hayan^{SK3}* mutants show wild type levels of *Drs* expression after protease injection, *psh^{SK1}* flies suppress *Drs* to ~35% of wild-type levels. Only in the simultaneous absence of both Hayan and Psh is *Drs* expression suppressed to *spz^{rm7}* levels (Fig. S4C). This suggests that Hayan also contributes to the Psh pathway. Thus, both Hayan and Psh, redundantly regulate the Toll pathway downstream of PRRs. We note that Hayan and Psh cluster phylogenetically with the SP Snake (Fig. 7A), a protease that in dorso-ventral Toll signaling cleaves the terminal SP that cleaves Spätzle (Dissing, 2001; LeMosy et al., 2001; Lindsay and Wasserman, 2014). This suggests Hayan and Psh could play a similar role upstream of SPE. Consistent with these observations, expression of an activated form of *Hayan* using a fat body Gal4 driver (*c564*) leads to upregulation of the Toll readout *Drs* in the absence of infection (Fig. 6 C), as previously shown for expression of *psh* (Jang et al., 2006)**.** Finally, re-introduction of the *Hayan-psh* genomic region in double mutants using a Flyfos transgene (Sarov et al., 2016) rescues *Drs* expression to wild-type levels after exposure to *M. luteus* (Fig. 6 D). Collectively, our double mutant analysis reveals an unexpected role of Hayan and Psh in the pattern recognition pathway regulating Toll activity.

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. While the *Drosophila* immune response has been studied intensely, our knowledge of the melanization reaction has lagged behind. This is in part because *Drosophila* immunity has traditionally focused on immune modules conserved in mammals, but the overwhelming complexity of serine protease cascades has also prevented a clear understanding. Indeed, insect genomes harbor an incredible diversity of serine proteases, whose function remains largely

uncharacterized (Cao et al., 2015; Ross et al., 2003b). In this study, we provide new insights on the PO cascade and uncover a new relationship between the Toll and melanization pathways.

Melanization is more than the blackening of the wound area

While 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 infection model using a low-dose inoculation of the Gram-positive bacteria *S. aureus*, which we find is especially appropriate to study melanization. Strikingly, flies deficient for PPOs, but not flies with impaired AMP production or lacking hemocytes, rapidly succumb to this challenge. The melanization response restricts the growth of *S. aureus* preventing its systemic dissemination. Furthermore, our study shows a disconnect between resistance to infection and the blackening of the wound site. While 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 in adults. This indicates that it is not the blackening *per se* that is involved in the control of *S. aureus*, but rather other reactions downstream of PPO activity. For instance, the melanization response is associated with the production of cytotoxic molecules like ROS (reviewed in 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 (Fig. 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 at the level of or downstream of *Grass* to regulate the melanization pathway

Studies in other insects such as *Manduca sexta* and *Tenebrio molitor* have shown that melanization and the Toll pathway share a common upstream activation mechanism that then separates downstream of SPE-like SPs that are capable of cleaving both Spz and PPO (An et al., 2009; Kan et al., 2008; Kim et al., 2008). Prior to our results, there was only indirect evidence in *D. melanogaster* that the Toll pathway activates melanization (Matskevich et al., 2010). It was however noted that Toll regulates many SPs and serpins involved in the melanization cascade at transcriptional levels (De Gregorio et al., 2001; Ligoxygakis, 2002). In addition, over-expression of several SPs functioning upstream of Spz, or a gain-of-function activation of the Toll pathway leads to spontaneous melanization (An et al., 2013; Gerttula et al., 1988; Lemaitre et al., 1995b; Tang et al., 2006). Some studies have also reported 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 PRR-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 after exposure to Gram-positive bacteria. Our double mutant analysis suggests that the PRR cascade diverges downstream of Grass to activate Sp7 and PPO1. 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. 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). At this stage, it cannot be excluded that MP1 contributes to melanization in a redundant way. Alternatively, results obtained with RNAi may be the consequence of off-target effects that shut down other SPs.

Hayan and Psh redundantly regulate Toll signaling downstream of the PRR pathway

The evidence that SPE in other insect species can lead to the activation of both the Toll and melanization pathways led us to verify the roles of two melanization SPs, Hayan and Sp7, in Toll activation. Single mutants for *Hayan* and *Sp7* showed no defect in Toll pathway activation as revealed by the level of *Drosomycin* induction. Surprisingly, we found that Hayan-psh^{Def} mutants fail to activate the Toll pathway regardless of the type of challenge. This points to a crucial function for these two SPs in the activation of the Toll pathway in

both the protease and PRR pathways. The Psh bait region motif ^{107}GRVDVPTFGS^{116} is critical for protease-dependent Psh cleavage in *Drosophila* S2 cells (Issa et al., 2018). This region is truncated but present in Hayan (Fig. S5), providing a mechanism for microbial proteases to cleave Hayan or Psh differently, leading to microbe-specific activation of downstream Toll. This contraction of the bait region may represent the early beginnings of sub-functionalization amongst *Hayan* and *psh* (He, 2005). Indeed, using RT-PCR, the shorter *psh*-like transcripts from *Hayan* appear less abundant in *D. melanogaster* than outgroup flies where *Hayan* alone should be responsible for Toll signaling (Figure S6C).

We also show that *Hayan* and *psh* arose from a recent duplication of an ancestral *Hayan* gene. Gene duplications can have varied evolutionary outcomes, but the strong reduction of *Drs* expression in *Hayan-psh^{Def}* mutants upon septic injury with *M. luteus*, but not in single mutants, suggests Hayan and Psh are redundant in regulating Toll signaling. Additionally, 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. As both SPs also share strong similarities in their catalytic domain, they likely cleave similar downstream targets. During embryonic dorso-ventral signaling, the SP Snake regulates the Spätzle processing SP Easter (Lindsay and Wasserman, 2014). The phylogenetic relatedness of Snake and Hayan/psh and the relatedness of Easter and SPE (Fig 7A) suggests Hayan and Psh act upstream of SPE. We note that *spz^{rm7}* deficient flies have lower *Drs* levels than any other mutants, except *Hayan-psh^{Def}* mutants. As *SPE* mutants still express a residual level of *Drs* upon septic injury with *M. luteus* (Fig. S4D), there is likely another SP with the ability to process Spz, as first proposed by Yamamoto-Hino and Goto (2016). CLIP-domain SPs related to Easter and SPE are promising candidates to fulfill this role. Intriguingly, MP1 clusters phylogenetically with Easter, and has been shown to cleave Spz in *Drosophila* S2 cells (Yamamoto-Hino and Goto, 2016). Further, biochemical characterization will be needed to fully clarify the positions of Hayan and Psh in the proteolytic cascade downstream of PRRs.

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: i) the existence of two different pathways activating melanization, ii) the involvement of the extracellular PRRs in the melanization reaction and,

iii) the implication of both Hayan and Psh in the extracellular PRR and the Psh pathway regulating Toll activity (Fig. 8). According to our model, cuticle injury activates Hayan by an unknown pathway, which results in the deposition of melanin (blackening reaction) around the wound area 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 Spätzle cleavage. In our model, the PRR pathway upstream of Toll branches at the level or downstream of Grass 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 pathway, to activate a common extracellular pathway upstream of Toll. Globally, we describe a crucial role for Hayan in Toll activation that is performed redundantly alongside Psh, and describe how these proteins act downstream of both PRRs and microbial proteases to activate Toll.

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. While 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 can pave the way to better characterize these cascades.

Methods

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; Brown et al., 2001; Buchon et al., 2009; Dudzic et al., 2015; Gobert, 2003; Gottar et al., 2006; Nam et al., 2012; Neyen et al., 2014b; Sinenko and Mathey-Prevot, 2004; Yamamoto-Hino and Goto, 2016). The *MP1*^{SK6}, *psh*^{SK1} and Hayan-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. 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-psh*^{Def} in the text). To rescue the *Hayan-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 their respective optical density of the pellet (O.D.) at 600 nm were: the DAP-type peptidoglycan-containing Gram-positive bacteria *Bacillus subtilis* (*B. subtilis*, O.D 5); the Lys-type peptidoglycan-containing Gram-positive bacteria *Micrococcus luteus* (*M. luteus*, O.D. 200), and *Staphylococcus aureus* (*S. aureus*, O.D. 0.5). Strains were cultured in Luria Broth (LB) at 29 °C (*M. luteus*) or 37 °C (other species). The yeast *Candida albicans* ATCC 2001 (*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 (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 three tubes of 20 flies were used for each survival experiment and survival was scored once or twice daily. 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.

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 thorax of the animal was pricked (as described in infection experiments) using a sterile needle (diameter: ~5 µm). Pictures were taken 16 hours post-pricking. Third instar larvae were pricked dorsally near the posterior end, using a sterile needle (diameter: ~5 µm). 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 by taking pictures.

Bacterial load of flies

Flies were infected with *S. aureus* as described above. At the appropriate time point, flies were sacrified by washing them in 70% ethanol. This treatment is expected to remove bacteria from the surface of the flies. Ethanol was washed off with sterile PBS and groups of five flies were homogenized with a PRECELLYS^{TM} 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. Only *S. aureus* bacterial colonies were recovered at this time point with this approach.

H_2O_2 assay

H_2O_2 levels were assessed with Fluorimetric Hydrogen Peroxide Assay Kit (Sigma MAK165) according to the manufacturer's documentation. Briefly, seven adult flies were homogenized in 0.12 ml PBS with a PRECELLYS and then centrifuged at 4°C and 13,000 RPM. Afterwards 0.1 ml homogenate was transferred into a new tube. Sample volumes of 30 µl were used for the assay in duplicate. Protein concentration of the samples was determined using the 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 10-15 adult flies by TRIzol reagent and dissolved in RNase-free water. 0.5 micrograms 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'-CGTGAGAACCTTTTCCAATATGAT-3', reverse 5'-TCCCAGGACCACCAGCAT-3'; *RpL32* (*Rp49*) forward 5'-GACGCTTCAAGGGACAGTATCTG-3', reverse 5'-AAACGCGGTTCTGCATGAG-3'.

SP sequence analysis

A list of *Drosophila melanogaster* CLIP-domain serine proteases was generated from Veillard et al. (2015), 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 orthologues 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. Supplementary Data File 1 accessible at http://bit.ly/2LKintJ.

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, and P-values are indicated in figure legends. Statistical significance of qPCR or ROS data was calculated with Two-way ANOVA or Mann-Whitney test. Melanization data was analyzed with Pearson's Chi-squared test. P-values of < 0.05 = *, < 0.005 = **, and < 0.0005 = *** were considered significant.

