Three clustered serine proteases, Hayan, Persephone and Skanda, regulate Toll pathway-mediated immunity in *Drosophila*

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by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled “Three clustered serine proteases, Hayan, Persephone and Skanda, regulate Toll pathway-mediated immunity in *Drosophila*” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sanjana Vasanth at École Polytechnique Fédérale de Lausanne (EPFL) under the supervision of Bruno Lemaitre, Professor, Global Health Institute, School of Life Sciences, EPFL, during the academic year 2023-2024.

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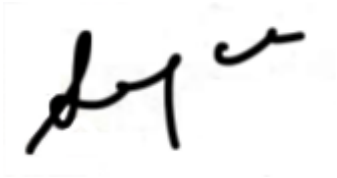
This thesis is dedicated to 6 year old Sanjana who wanted to be a scientist, and to her parents who never said no.

Declaration

I hereby declare that the matter embodied in the report entitled “Three clustered serine proteases, Hayan, Persephone and Skanda, regulate Toll pathway-mediated immunity in *Drosophila*” are the results of the work carried out by me at École Polytechnique Fédérale de Lausanne (EPFL), under the supervision of Prof Bruno Lemaitre, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.



Sanjana Vasanth

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Abstract

Drosophila melanogaster is an ideal model system for characterising innate immune pathways. Studying the immune response of *Drosophila* allows us to understand their functioning in mammals and other insects, providing us with a multitude of environmental and clinical applications. Two of these immune pathways, Toll signalling and melanisation, involved in combatting Gram-positive bacteria and fungi, are activated by extracellular serine protease cascades. Two clustered serine proteases, Hayan and Psh, play similar roles in regulating these cascades. Skanda (CG15046) is an uncharacterised serine protease homolog also clustered with Hayan and Psh. Its expression is upregulated in the fly upon infection, suggesting that it might be involved in the *Drosophila* immune response. In this study, we investigate whether Skanda, like its neighbours Hayan and Psh, does indeed function in *Drosophila* immunity. We reveal that Skanda is required for resistance to Gram-positive bacteria, particularly *Staphylococcus aureus*. We use compound mutants of the *Hayan-psh-Skanda* gene cluster to uncover interesting phenotypes that might be missed while using single mutants. We reveal that Skanda redundantly regulates the Toll pathway along with Psh, and *psh-Skanda* double mutants have no functional Toll response, similar to *Hayan-psh* double mutants. Therefore, we hypothesize that the *Hayan-psh-Skanda* cluster allows for the differential regulation and precise tuning of the Toll and melanisation pathways.

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Thank you all for being my village.

Contributions

Contributor name	Contributor role
Bruno Lemaitre, Sanjana Vasanth	Conceptualization Ideas
Bruno Lemaitre, Sanjana Vasanth	Methodology
-	Software
Sanjana Vasanth	Validation
Sanjana Vasanth	Formal analysis
Sanjana Vasanth	Investigation
Bruno Lemaitre, EPFL	Resources
-	Data Curation
Sanjana Vasanth	Writing - original draft preparation
Bruno Lemaitre, Sanjana Vasanth	Writing - review and editing
Sanjana Vasanth, Bruno Lemaitre	Visualization
Bruno Lemaitre	Supervision
Bruno Lemaitre	Project administration
Bruno Lemaitre	Funding acquisition

Chapter 1: Introduction

Drosophila melanogaster spends the majority of its life cycle in rotting fruit, among other decaying organic matter. As a consequence, the dipteran often comes in contact with many beneficial and pathogenic microorganisms that share its habitat. In order to combat infection by these bacteria, fungi, viruses, and even macroparasites (nematodes, wasps), *Drosophila* has evolved several innate immune pathways (*Figure 1*). The advantage of studying these immune responses in *Drosophila* is two-fold. First, these responses are evolutionarily conserved, making *Drosophila* a good model organism to characterise innate immune mechanisms in mammals (Tzou *et al.*, 2002). Second, studying the immune response of *Drosophila* also provides insight into the defence mechanisms of other insects that act as vectors for plant and animal diseases (Lemaitre and Hoffmann, 2007). Combined with the fact that *Drosophila* is a well-characterised classical model system, *Drosophila* immune research has major medical, agricultural and environmental impacts.

1.1 The *Drosophila* innate immune response

The insect's chitinous cuticle, tracheal and intestinal epithelium form the first line of defence against invasion by microorganisms. Breaching this physical barrier activates a **local immune response** involving the production of microbicidal anti-microbial peptides (AMPs) and reactive oxygen species (ROS) to eliminate the invaders (Davis and Engström, 2012). As the infection proceeds to the fly's hemolymph (blood), its systemic immune response is activated. This response can be divided into two parts – cellular immunity and humoral immunity.

Cellular immunity is provided by three types of hemocytes, or "blood cells". Plasmatocytes, which make up 90-95% of all hemocytes, function primarily in phagocytosis – engulfing both pathogens and host dead cells (Lanot *et al.*, 2001; Ulvila *et al.*, 2011). They also produce AMPs and clotting factors (Dimarcq *et al.*, 1997; Goto *et al.*, 2003). Crystal cells make up the remaining 5-10% of hemocytes (Lanot *et al.*, 2001). They are involved in melanisation, an arthropod-specific immune response that involves the deposition of melanin at wound sites to form a scab (Binggeli *et al.*, 2014). Lamellocytes are exclusive to larvae. They are usually very few in number but multiply rapidly upon parasite infection. They are involved in the encapsulation of parasite eggs (Dudzic *et al.*, 2015).

Humoral immunity involves the large-scale production of immune effectors by the fat body, a liver-like organ situated in the hemocoel of the fly (Imler and Bulet, 2005). AMPs are one of the main components of these effectors (Uttenweiler-Joseph *et al.*, 1998; Liu *et al.*, 2006). AMP production is triggered upon identification of bacterial and fungal cell wall components by the Pattern Recognition Receptors (PRRs) of Toll and Imd pathways (Lemaitre and Hoffmann, 2007). The peptides are then secreted into the hemolymph, where they kill invading pathogens (Hanson and Lemaitre, 2020).

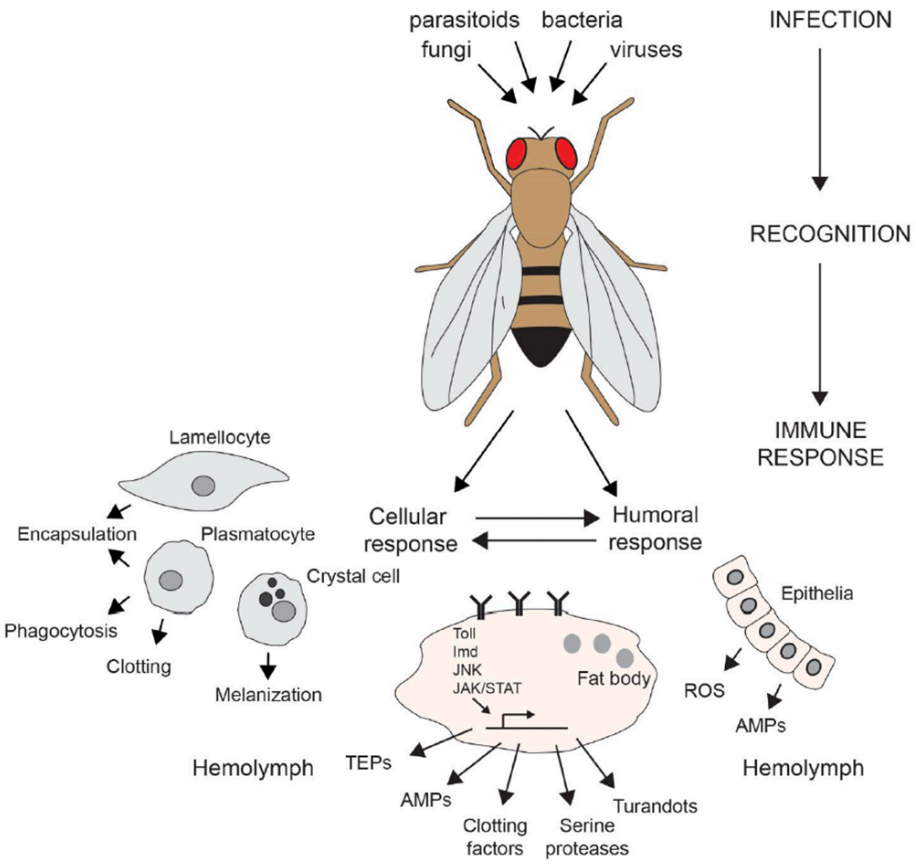


Figure 1. ***A summary of the innate immune response in*** **Drosophila**: *The cellular immune response (left) is carried out by the* Drosophila *hemocytes. Plasmatocytes take part in phagocytosis and clotting. Crystal cells take part in melanisation, and lamellocytes take part in encapsulation of parasite eggs. The humoral immune response (middle) mainly consists of the Toll and Imd pathways. These signalling cascades result in the secretion of multiple immune effectors including microbicidal AMPs by the fat body. The local immune response (right) carried out at the epithelia results in the release of ROS and AMPs to kill invading microorganisms. Adapted from* Vanha-aho *et al.*, 2016.

1.1.1 The Imd pathway

The immune deficiency (Imd) pathway is one of the two main NF-kB pathways that function in *Drosophila* humoral immunity. It is mainly involved in resistance to Gram-negative bacteria. The transmembrane receptor PGRP-LC and the cytosolic receptor PGRP-LE sense diaminopimelic acid (DAP)-type peptidoglycan that is present mostly in Gram-negative bacteria but also in a few Gram-positive bacteria (Gottar *et al.*, 2002; Leulier *et al.*, 2003; Takehana *et al.*, 2004; Kaneko *et al.*, 2006). These receptors recruit Imd, which goes on to activate the NF-kB protein Relish in two different ways. Imd recruits Fas-associated death domain (FADD), which activates caspase Death related ced-3/Nedd2-like (Dredd), which cleaves Relish. Imd also activates the TAK1/TAB2 complex, which activates the IKK complex. The IKK complex phosphorylates Relish. Cleavage allows Relish to translocate into the nucleus, and phosphorylation allows it to efficiently activate the transcription of Imd-induced genes (Kleino and Silverman, 2014). A

different set of AMPs are transcribed and secreted into the hemolymph, and a systemic immune response is mounted (Hanson *et al.*, 2019). The expression of AMP Diptericin A is commonly used as a readout of Imd activation.

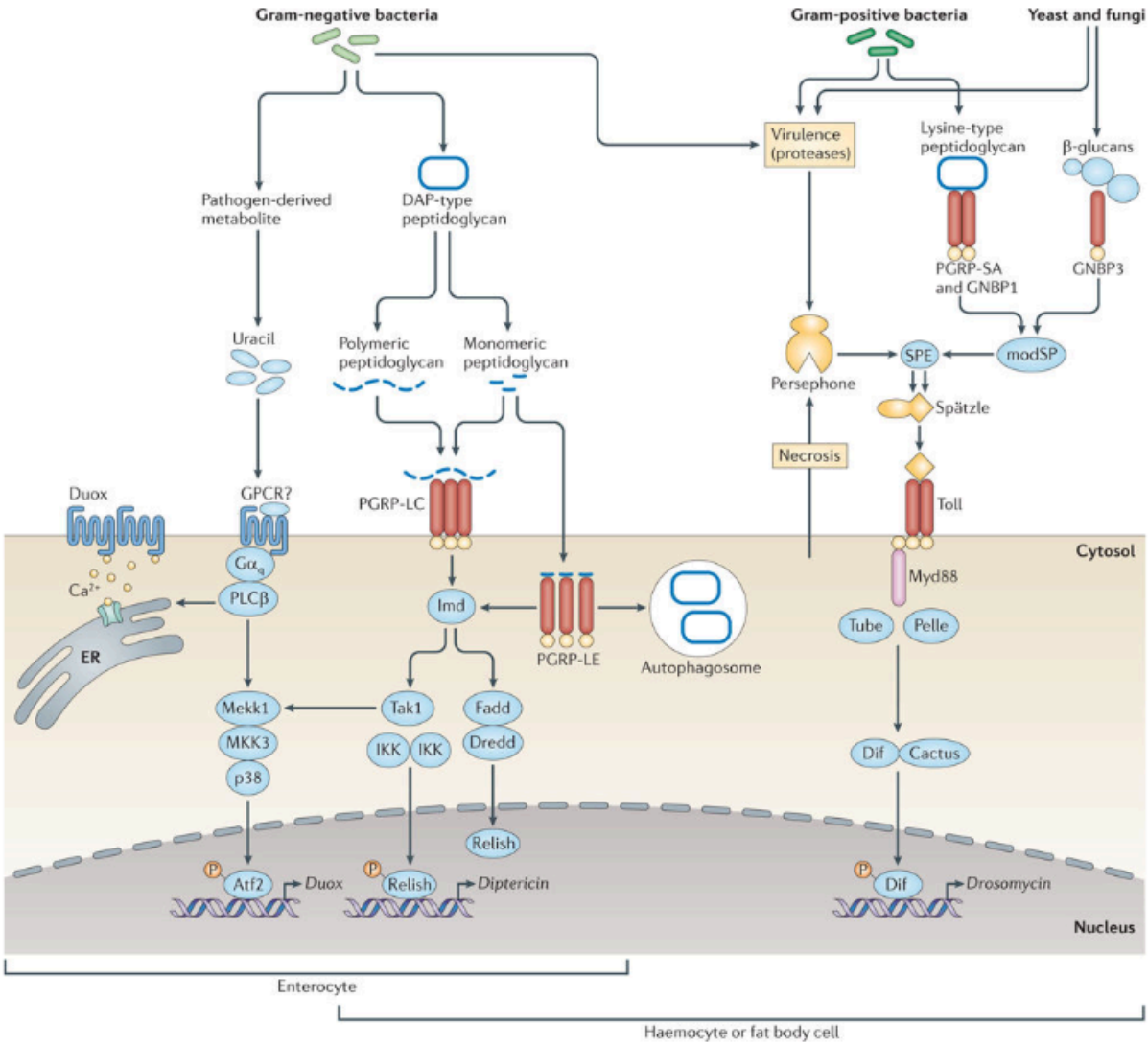


Figure 2. ***A summary of the pathways involved in humoral immunity in*** **Drosophila**: *Gram-negative bacteria are detected by the Imd pathway (left). DAP-type peptidoglycan is detected by PRRs PGRP-LC and PGRP-LE. They recruit Imd, which goes on to recruit the FADD/Dredd complex and TAK1/TAB2, which cleave and phosphorylate the NF-kB protein Relish. Activated Relish translocates into the nucleus to activate the transcription of Imd-regulated genes, including the AMP Diptericin. Gram-positive bacteria and fungi are detected by the Toll pathway (right). Lysine-type peptidoglycans and fungal β-glucans are detected by PRRs PGRP-SA, GNBP1 and GNBP3. They activate an extracellular serine protease cascade which results in the cleavege of cytokine Spz by the serine protease SPE. Spz binds to the Toll receptor, which then activates intracellular Toll signalling, resulting in the activation of NF-kB proteins Dif. Dif then translocates into the nucleus and activate the trnascription of Toll-regulated genes, including the AMP Drosomycin. Adapted from* Buchon *et al.*, 2014.

1.1.2 The Toll pathway

The Toll pathway is the other main NF-kB pathway functioning in *Drosophila* humoral immunity. First discovered in the context of embryonic development, Toll signalling was later found to play an important role in immunity against bacterial and fungal infections (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002). The pathway is activated upon recognition of certain pathogen-associated molecular pattern (PAMP) molecules. Lysine-type peptidoglycans, found in Gram-positive bacteria, are recognised by PRRs PGRP-SA and GNBP1 (Gobert *et al.*, 2003; Leulier *et al.*, 2003; Pili-Floury *et al.*, 2004). Fungal β-glucans are recognised by the PRR GNBP3 (Gottar *et al.*, 2006). This leads to the activation of an extracellular serine protease (SP) cascade. These SPs undergo zymogen activation upon cleavage by an upstream protease. Once activated, they cleave the corresponding downstream protease (Veillard *et al.*, 2016).

ModSP is autoactivated when peptidoglycans bind to PRRs, leading eventually to the activation of the SP Grass (Buchon *et al.*, 2009). A recent *in vitro* biochemical analysis found that ModSP cleaved CLIP-SP 48 (cSP48), which then cleaved Grass (Shan *et al.*, 2023). Hayan and Persephone (Psh) are two SPs that function redundantly downstream of Grass, and lead to the maturation of SP Spätzle processing enzyme (SPE) (Dudzic *et al.*, 2019; Shan *et al.*, 2023). SPE then cleaves the *Drosophila* cytokine Spätzle (Spz), which activates Toll intracellular signalling (Jang *et al.*, 2006). The Toll pathway has two branches. The first branch is the above-mentioned **PRR pathway**. The second branch is the **Psh pathway**, which is activated when microbial proteases cleave the Persephone bait region, directly activating SPE and Spätzle (Ligoxygakis *et al.*, 2002; Chamy *et al.*, 2008; Issa *et al.*, 2018). It has recently been shown that Hayan can also be activated by microbial proteases (Dudzic *et al.*, 2019; Shan *et al.*, 2023).

This SP cascade is negatively regulated by serine protease inhibitors, or serpins (Reichhart *et al.*, 2011). Necrotic (Spn43Ac/nec) has been shown to negatively regulate Psh activation (Levashina *et al.*, 1999). Serpin1 (Spn1) has been shown to work on the GNBP3 branch of the PRR pathway, upstream of Grass and SPE (Fullaondo *et al.*, 2011). Serpin5 (Spn5) is another known negative regulator of the Toll pathway (Ahmad *et al.*, 2009).

The mature form of Spz binds to the Toll receptor (Morisato and Anderson, 1994). The Toll receptor then activates intracellular signalling by recruiting and activating MyD88, Tube and Pelle (Galindo *et al.*, 1995; Tauszig-Delamasure *et al.*, 2002). Pelle then phosphorylates Cactus (IkB homolog), which leads to its degradation (Belvin and Anderson, 1996). The degradation of Cactus releases the NF-kB proteins Dif and Dorsal, which translocate into the nucleus (Ip *et al.*, 1993; Gillespie and Wasserman, 1994). This leads to the transcription and subsequent secretion of a specific set of microbicidal AMPs by the fat body into the hemocoel, mounting a systemic immune response (Lemaitre *et al.*, 1996). The expression of Drosomycin, a strongly induced antifungal peptide, is commonly used as a readout for Toll activation.

1.1.3 Melanisation

Melanisation is an arthropod-specific immune response that involves the deposition of melanin at wound sites. A scab is formed at the site of injury, contributing to wound healing and preventing the entry of new microbes into the hemocoel. Additionally, melanin is deposited around the surface of the invading microbes, sequestering them to wound sites. The ROS produced during the formation of melanin are also thought to be directly toxic to the microbes (Tang, 2009).

Melanisation, if not controlled, is a lethal process due to the high amount of toxic intermediates produced. This is evidenced by the lethality arising from mutations that cause excessive melanisation (De Gregorio *et al.*, 2002a; Scherfer *et al.*, 2008). Hence, its regulation is extremely important. A rate-limiting enzyme in the formation of melanin is phenoloxidase (PO), which catalyses the conversion of phenols to quinones. These quinones then polymerise to form melanin. POs are produced in an inactive form called prophenoloxidase (PPO) (Tang, 2009). Three PPOs have been identified in *Drosophila*; PPO1 and PPO2, produced by crystal cells, are involved in hemolymph melanisation, while PPO3, produced by lamellocytes, is involved in the encapsulation of parasite eggs (Binggeli *et al.*, 2014; Dudzic *et al.*, 2015).

Upon injury, crystal cells burst to release PPO1 and PPO2 into the hemolymph. PPO activation is regulated by multiple serine protease cascades (Tang *et al.*, 2006; Dudzic *et al.*, 2019). Melanisation is therefore a combination of cellular and humoral immunity. One cascade involves the **activation of Hayan** upon epithelial wounding, which eventually activates the PPOs (Nam *et al.*, 2012). Biochemical analysis showed that Hayan was able to cleave Melanisation Protease 2 (MP2 or Sp7) and Melanisation Protease 1 (MP1) *in vitro*. MP2 was then able to cleave PPO1 and PPO2 in vitro. MP1 might play a minor role in the PPO activation cascade, as MP1 was able to cleave PPO1 and PPO2 to a small extent (Shan *et al.*, 2023). A second cascade involves the activation of the Toll **PRR pathway**, where Psh regulates PPO1 activation through MP2 and possibly MP1 (Dudzic *et al.*, 2019; Shan *et al.*, 2023). There are also many negative regulators of this pathway in the form of serpins. Serpin27a (Spn27a) competes with PPO1 to bind to the SP that cleaves PPO1 (De Gregorio *et al.*, 2002a). Serpin28D (Spn28D) is another known negative regulator of this cascade (Scherfer *et al.*, 2008).

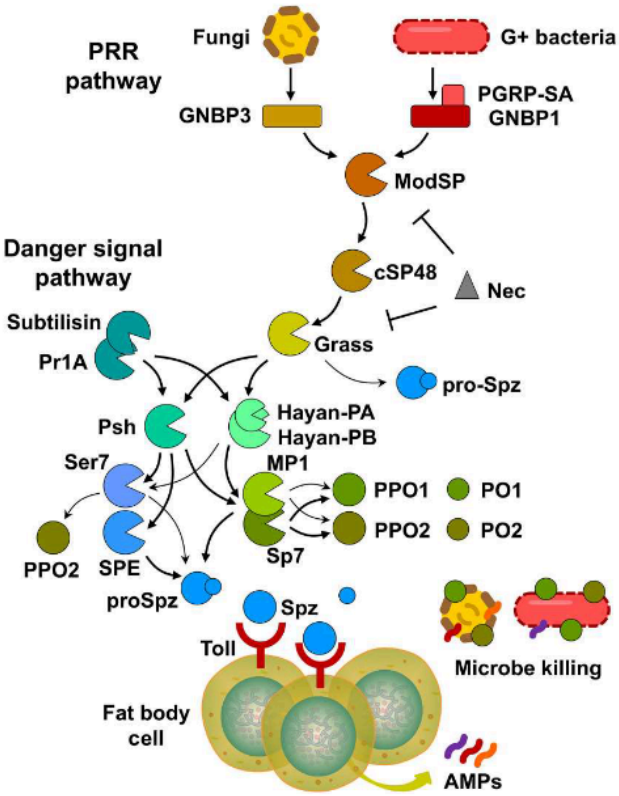


Figure 3. ***A summary of the extracellular serine protease cascade governing Toll signalling and melanisation in*** **Drosophila***: The invasion of pathogens into the* Drosophila *hemocoel initiates the activation of an extracellular serine protease network. Lysine-type peptidoglycans and fungal β-glucans are detected by PRRs PGRP-SA, GNBP1 and GNBP3. This triggers the sequential activation of ModSP, cSP48, Grass, and Psh/Hayan. Psh mediates the activation of SPE, which cleaves Spz, eventually activating intracellular Toll signalling. Psh can also be independently activated by various microbial proteases. Hayan and Psh also cleave MP1 and Sp7 (MP2), which leads to the cleavage of PPO1 and PPO2, activating the melanisation response. Adapted from* Shan *et al.*, 2023.

1.2 CLIP SPs and SPHs

Chymotrypsin-like serine proteases are amongst the most well-characterized enzyme families. They play essential roles in biological processes like digestion, embryonic development, and immunity, among others (Veillard *et al.*, 2016). They are usually produced as inactive zymogens and are activated upon proteolytic cleavage. Sequential cleavage of one SP by another can lead to the establishment of complex cascades (Veillard *et al.*, 2016). These proteins contain an S1A peptidase domain, characterized by the perpendicular arrangement of two adjacent β-barrel-like structures. Each barrel is made up of six antiparallel β-strands. The His-Asp-Ser catalytic triad responsible for its proteolytic ability is located in the cleft formed between the barrels (Huber *et al.*, 1974; Bode and Schwager, 1975; Wang *et al.*, 1985; Veillard *et al.*, 2016). SPs and their catalytically inactive homologs (SPHs) form one of the largest gene families in *Drosophila*, with 257 members (Cao and Jiang, 2018).

Depending on their function, SPs can have different N-terminal domains connected to their peptidase domain. Digestive SPs like trypsin and chymotrypsin contain only a short N-terminal peptide. SPs involved in more complex processes have domains necessary for protein-protein interactions. The interactions of these regulatory domains guide the sequential organisation of SP cascades along with their localisation and intensity (Veillard *et al.*, 2016). One such domain is the CLIP domain, called so because of the paper clip-like shape formed by the diisulfide linkages between its strictly conserved cysteine residues (Smith and DeLotto, 1992; Jiang and Kanost, 2000). Out of the 257 SPs and SPHs, 52 are associated with at least one CLIP domain (Cao and Jiang, 2018). CLIP domains are connected to the peptidase domains via a linker containing at least one cysteine. This cysteine forms a disulfide bond with a cysteine present in the peptidase domain. Due to this bond, the CLIP domain remains connected to the active SP after zymogen activation (Veillard *et al.*, 2016).