Authors' contributions

JD and BL designed the study. JD performed the experiments. MH performed bioinformatic analyses. JD, MH and BL analyzed the data and wrote the manuscript. II and SK supplied critical reagents. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tables:

Table 1. Melanization intensity after clean injury (CI) or septic injury with M. luteus in adult flies. Intensities ranging from +++ = strong to - = none. Of note: melanization is rescued by M. luteus in Hayan flies. This is dependent on the presence of PPO1.

	w^{1118}	$Hayan^{SK3}$	$Sp7^{SK6}$	$MP1^{SK6}$	$Hayan^{SK3}$;$PPO1^{\Delta}$	$Hayan^{SK3}$;$PPO2^{\Delta}$	$PPO1^{\Delta}$;$Sp7^{SK6}$	$PPO2^{\Delta}$;$Sp7^{SK6}$
CI	+++	-	++	+++	-	-	++	+
M. luteus	+++	+	++	+++	-	+	++	+

Figures:

Fig. 1

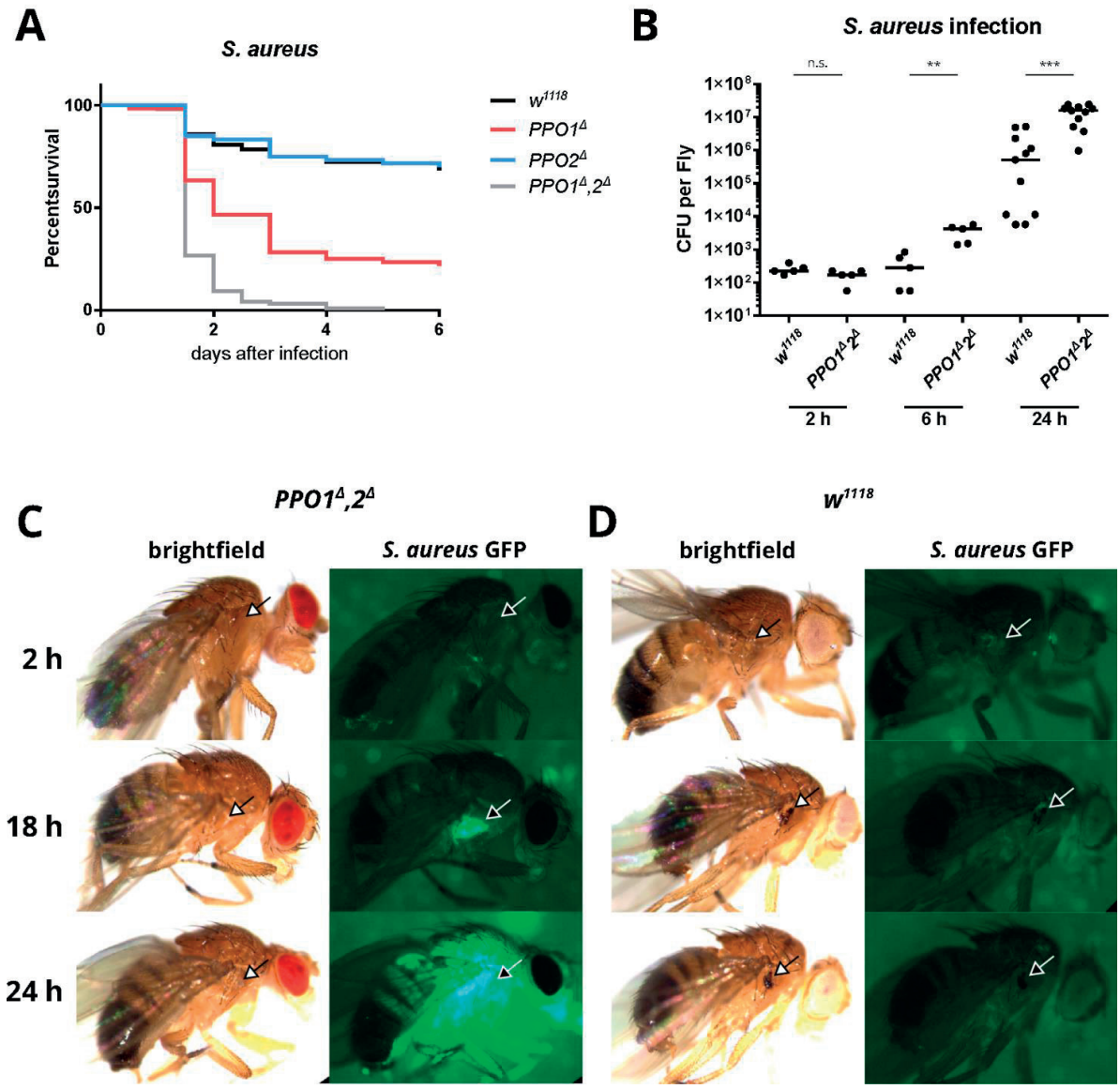

Fig. 1: PPOs contribute to the resistance of *Staphylococcus aureus*. A: Survival rates of flies following septic injury with Gram-positive *S. aureus*. Flies lacking *PPO1* ($P < 0.0001$) but not *PPO2* (n.s.) alone are less resistant than wild-type flies (w^{1118}). Flies lacking two PPO genes ($PPO1^{\Delta},2^{\Delta}$) ($P < 0.0001$) 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 hours post-infection. Increased *S. aureus* counts are found in $PPO1^{\Delta},2^{\Delta}$ flies after 6 and 24 hours. The number of colony forming units (CFU) per fly is shown on a logarithmic scale. Data were analyzed by Mann-Whitney test and significance is indicated in the graph. **C,D:** Growth of

GFP expressing *S. aureus* in *PPO1*$^{\Delta}$*,2*$^{\Delta}$ (C) or *w*^{1118} (D) flies after 2, 18 and 24 hours. *Wild-type* flies melanize the wound area (arrows) while *PPO1*$^{\Delta}$*,2*$^{\Delta}$ do not. No GFP signal is observed in *w*^{1118} flies while *PPO1*$^{\Delta}$*,2*$^{\Delta}$ exhibit a local GFP signal after 18 hours, and systemic GFP signal after 24 hours. Exemplary micrographs are shown.

Fig. 2

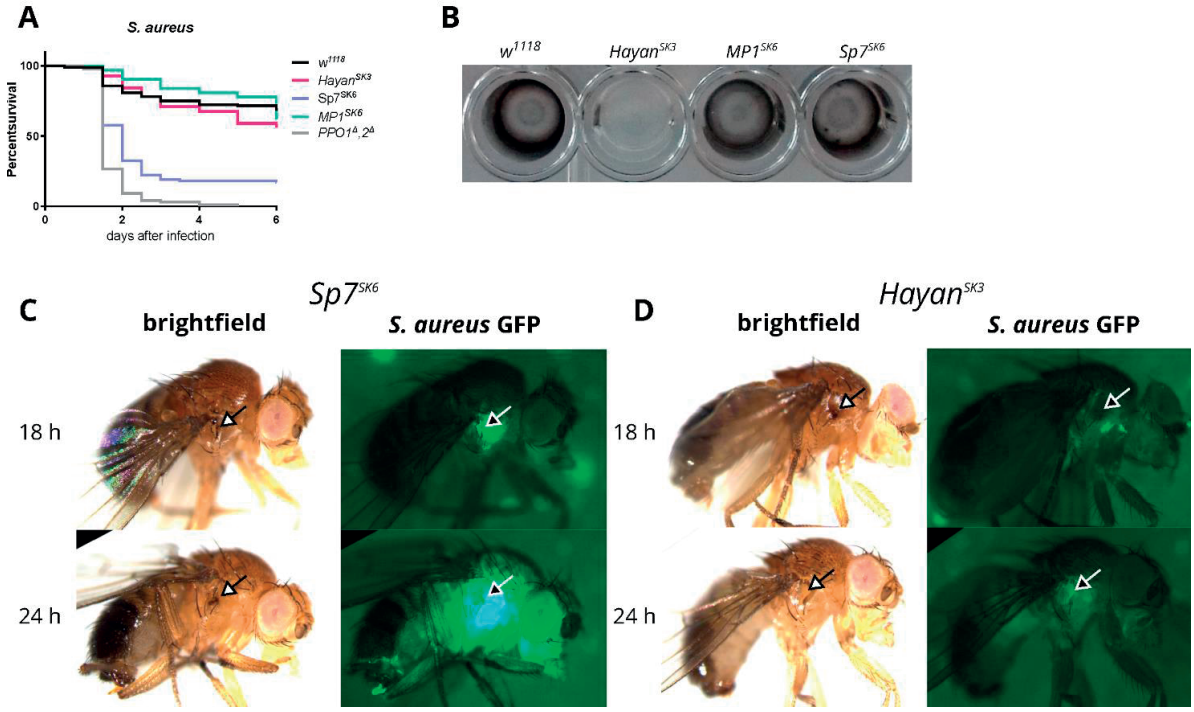

Fig. 2: *Sp7* but not *Hayan* or *MP1* is critical to resist *S. aureus* infections. A: Survival rates of flies following septic injury with Gram-positive *S. aureus*. Flies lacking *Sp7* ($P < 0.0001$) but not *Hayan* ($P = 0.0075$) or *MP1* ($P = 0.3156$) are less resistant than wild-type flies (*w*^{1118}). *PPO1*$^{\Delta}$*,2*$^{\Delta}$ flies were used as a positive control. P-values are from log-rank tests compared to wild-type flies. **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 while *Hayan*^{SK3} hemolymph fails to melanize. **C, D:** Growth of GFP producing *S. aureus* in *Sp7*^{SK6} (B) or *Hayan*^{SK3} (C) flies after 18 and 24 hours. *Sp7*^{SK6} flies show a local GFP signal at the wound area (arrows) while *Hayan*^{SK3} flies show a strong reduction. In *Sp7* mutants, this moves into a systemic GFP signal after 24 hours. Exemplary micrographs are shown.

Fig. 3

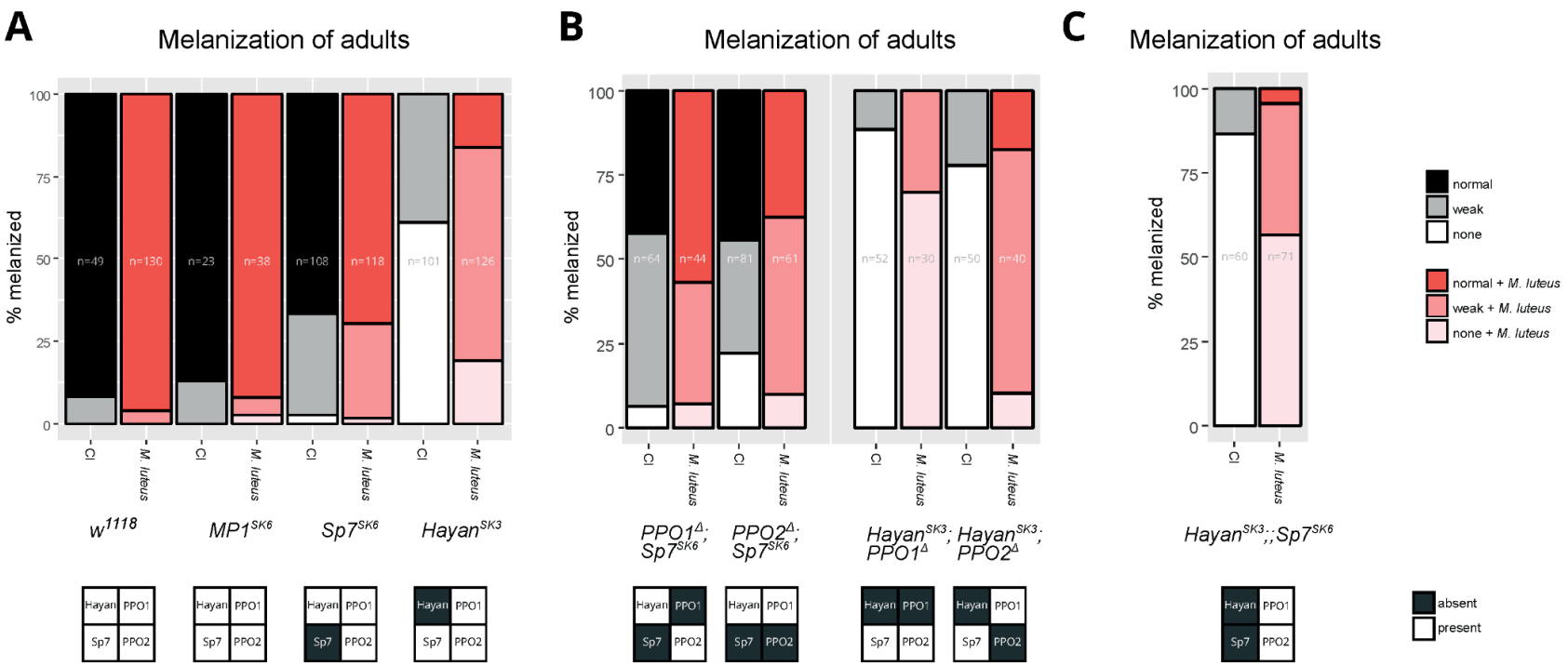
A
Melanization of adults
% melanized
100
75
50
25
0
n=49
n=130
n=23
n=38
n=108
n=118
n=101
n=126
Cl
M. luteus
w1118
MP1SK6
Sp7SK6
HayanSK3
Hayan
PPO1
Sp7
PPO2
B
Melanization of adults
n=64
n=44
n=81
n=61
n=52
n=30
n=50
n=40
PPO1Δ; Sp7SK6
PPO2Δ; Sp7SK6
HayanSK3; PPO1Δ
HayanSK3; PPO2Δ
C
Melanization of adults
n=60
n=71
HayanSK3;;Sp7SK6
normal
weak
none
normal + M. luteus
weak + M. luteus
none + M. luteus
absent
present