CLIP-SPs regulate some of the most important extracellular cascades in *Drosophila* – dorso-ventral patterning, Toll signalling and melanisation (Veillard *et al.*, 2016). The functions of CLIP SPHs, however, are a little more elusive. They have been implicated in somatic muscle attachment (Masquerade), morphogenesis of imaginal discs (scarface) and regulation of hemocyte proliferation (CG4793) among others (Murugasu-Oei *et al.*, 1995; Gordon *et al.*, 2008; Bina *et al.*, 2010). As they are catalytically inactive, they are imagined to act as co-factors or binding partners for CLIP SPs, and function as regulators of SP cascades. To this end, two CLIP SPHs, cSPH35 and cSPH242, have been found to act as co-factors for the activation of PPO1 by MP2 (Jin *et al.*, 2023).

1.3 Hayan and Psh

Hayan and *psh* are sister genes located only 751 bp apart on the *Drosophila melanogaster* X chromosome. They encode single CLIP domain SPs that play essential roles in the extracellular Toll and melanisation cascades (Dudzic *et al.*, 2019). Persephone (Psh) was first discovered as a suppressor of the *necrotic* phenotype. Removing *psh* in a *nec* deficient background abolishes the constitutive Toll activation seen when the serpin is removed (Ligoxygakis *et al.*, 2002). Psh can be activated by fungal and bacterial proteases (Gottar *et al.*, 2006; Chamy *et al.*, 2008). The inactive zymogen form of Psh contains a “bait region” that is prone to cleavage by microbial proteases regardless of their origin, type, or specificity. After processing by the microbial proteases, Psh is cleaved by the *Drosophila* cathepsin 26-29-p. This two-step process produces an active Psh which then goes on to cleave SPE, which finally cleaves Spz (Issa *et al.*, 2018). This pathway is called the effector triggered immunity or protease cascade, a second branch acting alongside the canonical PRR branch of the Toll signalling (Chamy *et al.*, 2008).

Hayan was first discovered as a regulator of the systemic wound response and melanisation in *Drosophila* (Nam *et al.*, 2012). Hayan mutant flies lack melanisation both in the hemolymph and at the wound site (Dudzic *et al.*, 2019). The excessive melanisation seen in Spn27a and Spn28D mutant flies is lost when Hayan is also removed (Nam *et al.*, 2012). The ROS produced

when Hayan activates melanisation at the wound site via the PPOs activates c-Jun N-terminal kinase (JNK)-dependent cytoprotection in neuronal tissues, protecting flies from stress and physical trauma (Nam *et al.*, 2012).

A recent study revealed that Hayan and Psh form a closely related monophyletic lineage among other CLIP SPs. Bioinformatic evidence indicates that *psh* evolved specifically in the Melanogaster group flies from a gene duplication event of an ancestral *Hayan* gene that is conserved across the *Drosophila* genus. Hayan also has a protease “bait region” similar to Psh (Dudzic *et al.*, 2019).

Hayan and Psh integrate signals from both the PRR branch and the protease branch of Toll signalling. Biochemical analysis revealed that like Psh, Hayan can also be cleaved by microbial proteases (Shan *et al.*, 2023). Hayan and Psh can be cleaved by Grass, and redundantly regulate the PRR branch of the Toll pathway as well (Shan *et al.*, 2023). Flies lacking both Hayan and Psh have no functional Toll response, similar to Spz mutants (Dudzic *et al.*, 2019). Psh is also required for melanisation upon septic injury with Gram-positive bacteria (Dudzic *et al.*, 2019). Therefore, Hayan and Psh are two CLIP SPs with similar structures and functions.

The gene CG15046 encodes a CLIP SPH and is located in the same gene locus as Hayan and Psh, only 529 bp away from Psh. Like its neighbours, CG15046 gene expression is upregulated in *Drosophila* upon infection with pathogens (De Gregorio *et al.*, 2002b). As Hayan and Psh are clustered with each other and function in Toll signalling and melanisation, we wondered if CG15046 had a similar role to play in *Drosophila* immunity.

Our characterisation of CG15046, hereafter named Skanda, reveals that it is involved in the *Drosophila* immune response. Skanda is required for resistance to Gram-positive bacteria, but does not affect AMP expression. Psh and Skanda redundantly regulate Toll signalling, similar to Hayan and Psh. Globally, we reveal that the *Hayan-psh-Skanda* cluster allows for differential regulation of the Toll and melanisation cascades in *Drosophila*.

Chapter 2: Materials and methods

2.1 Key Resources

Reagent or resource	Source	Identifier/Notes
Fly stocks		
w^{1118} (wild type)	Ferreira *et al.*, 2014	Drosdel background
Skanda^{1}	This study	N/A
Skanda$^{\Delta 107}$	This study	N/A
Hayan^{sk6}	This study	N/A
psh^{sk1}	Dudzic *et al*., 2019	N/A
Hayan-psh^{Def}	Dudzic *et al.*, 2019	N/A
Psh-Skanda^{Def}	This study	N/A
Hayan^{3414}*, Psh-Skanda*^{Def}	This study	N/A
spätzle^{rm7} (*spz*^{rm7})	Lemaitre *et al.*, 1996	N/A
PPO1$^{\Delta}$*,PPO2*$^{\Delta}$	Binggeli *et al.*, 2014	N/A
Bom$^{\Delta 55C}$	Clemmons *et al.*, 2015	N/A
Relish^{E20}	Hedengren *et al.*, 1999	N/A
Bacterial and fungal strains		
Staphylococcus aureus	Neyen *et al.*, 2014	N/A
Micrococcus luteus	Neyen *et al.*, 2014	N/A
Enterococcus faecalis	ATCC 29212	29212
Pectobacterium carotovora carotovora strain 15 (Ecc15)	Basset *et al.*, 2000	N/A
Commercial Assays		
Quantitative PCR reagent kit	Applied Biosystems SYBR Select Master Mix	4472908

Reverse Transcription reagent kit	PrimeScript™ RT Reagent Kit (Perfect Real Time)	RR037B
Oligonucleotides (qPCR)		
Rp49 F - 5’ TCTGCATGAGCAGGACCTC	This study	N/A
Rp49 R - 5’ ATCGGTTACGGATCGAACAA	This study	N/A
Drosomycin F - 5’ CTCTTCGCTGTCCTGATGCT	This study	N/A
Drosomycin R - 5’ ACAGGTCTCGTTGTCCCAGA	This study	N/A
Hayan F - 5’ CCAATCCCAATCCCTCCCG	This study	N/A
Hayan R - 5’ GATCGGATCTTCTCACACGCT	This study	N/A
Psh F - 5’ CCATTGAAGTGGTCCCTGCA	This study	N/A
Psh R - 5’ GGGCATGGTATCCGTCACC	This study	N/A
Skanda F - 5’ GGAGGTGGGTGATCCCTGT	This study	N/A
Skanda R - 5’ GACGCCAGAGTCTCACAATCC	This study	N/A
Softwares		
GraphPad Prism	GraphPad Prism version 10.2.1 for Windows, GraphPad Software, Boston, Massachusetts USA	http://www.graphpad.com
Jalview	Waterhouse *et al.*, 2009	https://www.jalview.org/
Chimera	Pettersen *et al.*, 2004	https://www.cgl.ucsf.edu/chimera/

Table 1. A description of the key resources used in this study

2.2 Insect stocks

Fly stocks were raised at 25°C on Yeast-Cornmeal food (6% cornmeal, 6% yeast, 0.62% agar, 0.1% fruit juice, supplemented with 10.6g/L Moldex and 4.9ml/L propionic acid). Experiments were performed on 5 to 10 day-old male flies. w^{1118} Drosdel flies were used as wild type unless stated otherwise. The psh^{sk1}, $Hayan\text{-}psh^{Def}$, $spätzle^{rm7}$, $PPO1^{\Delta},PPO2^{\Delta}$, $Bom^{\Delta 55C}$ and $Relish^{E20}$ lines are described previously (Lemaitre *et al.*, 1996; Hedengren *et al.*, 1999; Binggeli *et al.*, 2014; Clemmons *et al.*, 2015; Dudzic *et al.*, 2019). The $Hayan^{sk6}$ and $Psh\text{-}Skanda^{Def}$ mutant lines were generated using CRISPR/Cas9 as described in Kondo and Ueda, 2013. The $Hayan^{sk6}$ line has a 14 bp deletion from position 18482430 to 18482443 on the X chromosome. The $Psh\text{-}Skanda^{Def}$ line has a 4623 bp deletion from position 18484829 to 18489451 on the X chromosome. The $Skanda^{\Delta 107}$ and $Hayan^{3414}$, $Psh\text{-}Skanda^{Def}$ mutant lines were generated using CRISPR/Cas9 as described in Rommelaere *et al.*, 2019. The $Skanda^{\Delta 107}$ line contains an 8 bp deletion from position 18488965 to 18488972 on the X chromosome. The $Hayan^{3414}$, $Psh\text{-}Skanda^{Def}$ line was created using the $Psh\text{-}Skanda^{Def}$ line, and contains an additional 5bp deletion from position 18482347 to 18482351 on the X chromosome. The $Skanda^{1}$ line was generated by homologous recombination as described in Binggeli *et al.*, 2014. A copy of the mini-white gene was inserted at position 18488841 on the X chromosome, disrupting gene expression. All the mutant strains were isogenised in the w^{1118} Drosdel background.

2.3 Microorganism infection experiments

The bacterial strains and the optical densities of their pellets at 600 nm (OD_{600}) were: *Enterococcus faecalis* (*E. faecalis*, OD_{600} 5), *Micrococcus luteus* (*M. luteus*, OD 200), *Pectobacterium carotovora carotovora strain 15* (Ecc15, OD_{600} 200) and *Staphylococcus aureus* (*S. aureus*, OD 0.5). All strains were cultured in Luria Broth (LB) overnight. *E. faecalis*, *M. luteus* and *S. aureus* were cultured at 37°C, while Ecc15 was cultured at 29°C. Pellets were diluted in 1X PBS.

Systemic infection (septic injury) was performed by pricking 5 to 10-day-old adults in the thorax with a thin needle of diameter 0.2mm previously dipped into a concentrated bacterial pellet.

2.3.1 Survival

Infected flies were maintained at the conditions mentioned in Table 2 for survival experiments. Flies pricked with an ethanol-streilised needle (clean injury) were used as a control to distinguish between survival to bacterial infection and survival to wounding. 20 flies per genotype were used in each experiment and survival was scored once daily. 3 independent survival experiments were conducted per microorganism.

Bacterium/Challenge	Condition
E. faecalis	10 days at 25°C

Ecc15	10 days at 29°C
S. aureus	7 days at 25°C
Clean injury	10 days at 29°C

Table 2. Conditions for the maintenance of flies post infection with pathogens

2.3.2 Quantitative RT-PCR

Infected flies were maintained at 25°C and collected at indicated time points post-infection. 2 independent experiments were conducted per microorganism, with 2 replicates of 5 flies per genotype each time.

2.3.2 Bacterial load of flies

Infected flies were maintained at 25°C and collected 24 hours post-infection. 2 independent experiments were conducted, with 2 replicates of 5 flies per genotype each time.

2.4 Melanisation assessment by clean injury

Clean injury (CI) refers to an injury performed with an ethanol-sterilised needle. The thorax of 5 to 10-day-old adults were pricked using a sterile needle. Pictures of melanised flies were taken 16 hours post-injury, using a Leica M 205 FA microscope, a Leica DFC7000FT camera and the Leica Application Suite. 4 independent experiments were conducted using 20 flies per genotype each time.

2.5 Quantitative RT-PCR

Infected flies were collected at indicated time points post-infection along with unchallenged controls. Total RNA was isolated using TRIzol reagent and dissolved in DEPC-treated RNase-free water. 500 ng of total RNA was reverse-transcribed in 10 µL reactions using PrimeScript RT (TAKARA) with random hexamer and oligo dT primers to selectively amplify mRNA. Quantitative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using the Applied Biosystems SYBR Select Master Mix.

2.6 Bacterial load of flies

Flies were infected with *S. aureus* as described above. 24 hours post-infection, flies were surface-sterilized by washing them in 70% ethanol. Groups of five flies were homogenized by adding glass beads and the use of a PRECELLYSTM homogenizer in 0.5 mL LB. The homogenate was serially diluted and plated on LB agar. After incubation at 37°C overnight, colonies were counted and calculated to single fly CFUs; no colonies were recovered from uninfected flies using this approach.

2.7 Statistical analysis

Statistical analysis was only conducted for experiments that were performed 3 or more times. The statistical significance of survival data was calculated using a log-rank test compared to wild-type flies. The blackening strength (dark, medium, or light) of the adult cuticle was analysed using Chi-square test. P values of < 0.05 = *, < 0.01 = **, < 0.001 = ***, and < 0.0001 = **** were considered significant.