Fig. 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). Melanization was assessed in three categories: strong, weak or none (see Fig. S2). **A:** $MP1^{SK6}$ flies do not exhibit any defect in melanization compared to w^{1118} flies (p = 0.8219). $Sp7^{SK6}$ flies show a reduction in strong melanization although almost all flies melanize at least weakly (p = 0.0083). $Hayan^{SK3}$ flies show a strong defect in melanization after clean injury (p < 0.0001), which can partially be rescued by wounding with *M. luteus* (p < 0.0001). **B:** *Sp7* mutants still melanize the wound area to a certain extent with a simultaneous mutation in either *PPO1* or *PPO2*. In contrast, the partial melanization 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 melanization. Percentages of total flies (n) are displayed. Sample size (n) is indicated in each respective bar. Data analyzed with Pearson's Chi-squared.

Fig. 4

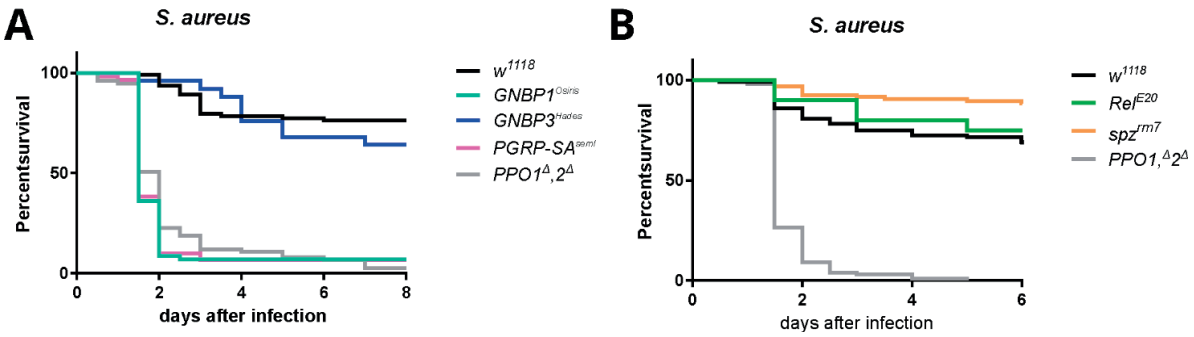

Fig. 4: PRRs regulating Toll signaling are critical to resist *S. aureus*. A: Survival rates of flies following septic injury with Gram-positive *S. aureus*. Flies lacking *GNBP1* (P < 0.0001) or *PGRP-SA* (P< 0.0001) but not *GNBP3* (P = 0.3141) are less resistant than wild-type flies (w^{1118}). **B:** Flies lacking the Toll ligand *Spätzle* (P = 0.0056) or IMD component *Relish* (P= 0.7845) resist *S. aureus* infection similar to wild-type flies (w^{1118}). Flies lacking two PPO genes ($PPO1^{\Delta},2^{\Delta}$) (P < 0.0001) act as the positive control for A and B.

Fig. 5

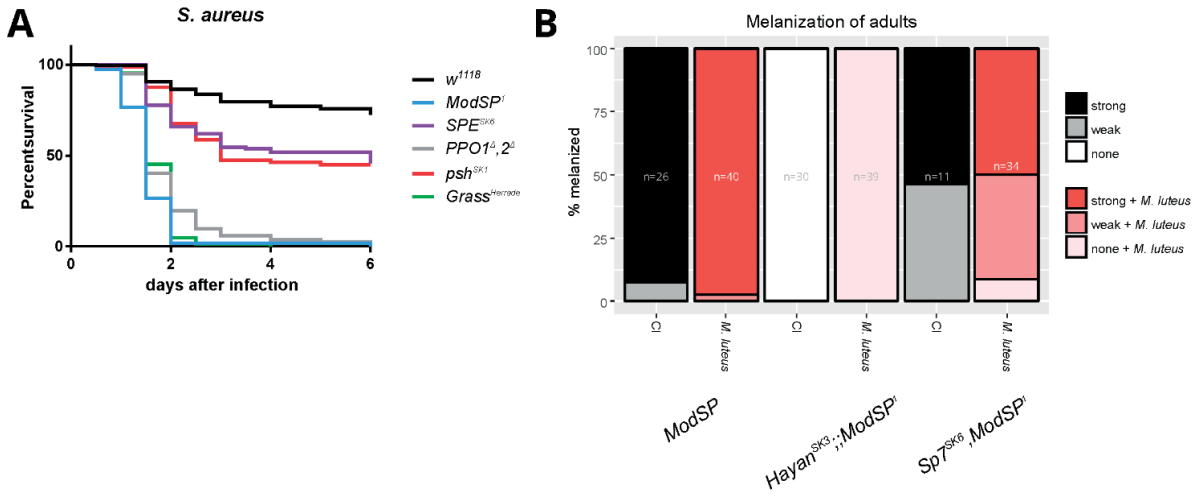

Fig. 5: The Toll pathway serine proteases ModSP and Grass are required to survive *S. aureus* infections. A: Survival rates of flies following septic injury with Gram-positive *S. aureus*. Flies lacking *ModSP*^{1} (P < 0.0001) or *Grass* (P< 0.0001) exhibit high susceptibility compared to wild-type flies (*w*^{1118}). The loss of *psh* (P < 0.0001), SPE or *Spirit* (P < 0.0001) results in a minor survival defect. P-values are from log-rank tests compared with wild-type flies. **B:** More than 90% of *ModSP*^{1} flies melanize normally. In contrast, flies mutant for *Hayan*^{SK3}*;;ModSP*^{1} do not melanize at all. This effect is dependent on the simultaneous absence of *Hayan* and *ModSP*^{1} because *Sp7*^{SK6}*,ModSP*^{1} flies do not exhibit a stronger phenotype than *Sp7*^{SK6} alone (see Fig. 4). Percentages of total flies are displayed, and n is indicated in each respective bar. Flies were either injured with a clean needle (black bars) or with a needle previously dipped in *M. luteus* solution (red bars). Melanization was assessed in three categories: normal, weak or none (see Fig. S2).

Fig. 6

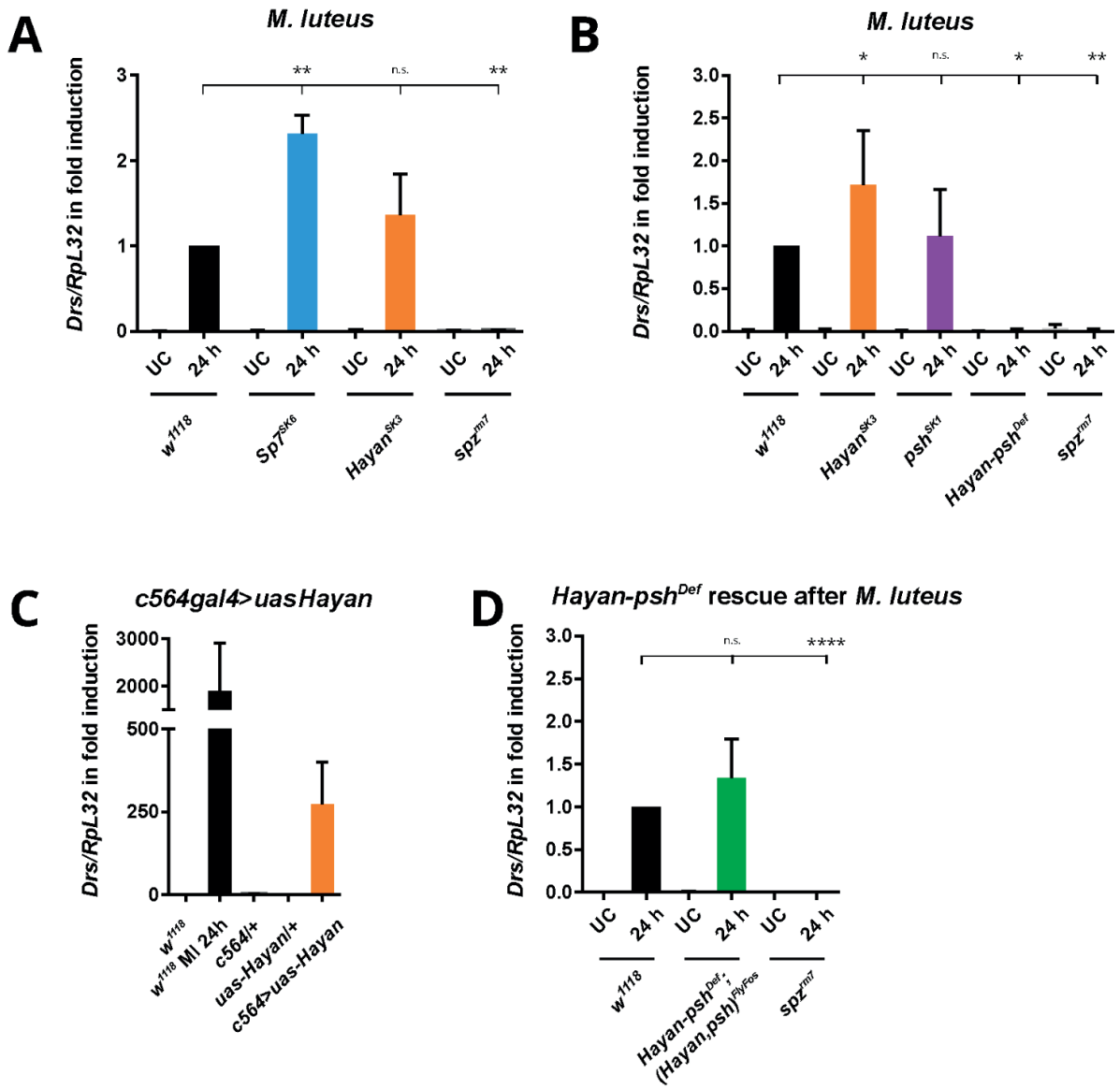

Fig. 6: *Hayan* contributes to Toll pathway activation. A: Single mutants for *Sp7* and *Hayan* show no loss of Toll pathway activity (*i.e*. expression of *Drosomycin* after *M. luteus* infection). $MP1^{SK6}$ and $Sp7^{SK6}$ flies show significantly higher levels of *Drs,* while *Hayan* mutants show no difference after 24 h compared to w^{1118} flies. **B:** Flies lacking *Hayan* and *psh* simultaneously fail to activate *Drs* expression similar to spz^{rm7} flies, while single mutants for *Hayan* or *psh* show no significant difference compared to w^{1118} flies. **C:** Expression of a constitutively active form of *Hayan* in the fat body is sufficient to induce *Drs* in otherwise unchallenged flies. **D:** Insertion of a *Hayan, psh* transgene (FlyFos) rescues to

wild-type levels of *Drs* expression in the *Hayan-psh*^{Def} double mutant background after *M. luteus* infection. Shown are the relative expression levels of *Drs* relative to *RpL32*. *w*^{1118} levels after 24h (A,B,D) or UC (C) are set to 1. *spz*^{rm7} flies act as a negative control. Data analyzed with Mann-Whitney test (A,B, C) or 2 way Anova (E).

Fig. 7

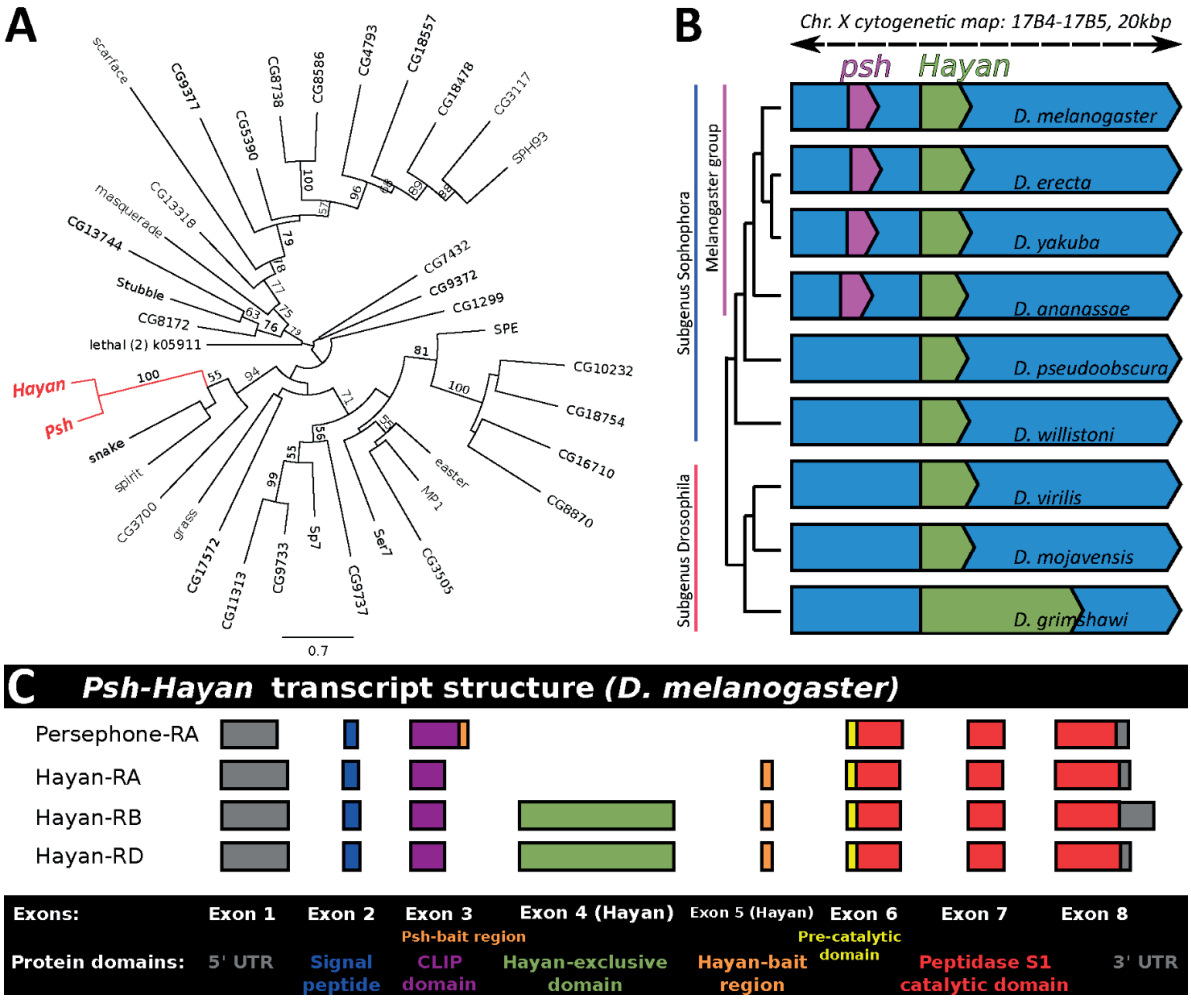

Fig. 7: Evolutionarily conserved Hayan isoforms are either similar to Psh, or unique to Hayan. A: Maximum likelihood phylogeny of catalytic domains from *Drosophila* CLIP-domain SPs from Veillard et al. (2015). Support values represent 100 bootstraps. Hayan and Psh form a monophyletic lineage within *D. melanogaster* CLIP-domain SPs. **B:** *psh* is a gene duplication restricted to Melanogaster group flies, derived from ancestral *Hayan* (also see Fig. S5 A). Annotations represent CDS gene regions. *Drosophila grimshawi's Hayan* region is elongated due to a 4000 bp intron between Dgri\GH12343-RB exons 3

and 4. **C:** *Hayan*-RA and *psh*-RA bear a striking resemblance in transcript structure, while the *Hayan* transcript isoforms *Hayan*-RB and *Hayan*-RD include a *Hayan*-exclusive domain not found in other CLIP-domain SPs in *D. melanogaster*.

Fig. 8

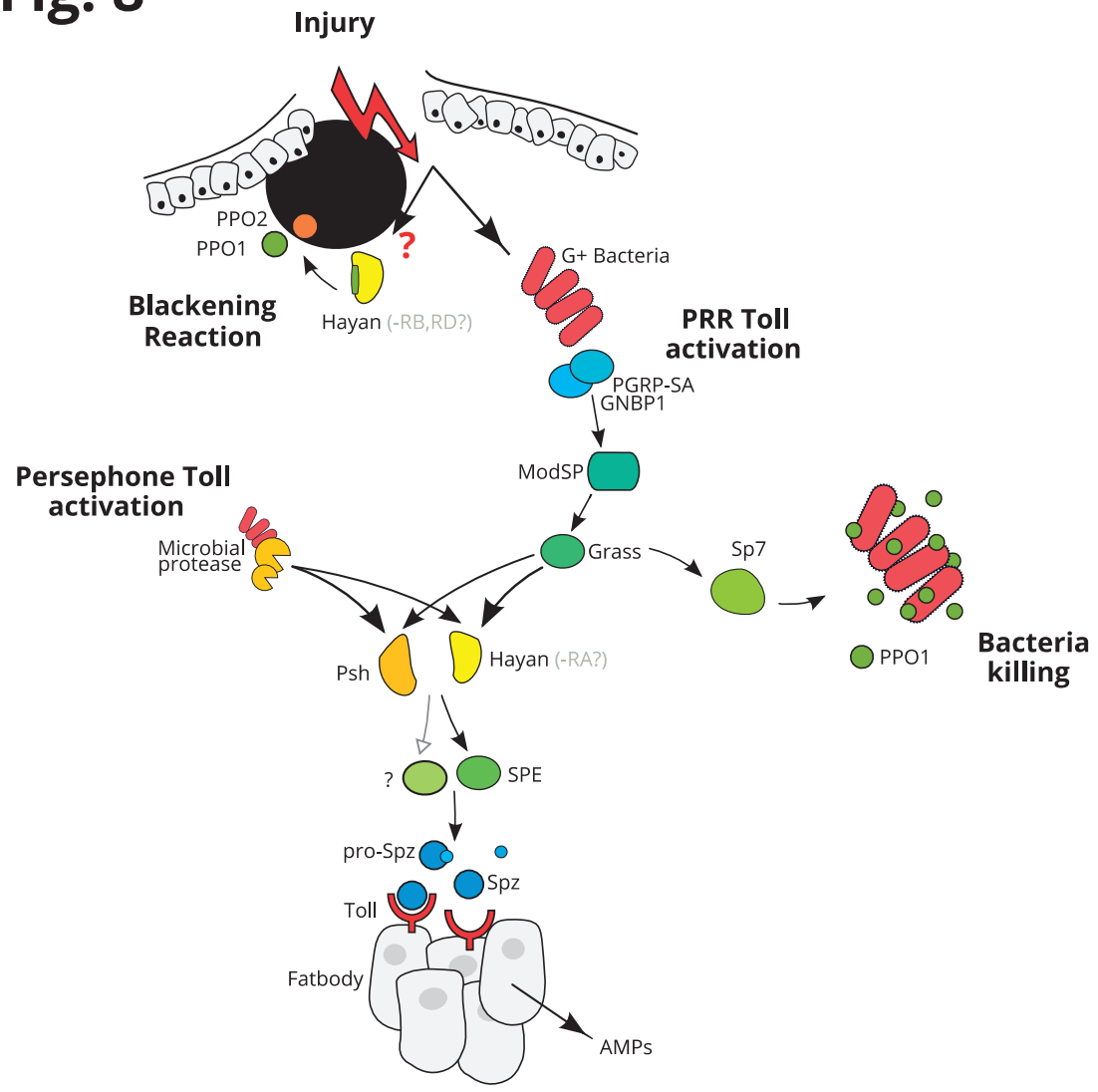

Fig. 8: A revised model of SPs regulating the Toll pathway and the melanization reaction. After the introduction of a wound through the cuticle and the underlying epithelium, Hayan can be activated by an unknown mechanism that results in the deposition of melanin around the wound area (left part). This 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 in the fat body (middle part). This extracellular SP pathway branches at the level of Grass to Sp7, activating PPO1 to combat invading bacteria, possibly via the production of cytotoxic intermediates but not melanin (right part). Microbial proteases can activate the Toll pathway through the Psh-SPE-Spz extracellular pathway. Although suggested by sequence homology, it is unclear whether microbial proteases can also activate Hayan but both Hayan and Psh regulate the Toll pathway down-stream 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.