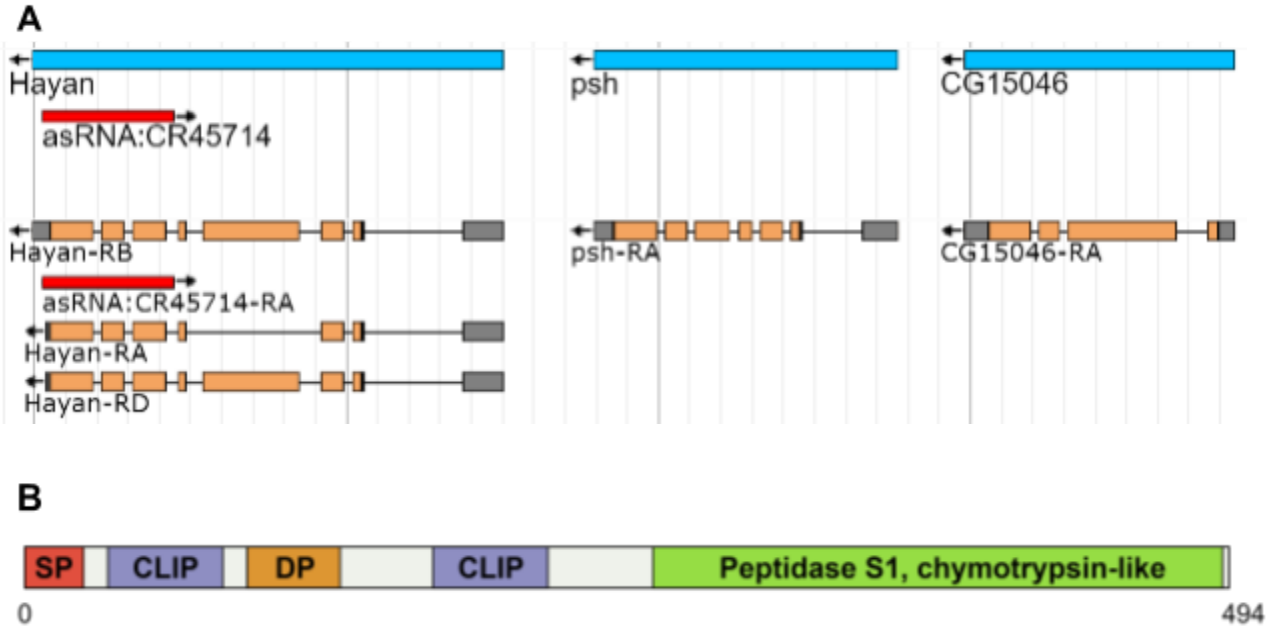
Graphs were plotted using GraphPad.

Chapter 3: Results

3.1 Skanda is a CLIP SPH clustered with Hayan and Psh

CG15046, hereafter named *Skanda*, encodes a CLIP-SPH and is located on the X chromosome in a cluster with two other genes encoding CLIP-SPs, *Hayan* and *persephone* (*Figure 4A*). Skanda is predicted to have a signal peptide, 2 CLIP domains and an S1A chymotrypsin-like peptidase domain (*Figure 4B*). To check whether Skanda is truly an SPH, we conducted a multiple sequence alignment using MUSCLE implemented on Jalview v2.11.3.2 (Edgar, 2004; Waterhouse *et al.*, 2009). As per Veillard *et al.*, 2016, we aligned the peptidase domains of Skanda with other known SPs and SPHs (*Figure 4C*). None of the residues in the catalytic triad seem to be conserved.

To verify the results of the alignment, we generated a 3D model of Skanda using Alphafold2 and compared it to the already-known structure of Grass using the matchmaker tool on Chimera v1.17.3 (*Figure 4D*) (Pettersen *et al.*, 2004; Kellenberger *et al.*, 2011; Jumper *et al.*, 2021). The peptidase domains of the two proteins overlapped to a great extent, confirming that Skanda is a member of the S1A peptidase family. Closely inspecting the catalytic triad next showed that while the aspartate and serine residues are conserved, the histidine is replaced by a glycine. This should abolish the peptidase activity of the protein. Aligning the two CLIP domains of Skanda with that of Grass revealed a great degree of overlap between the positions of the beta sheets. The helix-loop-helix motif connecting the beta sheets was smaller in Skanda compared to Grass (*Figure S1*). Therefore, Skanda is indeed a CLIP SPH clustered with Hayan and Psh.



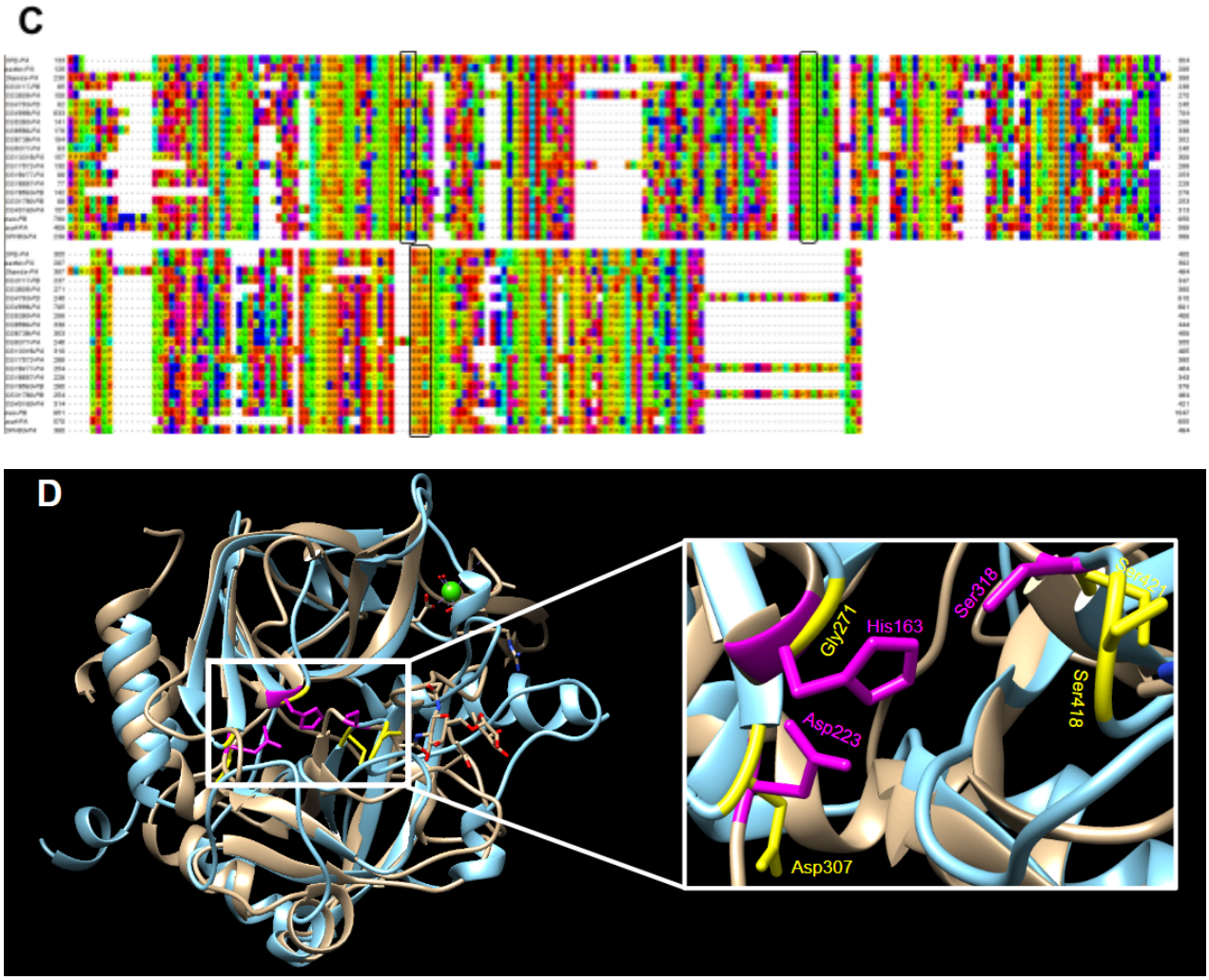


Figure 4. ***Structure of Skanda****: (A) The* Hayan-psh-Skanda *gene cluster is located on the X chromosome.* psh *is located 751 bp away from* Hayan*, and* Skanda *is located 529 bp away from* psh*. (from flybase.org) (B) Skanda is predicted to have a signal peptide (SP - red), 2 CLIP domains (CLIP - blue), a predicted disordered region (DP - orange) and an S1A chymotrypsin-like peptidase domain (Peptidase S1, chymotrypsin-like - green). Illustrated using IBS 2.0 (Xie et al., 2022). (C) MSA of peptidase domains of Skanda and other SPHs. Peptidase domains of two SPs, easter and SPE, have been used as a reference to identify key motifs. The black box around the MSA signifies the catalytic His, Asp and Ser residues. These residues do not seem to be conserved in Skanda (D) Overlap of the peptidase domains of Grass (brown) and Skanda (blue) visualised using Chimera v1.17.3. Inset: The catalytic triads of Grass (pink) and Skanda (yellow).*

3.2 *Skanda* expression is co-regulated by Toll and Imd pathways

Skanda is an early immune-induced gene, with expression peaking at 1.5 hours post-septic injury. Expression returns to baseline 6 hours post-pricking. Microarray data suggests that its expression is co-regulated by the Toll and Imd pathways, as *Skanda* expression is reduced three-fold in flies lacking *spätzle* and *Relish* (*Figure 5*). It is expressed throughout fly development, peaking in larval and pupal stages (*FIgure S2A*). It is highly expressed in the fat body, an important immune responsive organ in *Drosophila* (*Figure S2B*).

To study the role of Skanda in *Drosophila* innate immunity, the lab generated two mutant strains. *Skanda*^{1} was created by inserting a w+ cassette into the CG15046 gene locus in order to disrupt its expression. *Skanda*$^{\Delta 107}$, an 8 bp deletion mutant, was created using CRISPR/Cas9. The mutants were then isogenized for seven generations into the w^{1118} DrosDel isogenic genetic background. Both the strains are homozygous viable, fertile and show no obvious external deformities. To rule out any cis effects caused by the mutations, we checked to see whether *Hayan* and *psh* expression is affected in the mutant flies. Both the lines have wild-type expression of *Hayan*. *Skanda*^{1}, however, has a slight overexpression of *psh* (*Figure S3A*). The flies also have melanotic tumours, characteristic of over-active Toll signalling (*Figure S3B*).