Fig. S1

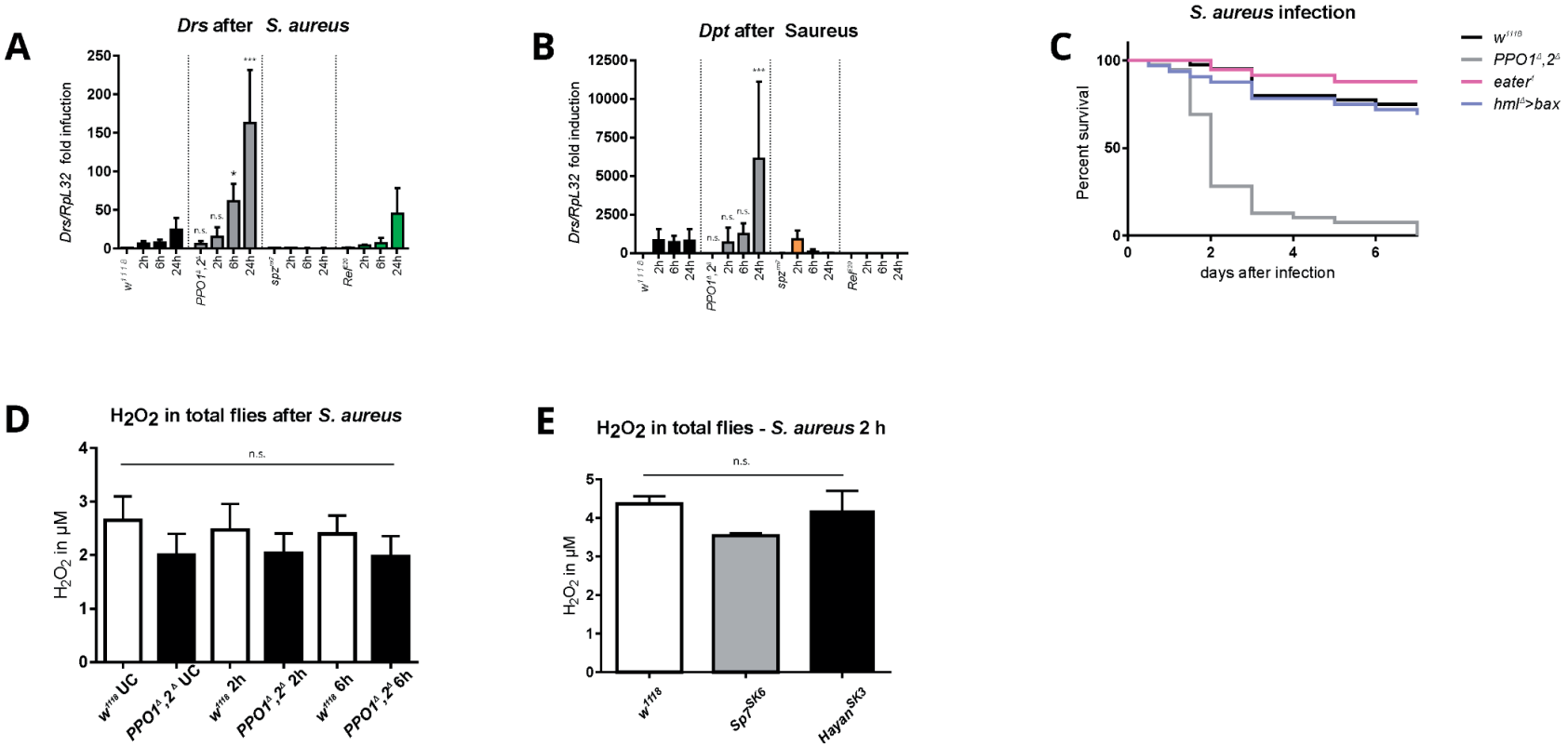
A
Drs after S. aureus
Drs/RpL32 fold infuction
B
Dpt after Saureus
Drs/RpL32 fold induction
C
S. aureus infection
Percent survival
days after infection
w1118
PPO1Δ,2Δ
eaterI
hmlΔ>bax
D
H2O2 in total flies after S. aureus
H2O2 in µM
n.s.
w1118 UC
PPO1Δ,2Δ UC
w1118 2h
PPO1Δ,2Δ 2h
w1118 6h
PPO1Δ,2Δ 6h
E
H2O2 in total flies - S. aureus 2 h
H2O2 in µM
n.s.
w1118
Sp7SK6
HayanSK3

Fig. S1: Resistance against low dose *S. aureus* infections is strongly dependent on melanization. A,B: *PPO1*$^{\Delta}$*,2*$^{\Delta}$ *flies* show reduction of Toll and IMD pathway activity. Expression of *Drosomycin* (A) or *Diptericin* (B) are given after septic injury with *S. aureus*. Shown are the expression levels of *Drs* or *Dpt* relative to *RpL32*. *PPO1*$^{\Delta}$*,2*$^{\Delta}$ *flies* show higher levels of *Drs* after 6 and 24 h, and *Dpt* after 24 h compared to *w*^{1118} flies. *Spätzle*^{rm7} and *Relish*^{E20} flies act as negative control. Data were analyzed with two-way ANOVA, only P-values for *PPO1*$^{\Delta}$*,2*$^{\Delta}$ compared to respective *w*^{1118} timepoints are indicated. Values represent the mean ± standard deviation of at least three independent experiments. **C:** Survival rates of flies following septic injury with Gram-positive *S. aureus*. Flies lacking the hemocyte receptor *Eater*, important for phagocytosis of *S. aureus* (P = 0.10) or flies lacking plasmatocytes by overexpressing the pro-apoptic gene *bax* (P = 0.5118) are as resistant as wild-type flies (*w*^{1118}). P-values are from log-rank tests compared to wild-type flies. **D:** H_2O_2 measured in whole fly lysates from flies infected with *S. aureus* after 2 and 6 hours compared to unchallenged flies. No significant differences were found between *PPO1*$^{\Delta}$*,2*$^{\Delta}$ and *w*^{1118} samples, although *PPO1*$^{\Delta}$*,2*$^{\Delta}$ samples show a trend towards lower values. **E:** H_2O_2 measured in whole fly lysates from flies infected with *S. aureus* after 2 hours. No significant differences were found between *w*^{1118}, *Sp7*^{SK6} and *Hayan*^{SK3} samples, although *Sp7*^{SK6} samples have lower values. Data analyzed with two-way ANOVA for D and E.

Fig. S2

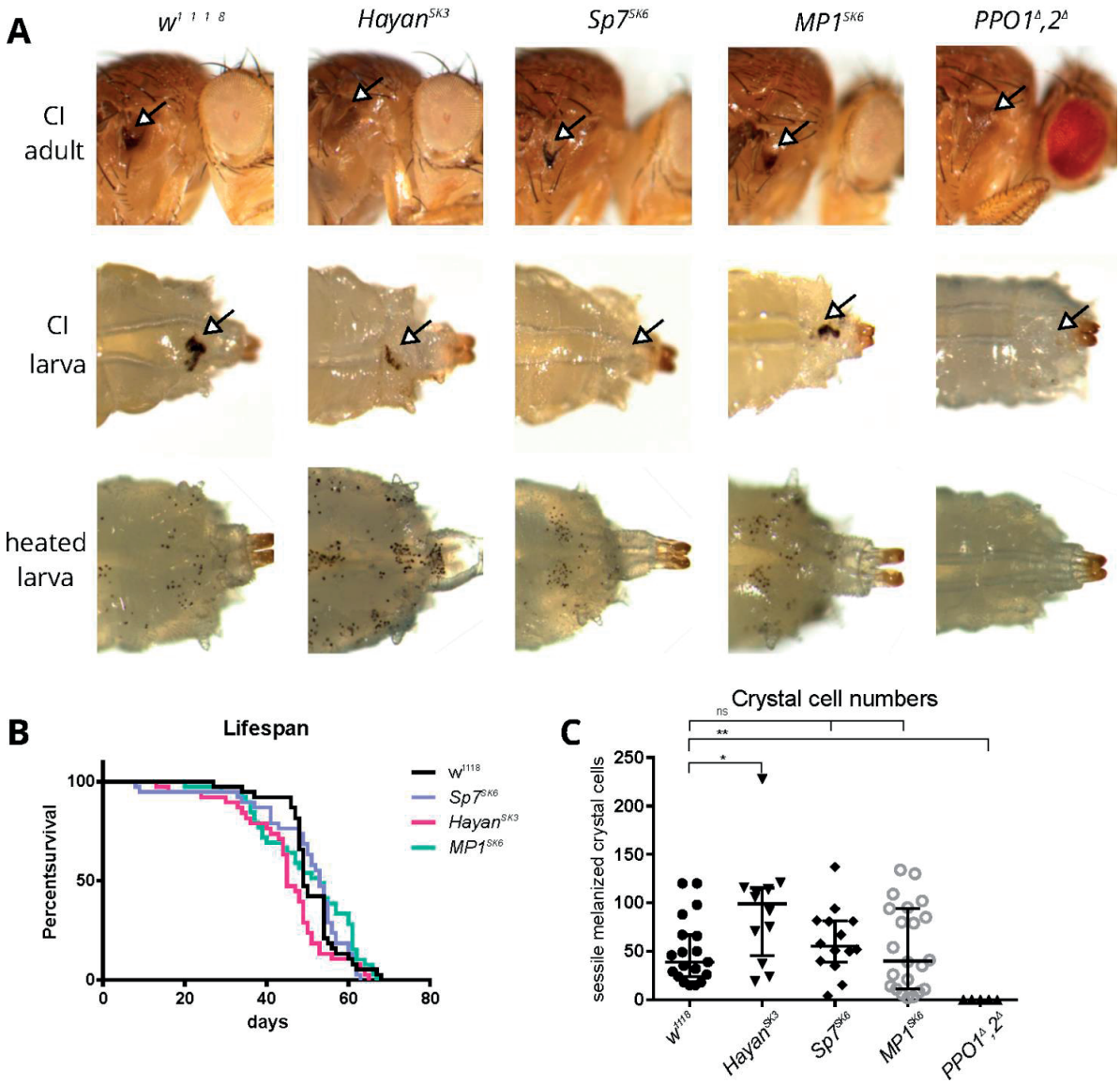

Fig. S2: *MP1* deficient flies show no over defect in Melanization A: Blackening of the wound area after clean injury in adult flies is only abolished in *Hayan*^{SK3} and *PPO1*$^{\Delta}$,*2*$^{\Delta}$ flies, while in larvae *Hayan* mutants show a strong defect and *Sp7* mutants a total loss of melanization. All SP mutants show melanized crystal cells after heating. **B:** No severe lifespan defect can be observed in all SPs mutant flies compared to *w*^{1118}. *Hayan*^{SK3} flies show a slight reduction in the median survival value from *w*^{1118} 49 days to *Hayan*^{SK3} 45 days (P = 0.0186), while differences between *MP1*^{SK6} or *Sp7*^{SK6} and *w*^{1118} do not reach significance. The x-axis is the survival time in days and the y-axis is the percentage of

living flies. P-values from log-rank test. **C:** Crystal cell counts after heating L3 larvae reveal no significant differences between *Sp7*^{SK6} or *MP1*^{SK6} and *w*^{1118} numbers, while *Hayan*^{SK3} crystal cell numbers are around twice as high compared to wild type. Data analyzed with unpaired t-test.

Fig. S3

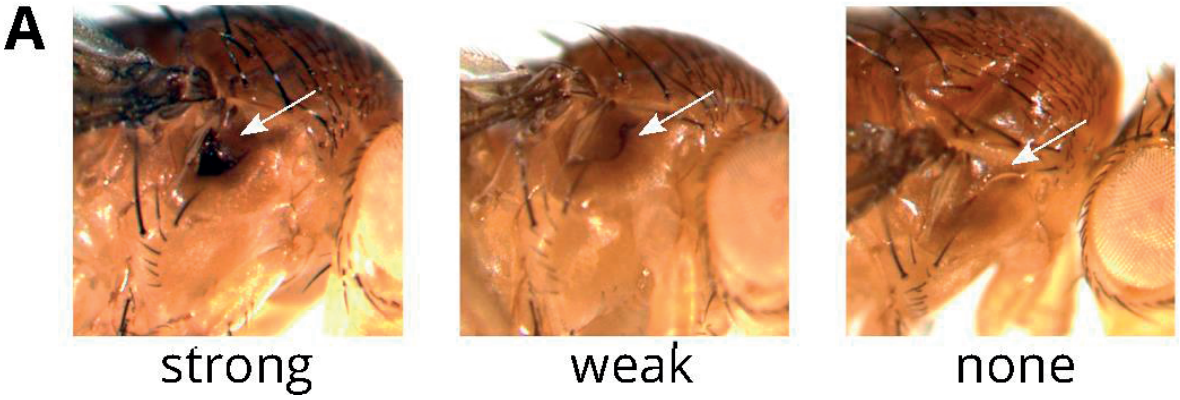

Fig. S3: Illustration of melanization intensities used for categories. Flies (*w*^{1118}*:* strong, weak or *PPO1*$^{\Delta}$*,2*$^{\Delta}$: none) were injured with a clean needle. Intensities are categorized as normal, weak, or no melanization.

Fig. S4

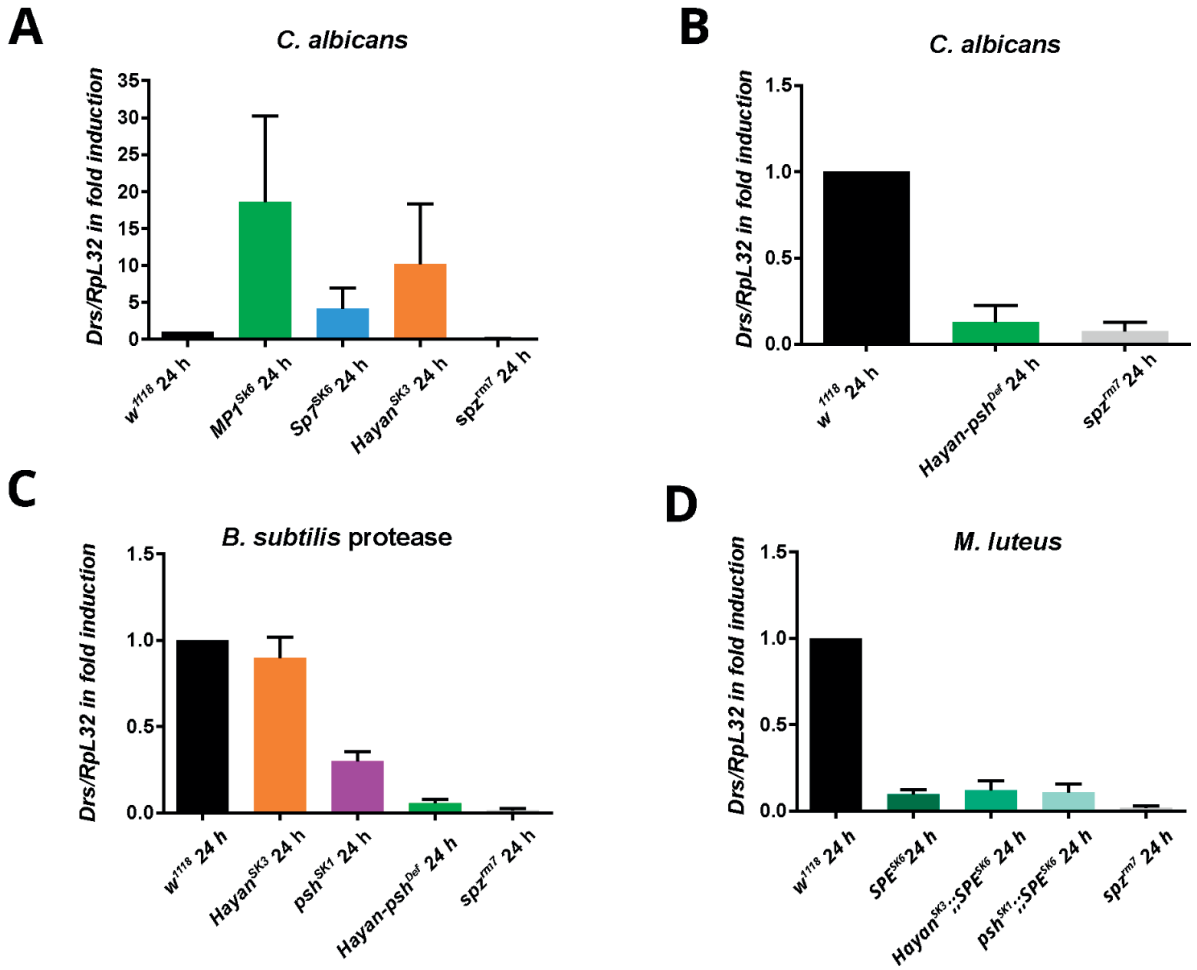

Fig. S4: *Hayan, psh* mutant flies have reduced *Drs* expression upon *C. albicans* or protease injection. A: Neither $MP1^{SK6}$, $Sp7^{SK6}$ nor $Hayan^{SK3}$ fail to activate *Drs* expression after being infected with *C. albicans* relative to w^{1118} induction (set to 1). **B:** *Hayan,psh* mutants fail to activate the Toll pathway after *C. albicans* infection. **C:** $Hayan^{SK3}$ shows w^{1118} levels of *Drs* expression after injection of purified *Bacillus sp.* protease. In contrast, psh^{SK1} flies suppress *Drs* to ~35% of wild-type levels. *Drs* expression in *Hayan-*psh^{Def} is suppressed to a level similar to spz^{rm7} flies. **D:** SPE^{SK6}, $Hayan^{SK3};;SPE^{SK6}$ and $psh^{SK1};;SPE^{SK6}$ mutants block *Drs* expression but not as completely as spz^{rm7} flies. Shown are the expression levels of *Drs* relative to *RpL32*. spz^{rm7} flies act as a negative control. Results for three (A,B,D) or two (C) individual experiments are shown.

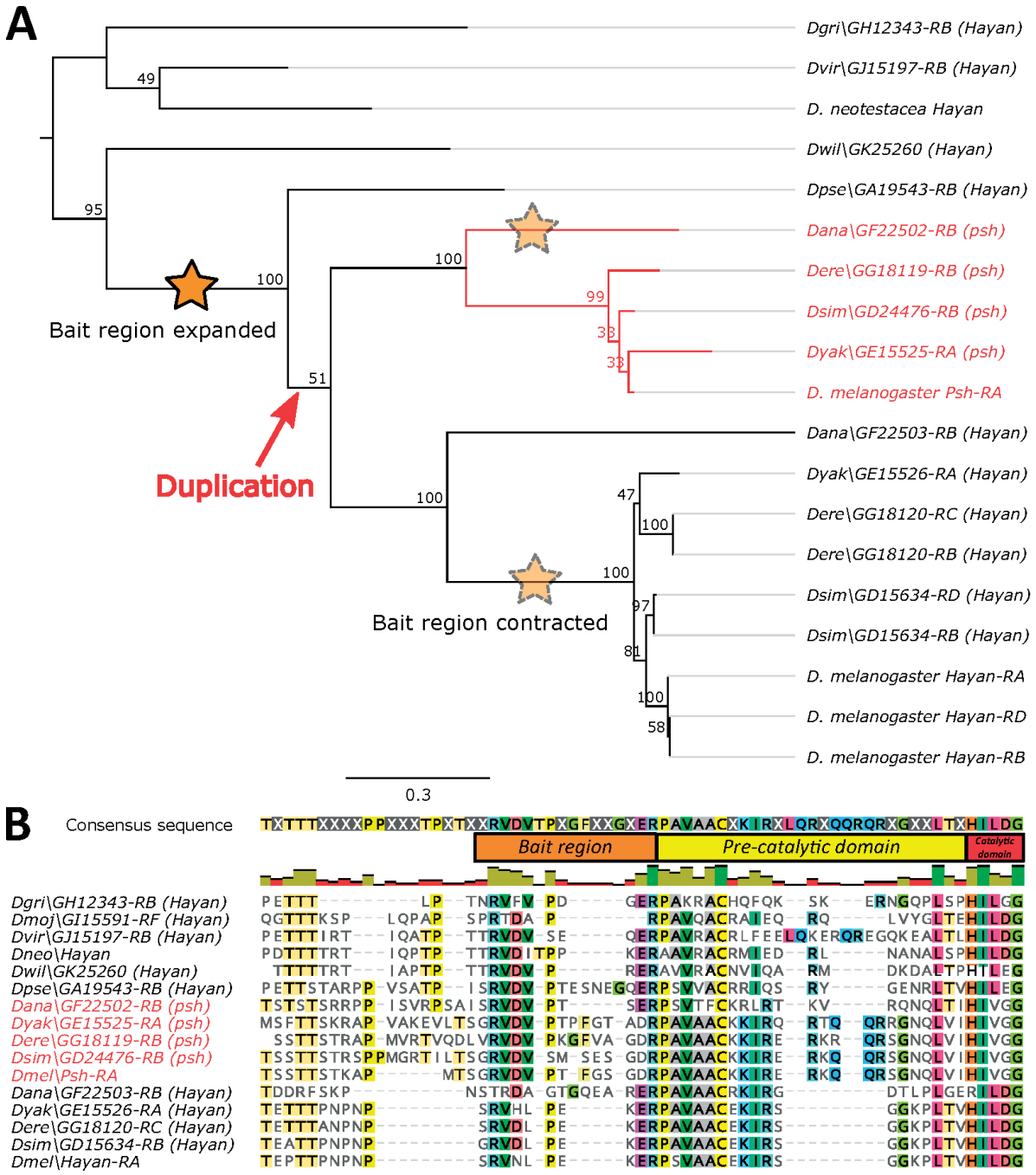

Fig. S5: Hayan and Psh are two related serine proteases. A: Maximum likelihood phylogeny of unique CDS regions from *Drosophila* Hayan and Psh isoforms. Support values represent 100 bootstraps. Psh forms a paraphyletic lineage nested within *Drosophila* Hayan, with branching patterns largely matching known sorting of *Drosophila* groups. Orange stars indicate the expansion of the microbial protease bait region seen in *D. melanogaster* Psh ^{114}FGS^{116} (Fig. S5 B), and translucent stars indicate reversion to the

ancestral Hayan bait region similar to *D. melanogaster* Hayan. **B:** Hayan also encodes the protease bait region described by Issa et al. (2018). The important bait motif corresponding to Psh ^{108}RVDVP^{112} is largely conserved in outgroup Hayan sequences, while Melanogaster group Hayan has diverged to an RVXLP motif. Interestingly, *D. ananassae* Psh has lost the expanded bait region (following the RVDVP motif), instead retaining these extra cleavage sites in *D. ananassae* Hayan. Following this bait region, the Hayan and Psh pre-catalytic domain is remarkably well-conserved, with some lineage-specific additional sites directly upstream of the Peptidase S1 catalytic domain.