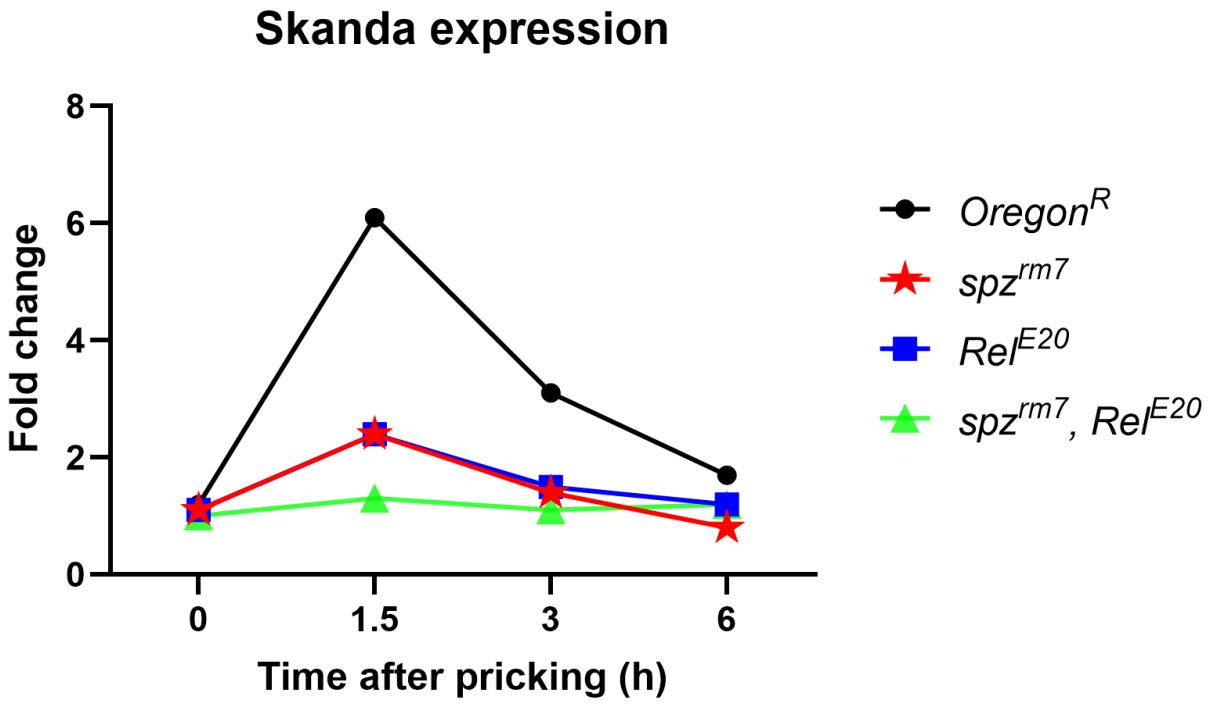
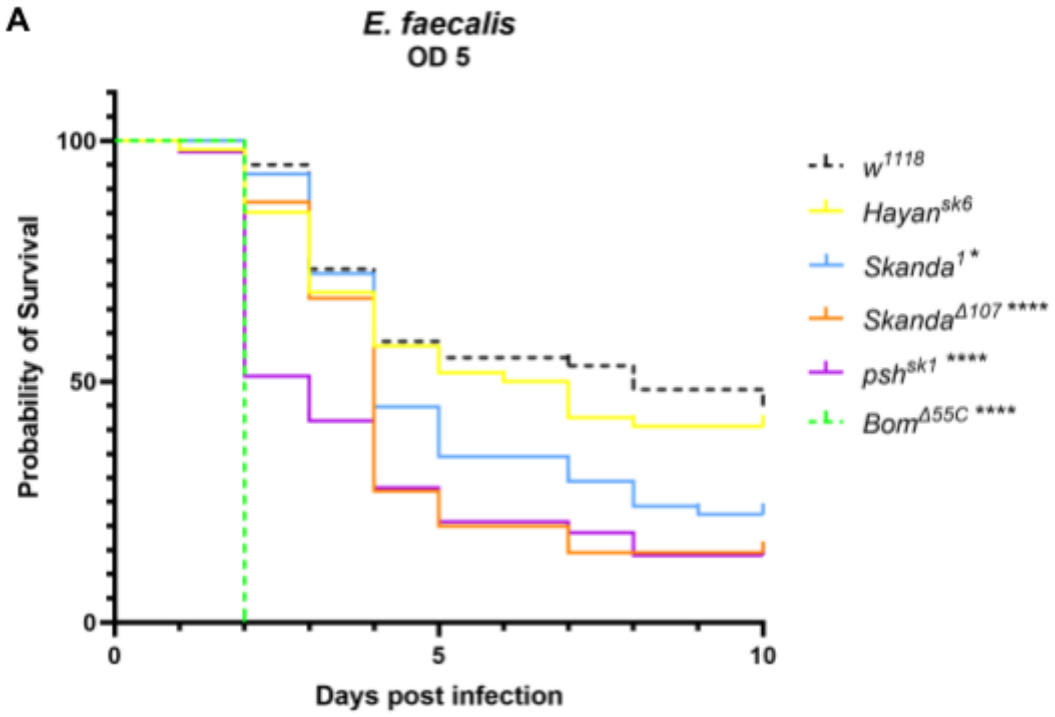


Figure 5. ***Regulation of* Skanda *expression by the Toll and Imd pathways****:* Skanda *is an early immune-induced gene, with expression peaking 1.5 hours post-pricking with pathogens.* Skanda *regulation is regulated by the Toll and Imd pathways, as* Skanda *upregulation upon pricking is lost in* spz^{rm7} *and* Relish^{E20} *flies.* Oregon^{R} *is the wild-type control used here. Data from (De Gregorio et al., 2002b)*

3.3 Skanda is required for resistance to Gram-positive bacteria

As *Skanda* expression is regulated by the Toll pathway, we tested whether it played a role in Toll signalling. Adult flies were pricked with *Enterococcus faecalis*, a Gram-positive bacterium, at OD_{600} 5. $Bom^{\Delta 55C}$ flies, lacking all ten Bomanin AMPs, were used as a positive control, as they are extremely susceptible to infection by Gram-positive bacteria and fungi (Clemmons ***et al.***, 2015). We also tested the response of *Hayan* ($Hayan^{sk6}$) and *psh* (psh^{sk1}) mutant flies. $Hayan^{sk6}$ mutants show wild-type susceptibility to the bacterium, while $Skanda^{1}$, $Skanda^{\Delta 107}$ and psh^{sk1} flies show increased susceptibility (*Figure 6A*). We then checked Toll activation in these flies by pricking with the avirulent Gram-positive bacterium *Micrococcus luteus* at OD_{600} 200. We monitored the expression of *Drosomycin* (*Drs*), an AMP whose expression is controlled by the Toll pathway. Spätzle (spz^{rm7}) mutant flies that lack a function Toll response were used as a positive control. $Skanda^{\Delta 107}$ and $Hayan^{sk6}$ flies show wild-type *Drs* expression, while $Skanda^{1}$ and psh^{sk1} flies show reduced *Drs* expression (*Figure 6B*).



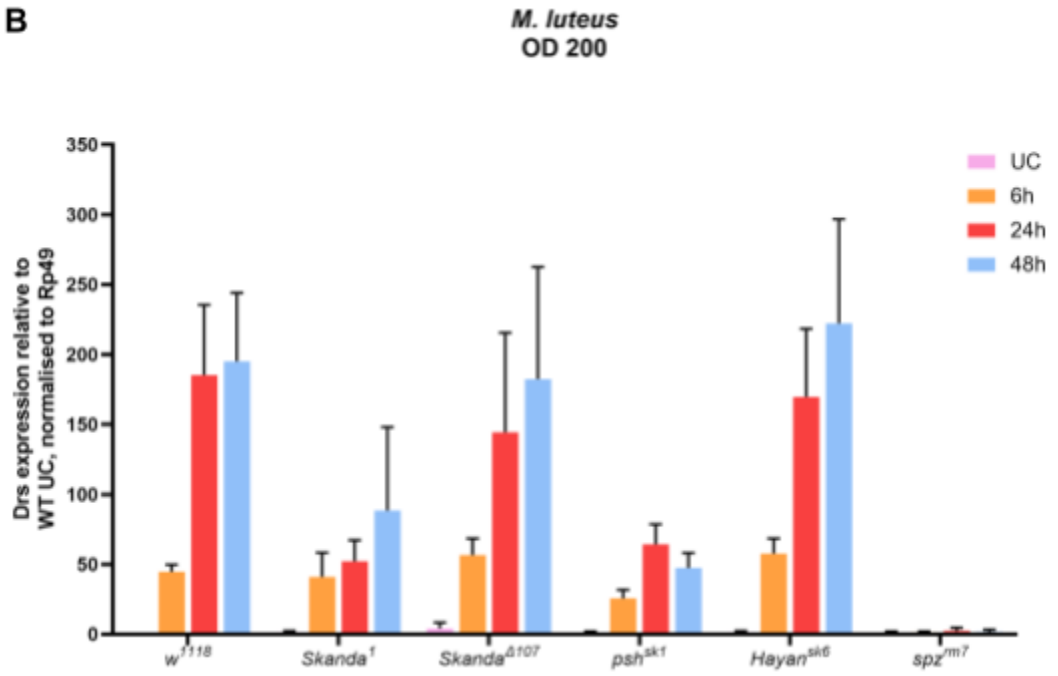


Figure 6. ***Response of* Hayan-psh-Skanda *single mutants to* E. faecalis *and* M. luteus***: (A) Survival of Hayan, psh and Skanda single mutants when pricked with* E. faecalis *OD 5. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (B)* Drs *expression of Hayan, psh and Skanda single mutants when pricked with* M. luteus *OD 200. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time.*

3.4 Skanda does not affect the Imd pathway

As *Skanda* expression is also regulated by the Imd pathway, we tested whether it played a role in Imd signalling. Adult flies were challenged by pricking with *Pectobacterium carotovora carotovora strain 15* (Ecc15), a Gram-negative bacterium, at OD_{600} 200. *Relish* (*Relish*^{E20}) mutant flies were used as a positive control as they lack a functional Imd response. Both *Skanda*^{1} and *Skanda*$^{\Delta 107}$ mutants show wild-type survival to Ecc15. *Hayan*^{sk6} and *psh*^{sk1} flies seem to rapidly die at day 7 post infection (*Figure 7A*). This is suspected to be due to their susceptibility to clean injury at 29°C (*Figure S4A*).

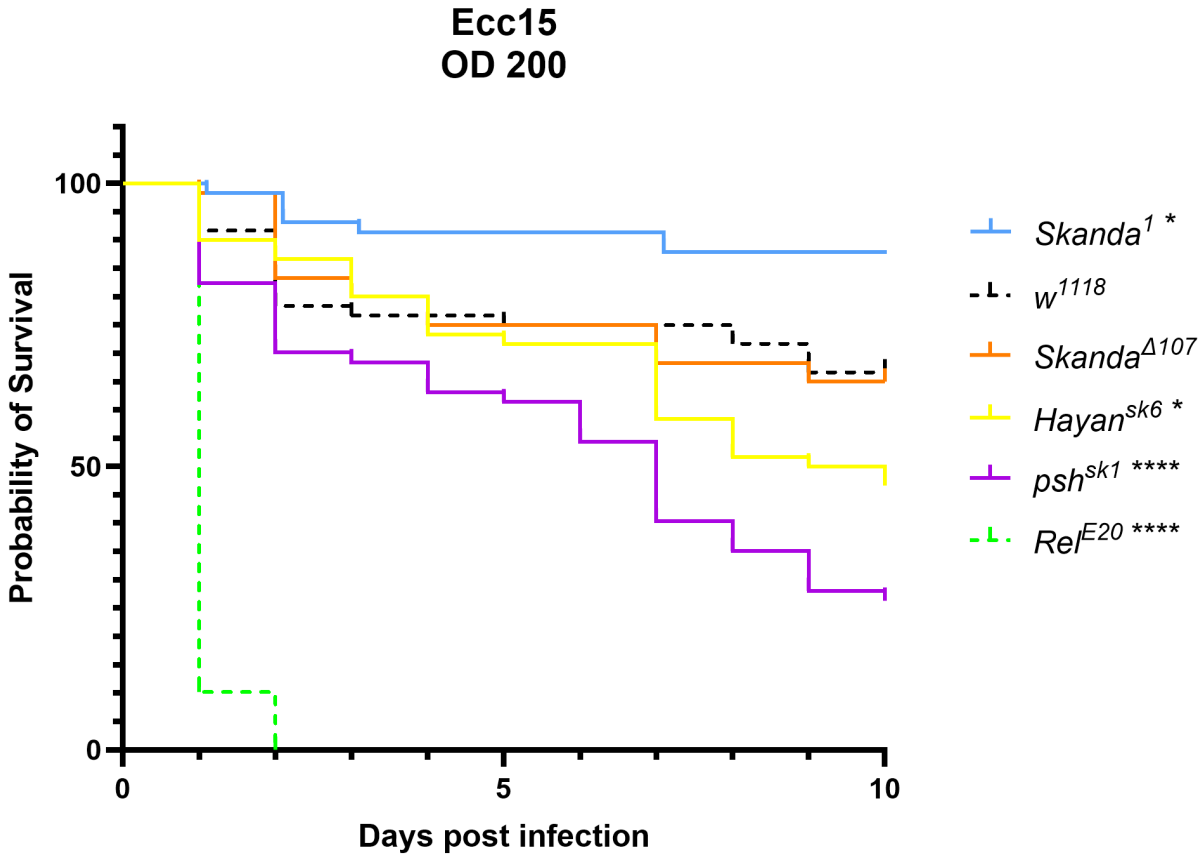


Figure 7. ***Response of* Hayan-psh-Skanda *single mutants to Ecc15****: (A) Survival of Hayan, psh and Skanda single mutants when pricked with Ecc15 OD 200. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

3.5 Skanda is not a critical component for melanization

Next, we tested whether Skanda is required for melanisation. Adult flies were pricked with a sterile needle of diameter 0.2mm. The injury triggers a blackening reaction at the wound site. Based on the size and colour of the black spot, flies were classified as having dark, medium, light or no melanisation (*Figure S5*). Wild-type flies have a roughly equal split between flies that showed dark, medium and light melanisation. The ratio shifts for *Skanda*$^{\Delta 107}$ and *psh*^{sk1} mutants, who have a slightly weaker melanisation response overall. *Skanda*^{1} flies have a much weaker melanisation response, with more than 75% of flies showing only light melanisation. As expected, *Hayan*^{sk6} flies show no melanisation (*Figure 8*).