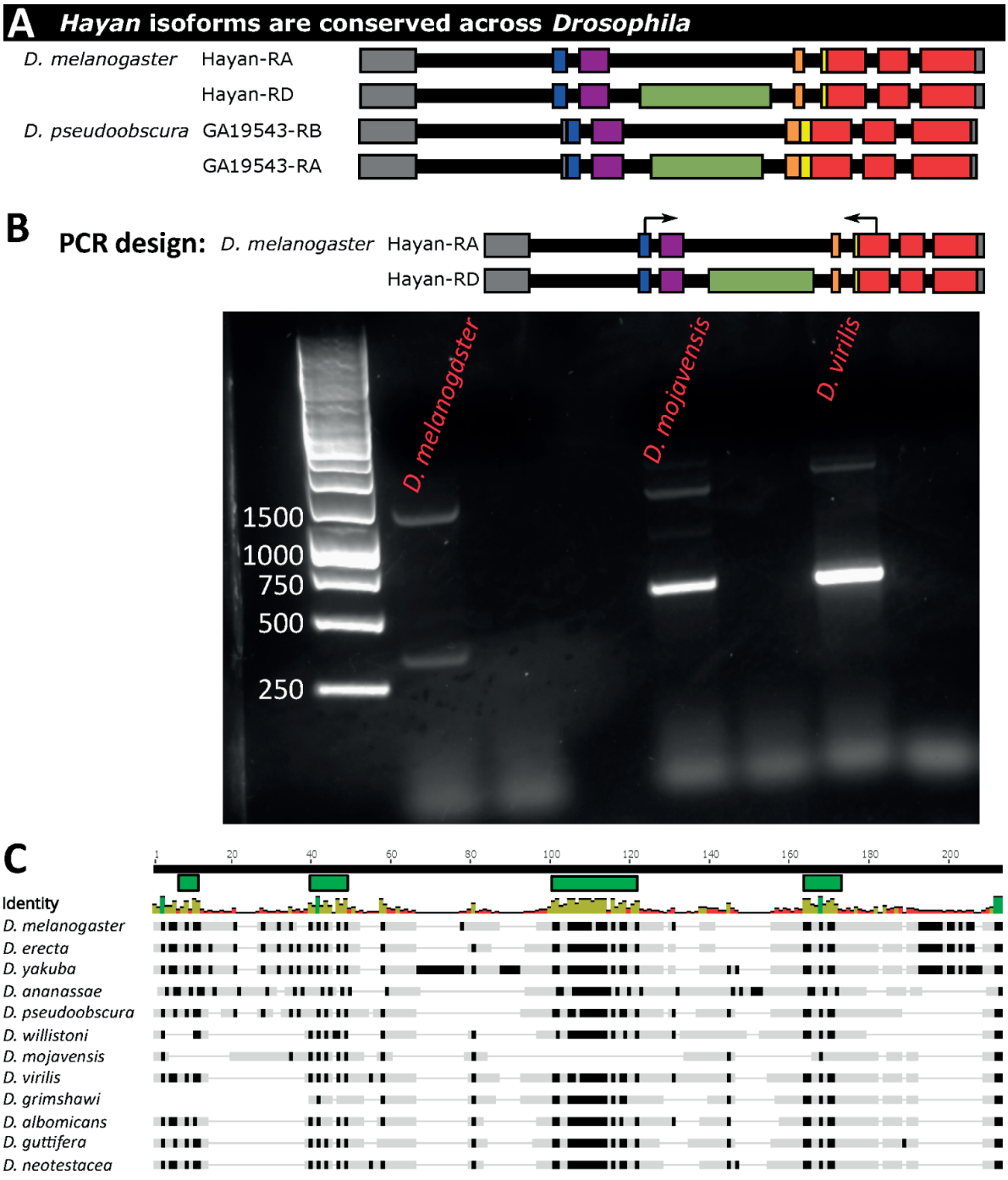

Fig. S6: Alternative isoforms of *Hayan* are conserved throughout *Drosophila*. A: Alternate *Hayan* isoforms are conserved outside the Melanogaster group in Flybase annotations for *D. pseudoobscura GA19543*. **B:** Independent RT-PCR of *Drosophila* species cDNA shows alternate *Hayan* isoforms in *D. melanogaster* and the subgenus Drosophila flies *D. mojavensis* and *D. virilis*. **C:** Codon-aligment of the Hayan-exclusive

domain from diverse *Drosophila*. Green bars represent motifs with many residues under purifying selection (Supplementary Data File 1), which also display strong conservation of sequence (black residues in alignment). Notably, *Drosophila mojavensis* lacks most conserved motifs in its Hayan-exclusive domain, and this region is difficult to interpret with available sequence data in the related *Drosophila buzzatii*.

9 CONCLUSION

The goal of this PhD thesis was to better understand the melanization reaction in *D. melanogaster*, focusing on two levels: i) the PPOs that directly contribute to melanogenesis and ii) on SPs regulating the activation of PPOs. In this section, I will summarize and discuss my main results.

The *D. melanogaster* genome contains three *PPO* genes, which are the rate limiting enzymes for melanogenesis. While the functions of PPO1 and PPO2 are well understood, we addressed the role of the third PPO, PPO3, in the first part of this thesis. While it was known that both PPO1 and PPO2 are produced in specialized hemocytes called crystal cells (Binggeli et al., 2014), the origin of PPO3 was debated in the literature. Some reports claim that PPO3 is also produced in crystal cells (Ferjoux et al., 2007; Waltzer et al., 2003), while others propose lamellocytes as the source of expression (Irving et al., 2005; Nam et al., 2008). With the help of newly generated PPO3 mutants, as well as genetic tools, we were able to clearly demonstrate that lamellocytes are the sole source of PPO3 in *D. melanogaster*. With the use the GAL4/UAS system, we identified the regulatory sequences upstream of *PPO3* to drive GFP expression, which resulted in GFP signal only in lamellocytes but not in crystal cells or plasmatocytes. We confirmed this result by heating larva, which results in the spontaneous melanization of crystal cells. This heating assay also revealed the presence of spontaneously melanized lamellocytes after wasp infestation. Fly larvae lacking PPO3 lose the latter pattern, confirming that lamellocytes but not crystal cells produce PPO3.

Binggeli and colleagues reported that *Drosophila* larvae mutant for both PPO1 and PPO2 lose the ability to produce a melanotic capsule around parasitoid wasps (Binggeli et al., 2014). These results were at first sight surprising, because it has been suggested that PPO3 plays a role in the encapsulation process. Astonishingly, we found a cryptic mutation of *PPO3* in the *PPO1,2* mutant used by Binggeli and colleagues, which prompted us to reinvestigate the role of all three PPOs in the encapsulation process. With the help of *PPO* compound mutants in all possible combinations, we found that the combined loss of *PPO1* and *PPO2* is not sufficient to abolish melanin production around a wasp egg. Interestingly, we found only when flies are simultaneously mutant for *PPO2* and *PPO3,* they cannot melanize the wasp egg anymore. This indicates overlapping functions of PPO2 and PPO3, and the contribution of both crystal cells and lamellocytes to the encapsulation

process. Our finding were in accordance with the previous reports of (Nam et al., 2008; Wertheim et al., 2005). While PPO2 is also important for the systemic melanization process after injury, we found no further role of PPO3 beyond encapsulation.

Finally, we analyzed these functional differences of the *PPO* genes in an evolutionary context. We found that while *PPO1* and *PPO2* are conserved throughout all 23 *Drosophila* species with a currently sequenced genome, this is not the case for *PPO3*. We found that *PPO3* is only present in the Melanogaster subgroup containing *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta* and *D. yakuba*. The indication that lamellocytes and the ability to cellularly encapsulate parasitoid wasp eggs is also found outside this group, points to the acquisition of *PPO3* dependent melanization after the evolution of the cellular basis to encapsulate wasp eggs. We also found that *PPO3* most likely arose from a gene duplication of *PPO2*, which is consistent with the functional overlap we present. Additionally, we present evidence that *PPO3* arose or is maintained under the evolutionary pressure of parasitoid wasps. We found that in *D. sechellia* the *PPO3* gene harbors a mutation which renders it nonfunctional. This is likely a consequence of the relaxation of evolutionary pressure on *PPO3* due to the fact that *D. sechellia* lays its eggs in 'noni' fruits, which are reported to be toxic to parasitoid wasps. Therefore, *D. sechellia's* niche might be parasitoid wasp-free, and this could have led to the loss of immune functions against them (Salazar-Jaramillo et al., 2014; Yassin et al., 2016). The existence of such a cryptic mutation in the laboratory stock used by Binggeli and colleagues is fascinating, but can be explained by a decrease in the selective pressure of parasitism in laboratory strains. Of note, the original *imd1* mutation was also found in a lab stock (#1046 Bloomington center). The existence of cryptic mutations like these, challenge the term "wild-type" used for comparisons with mutant strains. To address the problem of the diversity of various wild-types and to minimize the possibility of cryptic mutations, we are now using several wild-types for comparisons, and also isogenize our mutants into the same genetic background.

The second objective of my PhD addressed the regulation of the melanization reaction through SPs. We generated new loss-of-function mutants for three SPs (MP1, Sp7, Hayan) involved in *D. melanogaster* melanization. MP1 and Sp7 were previously reported to regulate the melanization reaction, however previous results were obtained using mainly RNAi experiments or hypomorph mutants (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Tang et al., 2006). RNAi can lead

to off-target effects, and the use of a hypomorphic mutation can mask phenotypes through residual expression or protein function. For Hayan, a null mutant was generated and reported to have a total loss of melanization (Nam et al., 2012). Surprisingly, with the use of our newly generated null mutants, we observed phenotypes that partially contradict the already published reports. We were able to confirm that both Hayan and Sp7 affect melanization in general (Dudzic et al., 2015). Interestingly none of the single mutations in *Sp7* or *Hayan* abolished melanization completely. While *Sp7* mutant flies melanize the wound area to almost wild-type levels, *Hayan* mutants show no melanin at the wound. In larvae we found opposite phenotypes, while *Sp7* mutants do not show melanin at the wound site, *Hayan* flies produce residual melanin and are therefore not deficient for melanization. These results prompted us to investigate the roles of those three SPs in more detail.

We first developed a low-dose infection model with S. *aureus*, which is especially appropriate to study melanization. Flies lacking the ability to melanize (mutant for *PPO1* and *PPO2*) cannot survive the infection with *S. aureus*. We found that melanization-deficient flies are unable to control *S. aureus* growth and cannot prevent its systemic dissemination. This susceptibility can be clearly attributed to the melanization reaction, because flies with impaired AMP production or lacking hemocytes survive this infection. We then used this infection model to investigate the role of MP1, Sp7 and Hayan in the activation of the melanization reaction. To our surprise, we found that only *Sp7* but not *MP1* or *Hayan* deficient flies phenocopied the *PPO1,2* mutants. This result is even more puzzling if one considers that the susceptible *Sp7* mutant flies show normal levels of hemolymph and wound melanization, while the resistant *Hayan* deficient flies show no melanization of hemolymph or wound areas. Thus, the susceptibility to *S. aureus* is linked to the melanization cascade, but not to the melanin deposition. Therefore, we disconnect the production of melanin from the septic clearance effects mediated via PO activity. We investigated the role of cytotoxic compounds produced by PO activity by measuring H_2O_2 and found a reduction of H_2O_2 levels in the *S. aureus* sensitive mutants (*PPO1,2* and *Sp7*), but not the resistant flies (*wild-type*, *Hayan*). This indicates that indeed PO activity might be able to produce cytotoxic compounds independently of melanin polymerization. Unfortunately, the differences in ROS production are minor, and future studies should better characterize the mechanism by which the melanization cascade eliminates *S. aureus*.

While *Hayan* (impaired melanogenesis) and *Sp7* (impaired survival) mutants both exhibit the above-mentioned phenotypes affecting the melanization reaction, we found no clear role for *MP1* mutants. Flies as well as larvae deficient for *MP1* show no defect in hemolymph melanization, wound melanization or survival, which stands in contrast to previous reports based on RNAi (Tang et al., 2006). We cannot exclude a minor role of MP1 in melanin production around wound sites, because flies simultaneously mutant for *Hayan* and *Sp7* still exhibit a residual melanization. Interestingly, we found that we can provoke wound melanization in *Hayan* mutant flies when we introduce Gram-positive bacteria into the wound: after septic injury with *M. luteus*, almost all *Hayan* deficient flies melanize the wound site at least weakly. This pointed to the possible involvement of PRRs for Gram-positive bacteria in the activation of POs, and therefore indicated a role of Toll in the melanization reaction, as demonstrated in many other insects (An et al., 2009; Kan et al., 2008; Kim et al., 2008).

Indeed, when we used our newly developed low-dose infection model with *S. aureus*, we found that the extracellular PRRs involved upstream of Toll in the recognition of Gram-positive bacteria (PGRP-SA and GNBP1), but not the fungal receptor (GNBP3), exhibit similar susceptibility to *S. aureus* like melanization deficient flies. We identified other components of the extracellular SP cascade involved in the regulation of the Toll pathway that share this survival phenotype. More precisely, we demonstrate that *ModSP* (downstream of PRRs) and *Grass* (downstream of ModSP) deficient flies, but not flies lacking *SPE* (downstream of Grass), die at similar rates like flies lacking PPOs. This indicates that Grass has two roles: i) activating SPE or an effector SP upstream of SPE and ii) activating Sp7 (directly or an upstream effector) and therefore initiating the PPO mediated clearance of *S. aureus*. This study therefore clearly demonstrates a direct connection between the extracellular SPs that regulate the activation of Toll and the melanization reaction in *Drosophila*.