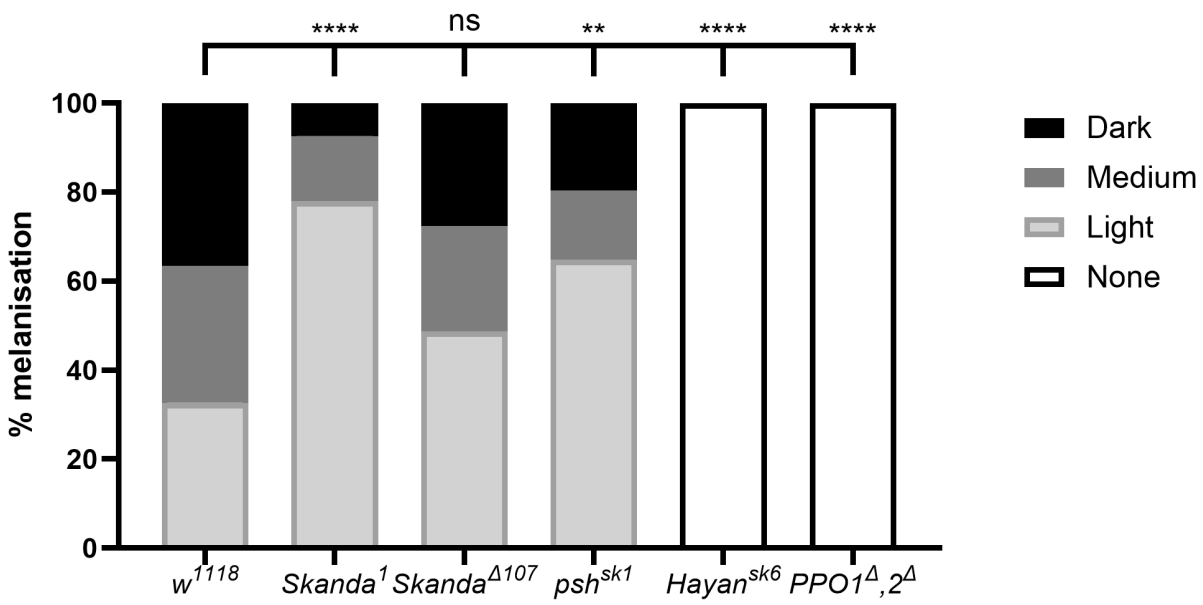
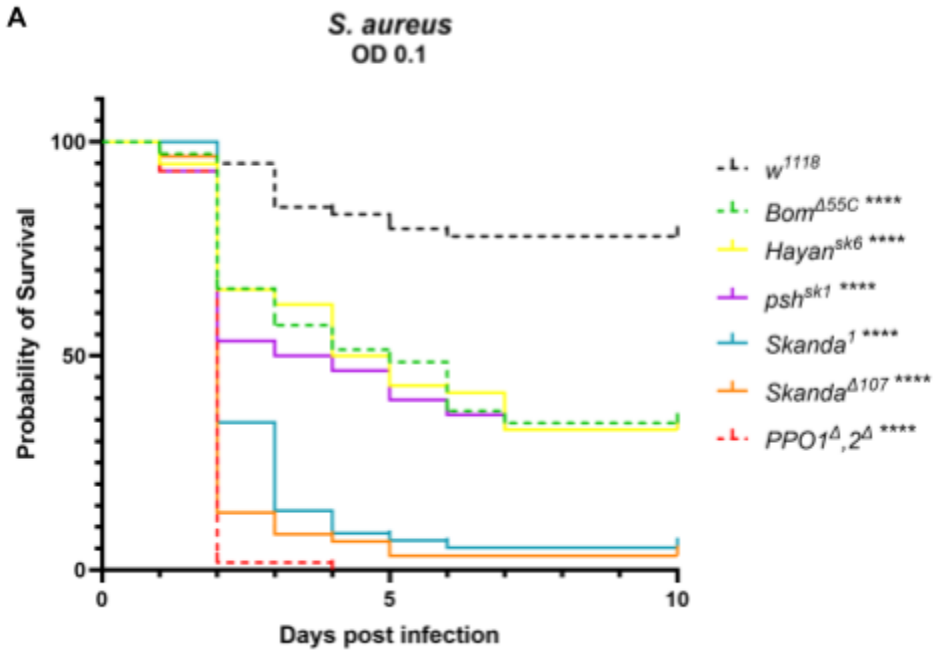
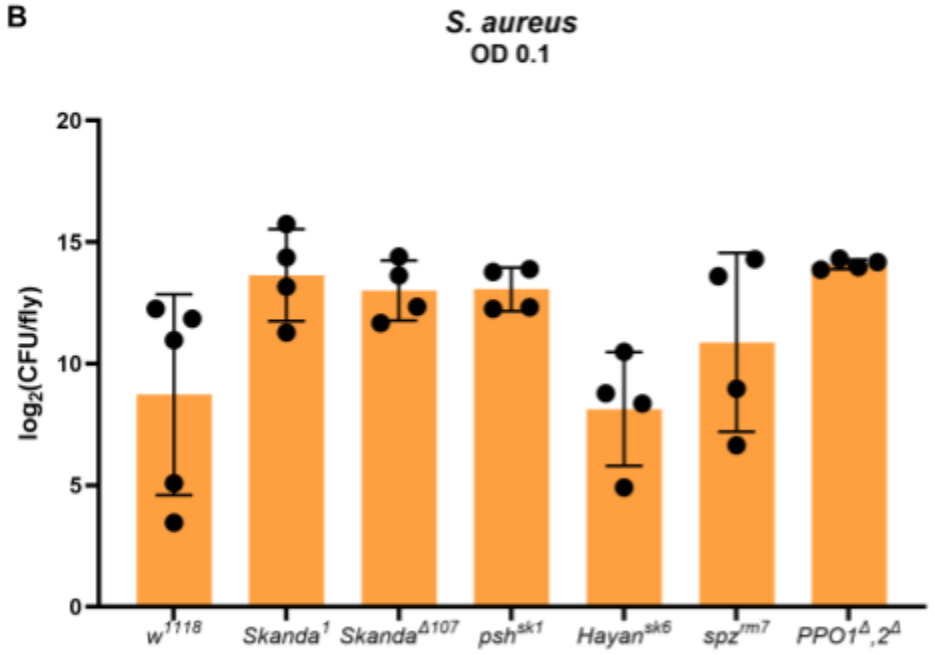


Figure 8. ***Melanisation response of*** **Hayan-psh-Skanda** ***single mutants****: Melanisation of Hayan, psh and Skanda single mutants 16 hours post clean injury with a sterile needle. Dark, medium and light refers to the size and colour of the scab. The experiment was repeated 4 times, with 20 flies per genotype each time. ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

3.6 Skanda mutants are highly susceptible to *S. aureus* infection

Staphylococcus aureus is a Gram-positive bacterium with lysine-type peptidoglycan. Its infection is combatted by a combination of melanisation and Toll signalling (Dudzic *et al.*, 2019). Hemocytes are also known to be critical to survive *S. aureus* infection (Defaye *et al.*, 2009). A previous study noted the overexpression of Skanda in hemocytes upon *S. aureus* infection (Nazario-Toole, 2016). Using this as a starting point, we tested whether Skanda mediates immune response to *S. aureus*. Adult flies were pricked with *S. aureus* at OD_{600} 0.1. $PPO1^{\Delta},2^{\Delta}$ flies, completely lacking melanisation, were used as a positive control as they are known to rapidly succumb to *S. aureus* infection. $Bom^{\Delta 55C}$ flies were used as a control for the Toll response. They are as often susceptible as spz^{rm7} flies when infected with Gram-positive bacterium. Both *Skanda* mutants are extremely susceptible to *S. aureus* infection, similar to $PPO1^{\Delta},2^{\Delta}$ flies. This was a surprising result, as these flies have mostly normal Toll and melanisation responses. $Hayan^{sk6}$ and psh^{sk1} flies are as susceptible as $Bom^{\Delta 55C}$ flies (*Figure 9A*). Comparing *S. aureus* bacterial load 24h post-infection revealed that the *Skanda* mutants, psh^{sk1} flies and $PPO1^{\Delta},2^{\Delta}$ flies have a much higher bacterial burden when compared to wild type and $Hayan^{sk6}$ flies, indicating their inability to control bacterial growth (*Figure 9B*). When we checked *Drs* expression upon *S. aureus* infection at various time points, we observed that both *Skanda* mutants show a wild-type Toll response. *psh* mutants have a weaker Toll response despite having a higher bacterial load, probably because the Toll response itself is stunted in these flies. *Hayan* mutants have a higher Toll response despite having wild-type bacterial load (*Figure 9C*).


A
S. aureus
OD 0.1
Probability of Survival
100
50
0
0
5
10
Days post infection
w1118
Bom55C ****
Hayansk6 ****
pshsk1 ****
Skanda1 ****
SkandaΔ107 ****
PPO1Δ,2Δ ****


B
S. aureus
OD 0.1
log2(CFU/fly)
20
15
10
5
0
w1118
Skanda1
SkandaΔ107
pshsk1
Hayansk6
spzrm7
PPO1Δ,2Δ

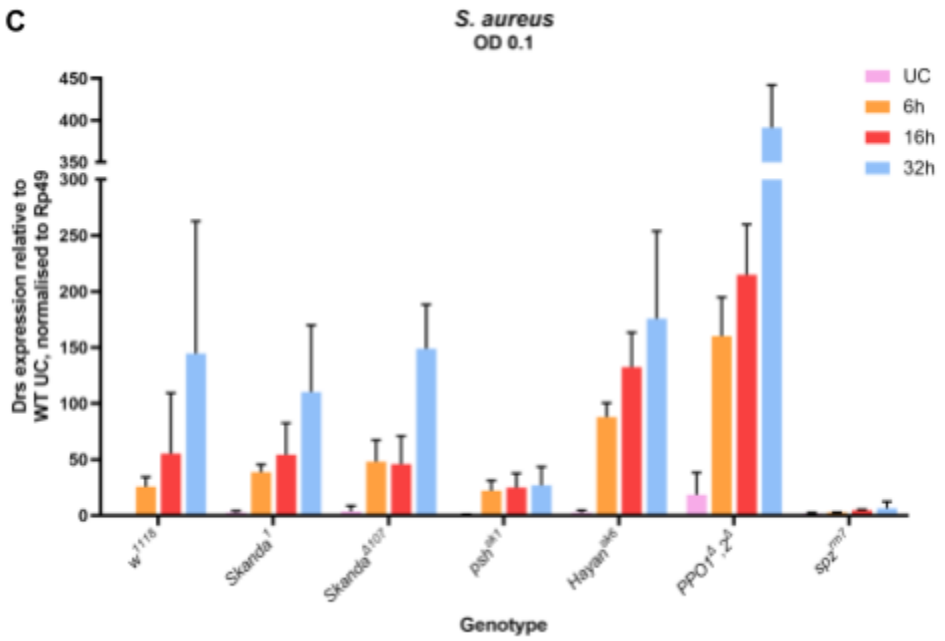


Figure 9. ***Response of* Hayan-psh-Skanda *mutants to* S. aureus***: (A) Survival of Hayan, psh and Skanda single mutants when pricked with* S. aureus *OD 0.1. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (B) Bacterial load of Hayan, psh and Skanda single mutants 24 hours post-pricking with* S. aureus *OD 0.1. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time. (C)* Drs *expression of Hayan, psh and Skanda single mutants when pricked with* S. aureus *OD 0.1. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time.*

3.7 *Hayan-psh-Skanda* compound mutants lack functional Toll response

A previous study revealed that sister CLIP-SPs Hayan and Psh regulate the Toll pathway redundantly (Dudzic *et al.*, 2019). Flies lacking both Hayan and Psh show a much lower level of Toll activation upon pricking with *M. luteus* in comparison to flies lacking only one of the SPs. Their *Drs* activation profile and survival to Gram-positive bacteria is similar to spz^{rm7} mutants. We were interested in generating more compound mutants of the *Hayan-psh-Skanda* cluster to check if other such redundancies exist. We made a small deletion to generate a $Psh\text{-}Skanda^{Def}$ mutant and used those flies to create a $Hayan^{3414}$, $Psh\text{-}Skanda^{Def}$ mutant by making an additional deletion in the *Hayan* gene. To our surprise, $Psh\text{-}Skanda^{Def}$ mutants also showed a much lower level of Toll activation compared to *psh* and *Skanda* single mutants (*Figure 10B*). These flies were extremely susceptible to *E. faecalis* infection (*Figure 10A*). $Hayan^{3414}$, $Psh\text{-}Skanda^{Def}$ flies also lacked a functional Toll response like the other compound mutants, and were also susceptible to infection by *E. faecalis* (*Figure 10A-B*).

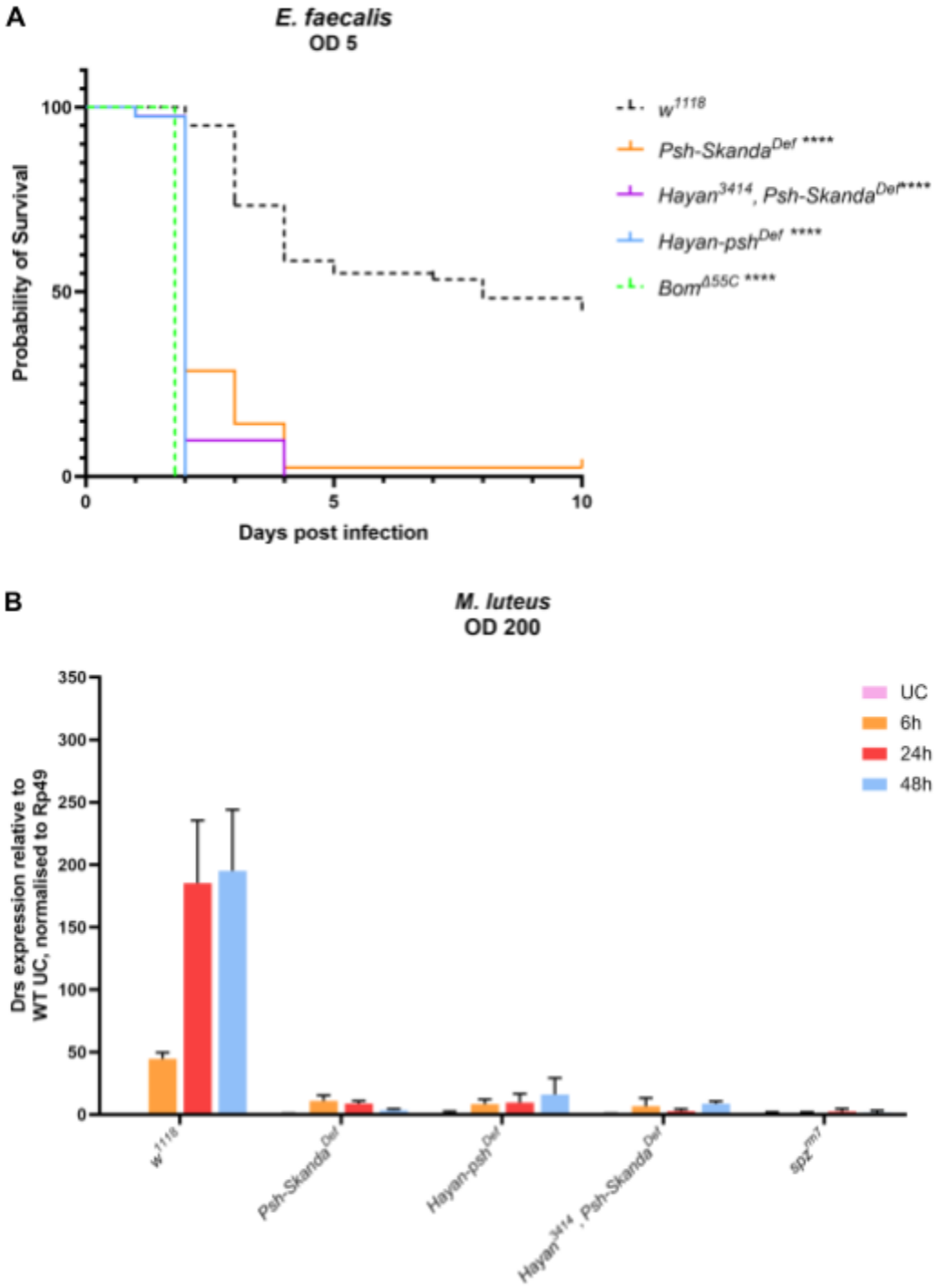


Figure 10. ***Response of* Hayan-psh-Skanda *compound mutants to* E. faecalis *and* M. luteus**: *(A) Survival of Hayan, Psh Skanda compound mutants when pricked with* E. faecalis *OD 5. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (B)* Drs *expression of Hayan, Psh Skanda compound mutants when pricked with* M. luteus *OD 200. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time.*

3.8 *Hayan-psh-Skanda* cluster does not regulate the Imd pathway

Adult flies were pricked with Ecc15 at OD_{600} 200. *Relish* (*Relish*^{E20}) mutant flies were used as a positive control as they lack a functional Imd response. *Psh-Skanda*^{Def} flies show wild type susceptibility to the bacterium. *Hayan-psh*^{Def} and *Hayan*^{3414}*, Psh-Skanda*^{Def} flies seem slightly more susceptible than wild type fles, with mortality particularly increasing between days five and seven (*Figure 11A*). This is suspected to be due to their susceptibility to clean injury at 29°C (*Figure S4B*).

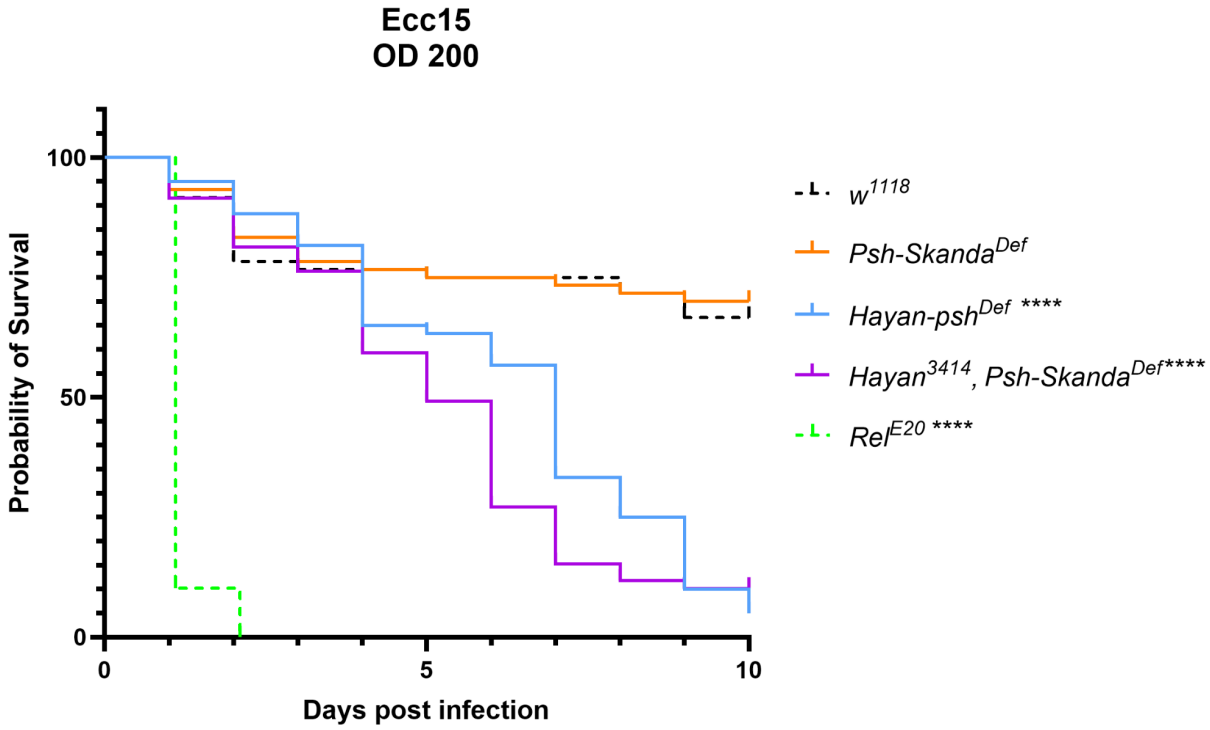


Figure 11. ***Response of* Hayan-psh-Skanda *compound mutants to Ecc15****: Survival of Hayan, Psh Skanda compound mutants when pricked with Ecc15 OD 200. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

3.9 Hayan is the major regulator of melanisation upon clean injury

Next, we tested the melanisation response of these compound mutants by pricking adult flies with a sterile needle of diameter 0.2mm. *Psh-Skanda*^{Def} flies have a slightly weaker overall response than wild type flies, similar to the *Skanda*$^{\Delta 107}$ and *psh*^{sk1} single mutants. *Hayan-psh*^{Def} and *Hayan*^{3414}*, Psh-Skanda*^{Def} flies show almost no melanisation, showing that Hayan is the major component regulating melanisation upon clean injury (*Figure 12*).

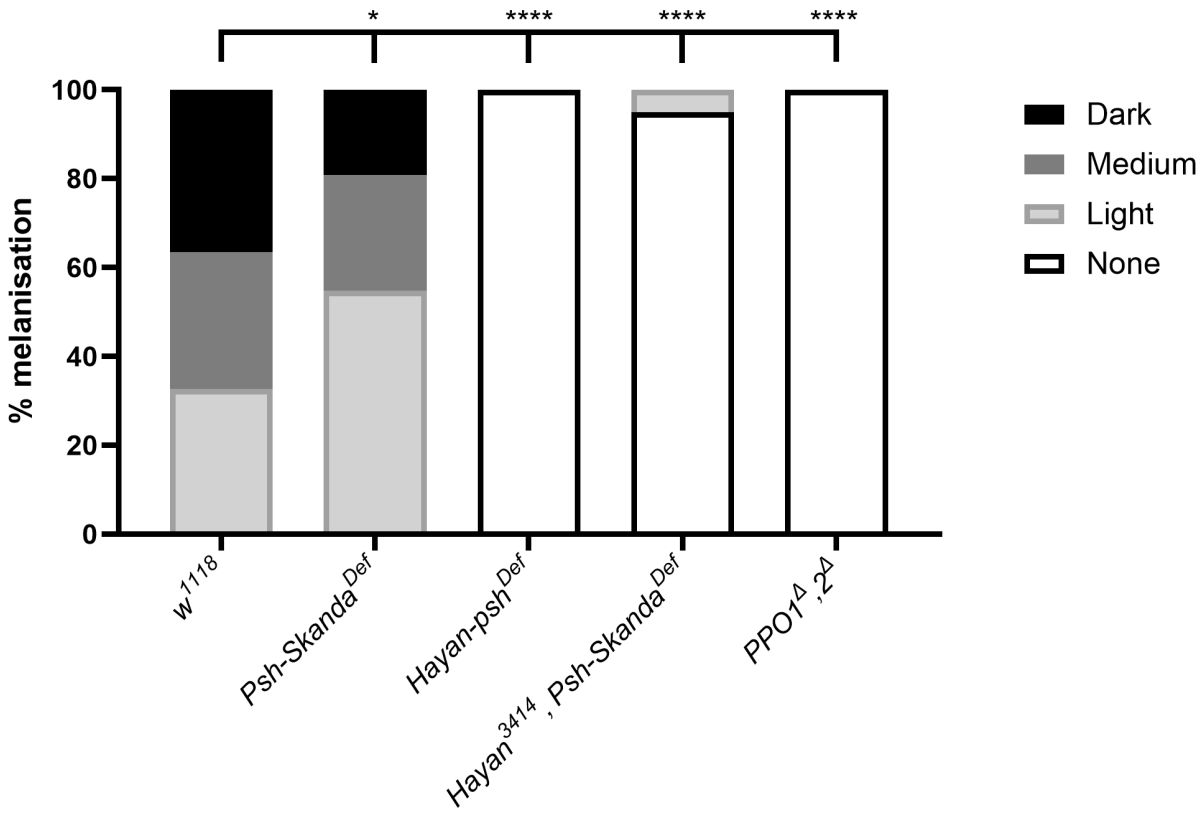
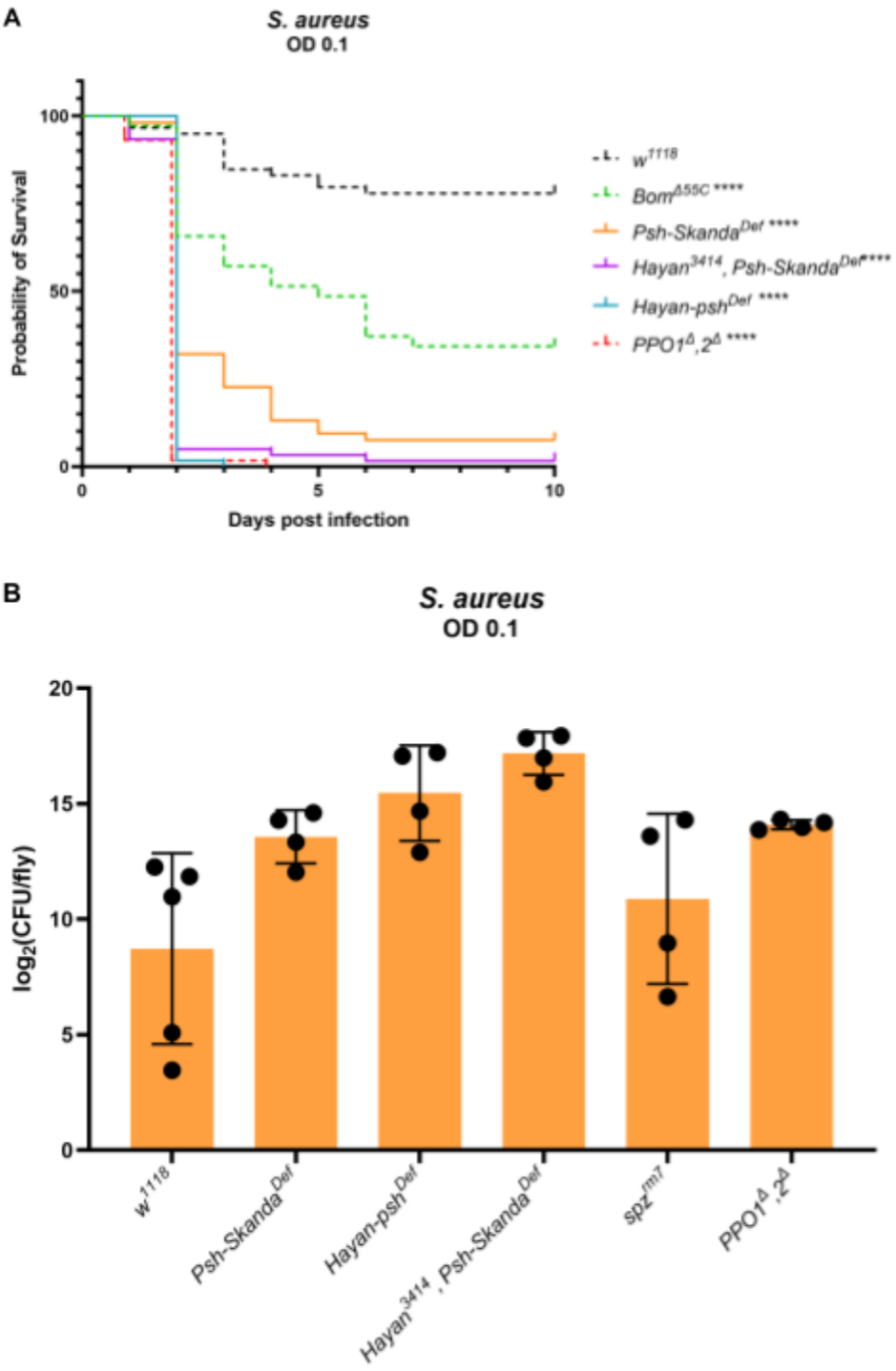