After finding this connection, we focused on the possibility that one of the two SPs involved in activating the melanization reaction might also have a role in Toll activation. By analyzing the activation of Toll after exposure to Gram-positive *M. luteus* via measuring *Drosomycin* expression as a reporter of Toll activity, we found no defect in *Drosomycin* induction in *Sp7, Hayan* and *MP1* single mutant flies. Interestingly, *Hayan* is the direct genomic neighbor of the Toll-regulating SP *persephone* on the X-chromosome of *D. melanogaster*. Our phylogenetic analysis revealed that Hayan and Psh are closely related, and that *psh* appears to be the result of a gene duplication

event of an ancestral *Hayan* gene. We therefore generated a double mutant for both *Hayan* and *psh* to investigate the possibility that both SPs still have overlapping roles, and the loss of only one might therefore hide a possible phenotype. Indeed, we found that flies harboring the *Hayan-psh* deficiency fail to activate the Toll pathway after infection with the Gram-positive bacteria *M. luteus*, the yeast *C. albicans*, as well as the injection of purified protease from *Bacillus subtilis*. This compellingly suggests a role of both *Hayan* and *psh* in the activation of the Toll, via both the Psh and PRR pathway. We propose that both Hayan and Psh stem from a recent gene-duplication event and still have overlapping functions, but already show signs of the early beginnings of sub-functionalization. Further phylogenetic investigations reveal that both Hayan and Psh are related to the SP Snake, which regulates the Spätzle processing SP Easter during development (Lindsay and Wasserman, 2014). We suggest therefore that both Hayan and Psh are likely to directly act upstream of SPE in the immunity related Toll activation. Our study was possible due to the ability to generate loss of function mutants for single or multiple genes by using a CRISPR approach. Whether it is common to observe phenotypes only in compound mutants, which are otherwise masked in single mutations, can be answered with the further generation of compound mutants that affect multiple SPs. SP genes are often found in clusters and a future project deleting those clusters one by one seems appealing.

Taken together, the presented evidence from the second part of this thesis reshapes our knowledge of the SPs regulating the Toll pathway and melanization. First, we present strong evidence of a connection between Toll and the melanization reaction, and secondly, we propose that Hayan has a critical role in Toll activation upstream of SPE. Additionally, we disconnect the visible melanogenesis process operated through POs from an “invisible” septic clearance mechanism, also orchestrated via POs probably through the production of cytotoxic compounds. We conclude therefore that the melanization reaction in *D. melanogaster* is more than black or white.

9.1 Outlook

Currently, it is unclear if the monomeric form of activated PO can produce melanin on its own. In several insects it was found that POs have to interact with other proteins to form so called “activation complexes” to produce melanin (Whitten and Coates, 2017). Gupta and colleagues

found that when PPO is cleaved by its PPAF PAP-1, the resulting PO activity is relatively low in the lepidopteran *M. sexta*. They observed a 20-fold higher PO activity in the additional presence of two SPHs, SPH1/2, which work as co-factors necessary for optimal melanin production. This boost in activity is not dependent on the cleavage of PPO to PO, because they observed that even in the absence of the SPHs a high proportion of PPO is cleaved to PO, but shows only low activity (Gupta et al., 2005). POs are not only found to interact with SPHs but can also interact with or react to other macromolecules. Bidla and colleagues for instance report, that *D. melanogaster* exhibits two different forms of melanization. They differentiate between a systemic melanization response after bacterial sepsis and a clot melanization. The latter one can be induced by the presence of the apoptotic marker phosphatidylserine (Bidla et al., 2009). They propose that PPOs or POs can form complexes with their activating proteases and lipids to form melanin producing complexes in the clot, which result in a super-induction of PO activity (Bidla et al., 2009). Recent reports indicate the presence of multi-functional Immune Complexes (ICs) in the hemolymph of *Bombyx mori* and *Aedes aegypti* (Clark and Strand, 2013; Phillips and Clark, 2017). These are high molecular weight protein complexes consisting of a variety of proteins. By using mass spectrometry, the presence of pathogen recognition proteins, coagulants, and melanization components were confirmed in these complexes. Interestingly, while monomeric PO fractions showed little activity after HPLC elution, the fractions containing the immune complexes showed an almost instant and strong melanization (Clark and Strand, 2013). Taken together, these results suggest a more complex mode of regulation for the melanization reaction than simply activating the POs. While the function of those multi-protein complexes is not fully understood yet, it is tempting to speculate that those immune complexes could act as molecular scaffolds to control and locate melanization to specific locations, such as wound sites or microbial surfaces and regulate the activity of POs.

To gain further insights into the melanization reaction in *D. melanogaster*, an investigation of *Drosophila's* immune complexes seems promising. First, the presence of the immune complexes in Drosophila, although already indicated by others (Matskevich et al., 2010), has to be confirmed. The confirmation can be followed with a strategic approach to first identify the proteins incorporated in these immune complexes, and then systematically use RNAi or mutants of those proteins to identify their roles in the formation of the immune complexes and especially in the activation of the melanization reaction. Furthermore, it would be tempting to investigate whether

the protein-composition of the immune complexes differs from complexes formed in the hemolymph, at wound sites or attached to microbes. These investigations could aid in a better understanding in the melanization reaction and create a more complete picture of *Drosophila* and therefore invertebrate immunity. Thus, a future proteomic approach to analyze the mechanism of PO activation is required to understand how this cascade functions in the hemolymph.

10 ABBREVIATIONS

AMP	antimicrobial peptide
clip-SP	clip-serine protease
DCE	dopachrome conversion enzyme
DHI	5,6-dihydroxyindole
GNBP	Gram-negative bacteria binding protein
Grass	Gram-positive-specific serine protease
Imd	immune deficiency
MAMP	microbe associated molecular patterns
ModSP	modular serine protease
PGN	peptidoglycan
PO	phenoloxidase
PPAE	PPO-activating enzyme
PPAF	PPO-activating factor
PPO	prophenoloxidase
PGRP	peptidoglycan recognition protein
PRR	pattern recognition receptor
Psh	persephone
ROS	reactive oxygen species
serpin	serine protease inhibitor
SP	serine protease
SPE	spätzle processing enzyme
SPH	serine protease homolog
Spn	serpin
Spz	spätzle
SWD	spotted wing drosophila
WSSV	white spot syndrome virus

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12 CURRICULUM VITAE

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Education:

Ph.D.	August 2014 - to be completed end of 2018 Title: "The melanization reaction in Drosophila: More than black or white" Supervisor: Bruno Lemaitre École polytechnique fédérale de Lausanne EPFL, Switzerland
M. Sc. Biology	July 2014 Title: "Characterization of *Manduca sexta* peptidoglycan recognition proteins" Supervisor: Tina Trenczek Justus-Liebig-University Giessen, Germany
B. Sc. Biology	October 2012 Title: "Characterization of antigens from monoclonal *Manduca sexta* antibodies" Supervisor: Tina Trenczek Justus-Liebig-University Giessen, Germany

Publications:

Under review in Cell Reports (09/2018)	More than black and white: complex relationships involving serine proteases regulate the Toll pathway and the melanization response in Drosophila **J.P. Dudzic**, M.A. Hanson, I. Iatsenko, S. Kondo, and B. Lemaitre Pre-print available: https://doi.org/10.1101/383257
Under review in Developmental Cell (09/2018)	Blood cells have a metabolic cost: identification of a new adipokine to regulate hemocyte proliferation E. Ramond, **J.P. Dudzic**, B. Petrignani, J.P. Boquete, M. Poidevin, S. Kondo, B. Lemaitre
2015	Remote control of intestinal stem cell activity by haemocytes in *Drosophila* S. Chakrabarti, **J.P. Dudzic**, X. Li, E.J. Collas, J.P. Boquete, B. Lemaitre PLoS Genetics 12 (5), e1006089
2014	*Drosophila* innate immunity: regional and functional specialization of prophenoloxidases **J.P. Dudzic**, S. Kondo, R. Ueda, C.M. Bergman, B. Lemaitre BMC Biology 13 (1), 81

Conference talks and Posters:

2017	More than black or white: The variable roles of the melanization reaction in Drosophila innate immunity **J.P. Dudzic**, D. Main, B. Lemaitre Poster, European Drosophila Research Conference 2017, London, U.K.
2016	Interplay between gut and systemic immune response in Drosophila **J.P. Dudzic**, S. Chakrabarti, X. Li, E.J. Collas, J.P. Boquete, B. Lemaitre Conference talk, International Conference of Entomology 2016, Orlando, U.S.
2015	Drosophila innate immunity: regional and functional specialization of prophenoloxidases **J.P. Dudzic**, S. Kondo, R. Ueda, C.M. Bergman, B. Lemaitre Poster, European Drosophila Research Conference 2015, Heidelberg, Germany

Awards & Grants:

2018	SNF Early Postdoc.Mobility – Host laboratory: Steve Perlman, University of Victoria, British Columbia, Canada
2015	LS2 Swiss Academy of Sciences Travel Grant for PhD students
2014	Best Poster Award, European Drosophila Research Conference 2015, Heidelberg, Germany

Teaching:

2018,17,16	Teaching assistant for Biotechnology lab
2015,14	Teaching assistant for Immunology

Supervision:

2017	Supervision of lab technician trainee (6 months)
2017,16,15	Short term Bachelor projects

Qualification courses:

- Advanced Microscopy for Life Science (Bioimaging and optics platform EPFL)
- Gene transfer and recombinant protein expression in animal cells
- Responsible Conduct in Biomedical Research
- Transcriptomics Analysis: RNA-seq (Swiss Institute of Bioinformatics)
- Mass-Spectrometry based Proteomics (Swiss Institute of Bioinformatics)

Key skills:

- Molecular biology (molecular cloning, Real-Time PCR, genotyping)
- Protein biochemistry (recombinant protein production, purification and analysis in pro- and eukaryotic cells)
- Analysis of next-generation sequencing data (RNA-Seq)
- Advanced light microscopy (fluorescence, CLSM)

Further relevant professional experience:

2013 - 2014	Graduate Research Assistant Fraunhofer Institute for Molecular Biology and Applied Ecology, Giessen - Bio-Resources - Insects as model for human disease - Risk assessment of transgenic insects - Molecular Biocontrol
December 2012	Graduate Research Assistant Institute for General Zoology and Developmental Biology, Justus-Liebig Universität Giessen, Germany
December 2012	DAAD Exchange – German Academic Exchange Service Academy of Sciences of the Czech Republic, Prague (8 days) Laboratory of Cellular and Molecular Immunology Project „Mucosa associated natural Immunity of Invertebrates“
2012 – 2013	Graduate Research Assistant Institute for Plant Ecology, Justus-Liebig Universität Giessen, Germany Trace gas monitoring and sample ionization for mass spectrometry
2011 – 2013	Student Assistant IT Service Centre of Justus Liebig University Giessen, Germany Project „Lecture Recordings“
September 2011	Student Assistant for teaching Institute for Genetics, Justus-Liebig Universität Giessen, Germany
2011 – 2014	Student Assistant Institute for General Zoology and Developmental Biology Lab of Prof. T. Trenczek – Cellular-recognition and –defense mechanisms