Figure 12. ***Melanisation response of*** **Hayan-psh-Skanda** ***compound mutants****: Melanisation of Hayan, Psh Skanda compound mutants 16 hours post clean injury with a sterile needle. Dark, medium and light refers to the size and colour of the scab. The experiment was repeated 4 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

3.10 *Hayan-psh-Skanda* compound mutants are highly susceptible to *S. aureus*

All compound mutants are highly susceptible to infection with *S. aureus* at OD_{600} 0.1 (*Figure 13A*). Comparing *S. aureus* bacterial load 24h post-infection showed that all compound mutants have a significantly higher burden when compared to wild-type flies, similar to $PPO1^{\Delta},2^{\Delta}$ flies. $Hayan^{3414}$, $Psh\text{-}Skanda^{Def}$ flies seem to have a higher bacterial load than both double mutants and $PPO1^{\Delta},2^{\Delta}$ mutants (*Figure 13B*). The *Drs* expression profile of these mutants revealed something interesting. When pricked with *M. luteus*, $Psh\text{-}Skanda^{Def}$ flies show almost no expression of the AMP. However, when pricked with *S. aureus*, they show a much higher expression of *Drs*, similar to the expression levels of psh^{sk1} flies. Additionally, all compound mutants showed a strange peak in expression at 32h post infection, which was not observed in spz^{rm7} mutants (*Figure 13C*).


A
S. aureus
OD 0.1
Probability of Survival
100
50
0
0
5
10
Days post infection
w1118
BomΔ55C ****
Psh-SkandaDef ****
Hayan3414, Psh-SkandaDef****
Hayan-pshDef ****
PPO1Δ,2Δ ****
B
S. aureus
OD 0.1
log2(CFU/fly)
20
15
10
5
0
w1118
Psh-SkandaDef
Hayan-pshDef
Hayan3414, Psh-SkandaDef
spzrm7
PPO1Δ,2Δ

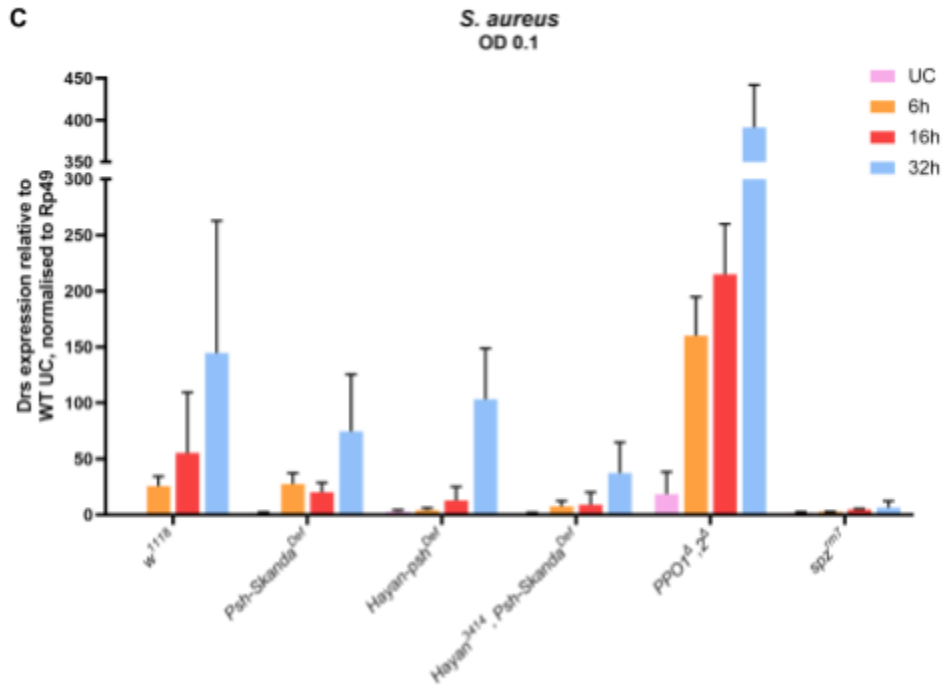


Figure 13. ***Response of* Hayan-psh-Skanda *compound mutants to* S. aureus***: (A) Survival of Hayan, Psh Skanda compound mutants when pricked with* S. aureus *OD 0.1. The experiment was repeated 3 times, with 20 flies per genotype each time. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (B) Bacterial load of Hayan, Psh Skanda compound mutants 24 hours post-pricking with* S. aureus *OD 0.1. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time. (C)* Drs *expression of Hayan, Psh Skanda compound mutants when pricked with* S. aureus *OD 0.1. The experiment was repeated 2 times, with 2 replicates of 5 flies per genotype each time.*

Chapter 4: Discussion

4.1 Skanda is a CLIP SPH required for resistance to Gram-positive bacteria

Skanda encodes a CLIP SPH and is located next to *Hayan* and *psh* on the X chromosome. As Hayan and Psh have important functions in Drosophila immunity and regulate Toll signalling redundantly, we decided to investigate whether Skanda has a similar role to play as well. We obtained two mutants to characterise Skanda. $Skanda^{1}$ was created by a w+ insertion in the *Skanda* locus, and $Skanda^{\Delta 107}$ was created by making an 8 bp deletion at the *Skanda* locus using CRISPR/Cas9. While checking for cis effects of the mutations, we discovered that $Skanda^{1}$ mutants overexpress *psh*. They also exhibit melanotic tumors, which are characteristic of over-active Toll signalling. We checked the *psh* locus in the $Skanda^{1}$ background and were unable to amplify the 5' UTR of *psh*. The repair process during homologous recombination might have accidentally caused a deletion in this region. We hypothesize that there might have been a regulatory element for *psh* here, and its deletion is what leads to the observed overexpression. $Skanda^{\Delta 107}$ mutants, on the other hand, have wild-type expression levels of *Hayan* and *psh*, indicating that the mutation did not affect these loci.

There is some discrepancy between the results obtained with the two *Skanda* mutants. While $Skanda^{\Delta 107}$ mutants have wild-type Toll signalling and nearly wild-type melanisation, $Skanda^{1}$ mutants have weaker Toll signalling and melanisation. We are unable to explain why $Skanda^{1}$ mutants have weaker Toll signalling. However, they might have a weaker melanisation response as the melanotic tumours might have exhausted all the PPO in the hemolymph. $Skanda^{1}$ mutants are only slightly more susceptible to *E. faecalis* infection than wild type flies, while $Skanda^{\Delta 107}$ mutants are significantly more susceptible. This is strange, given how $Skanda^{\Delta 107}$ mutants have normal Toll signalling and near-normal melanisation. It is worth noting, however, that we only inspected melanisation at the cuticle upon injury. This is different from melanisation around the bacteria, in the hemolymph. It is possible that $Skanda^{\Delta 107}$ mutants are unable to sequester pathogens by melanin deposition. It would also be interesting to check whether their hemocytes function normally, as phagocytosis play a role in immunity to *E. faecalis* (Defaye *et al.*, 2009). Given that the $Skanda^{\Delta 107}$ mutation has no cis effects, it might be better to consider results with this mutant as representative results.

Both of the *Skanda* mutants, however, respond similarly to *S. aureus* infection. They die rapidly when pricked with *S. aureus*. 24 hours post pricking, they show a much higher bacterial load compared to wild type flies, an indication that they are unable to control *S. aureus* growth. *Skanda* mutants show wild type Toll signalling upon *S. aureus* infection. Combined with the finding that *Skanda* expression is upregulated in Drosophila hemocytes upon *S. aureus* infection, it seems that this gene has a key role to play in the immune response to *S. aureus* (Nazario-Toole, 2016). It would be interesting to once again check if the hemocytes function

normally in the mutants, as they play an important role in immunity to *S. aureus* (Defaye *et al.*, 2009).

4.2 Psh and Skanda regulate the Toll pathway redundantly

A striking result from this study was that flies lacking both Psh and Skanda have no functional Toll response, similar to *Hayan-psh* double mutants. *Psh-Skanda^{Def}* flies are extremely susceptible to *E. faecalis* infection, and show almost no *Drs* expression upon pricking with *M. luteus*, similar to *spz^{rm7}* flies. It was easy to rationalize how Hayan and Psh might be redundantly regulating the Toll pathway, as they both are closely related CLIP SPs. Skanda, however, is catalytically inactive. Similar to how cSPH35 and cSPH242 act as co-factors during PPO1 activation, we hypothesize that Skanda might function as a co-factor to help Psh cleave SPE more efficiently (Jin *et al.*, 2023). This might explain why *Skanda$^{\Delta 107}$* mutants show intact Toll signalling, as SPE might still be cleaved, just less efficiently. There might also be compensatory signalling by Hayan. Confirming the Psh-Skanda interaction *in vivo* via co-immunoprecipitation would lend credibility to this hypothesis. *In vitro* experiments have shown that when Psh and SPE were incubated together, Psh was able to cleave some of the SPE (Shan *et al.*, 2023). It would be interesting to check whether incubating Skanda with activated Psh might allow it to cleave more of SPE.

We were unable to generate a *Hayan-Skanda* double mutant despite using multiple different strategies. This might be an indication that removing both genes together is embryonically lethal. However, this might not be the case as the *Hayan-psh-Skanda* triple mutant is perfectly viable. It might also be that removing *psh* along with *Hayan* and *Skanda* somehow rescues this lethality. Given that Hayan has a very similar structure to Psh, it is possible that Skanda could also act as a co-factor for Hayan, and that *Hayan-Skanda* double mutants phenocopy the other two double mutants. This co-factor hypothesis could be confirmed by checking if Hayan can pull down Skanda in a co-immunoprecipitation assay. It was seen that activated Hayan was unable to cleave SPE *in vitro* (Shan *et al.*, 2023). It would be worth checking whether the addition of Skanda would enable Hayan to cleave SPE. The *Hayan-psh-Skanda* triple mutant does not perform worse than the two double mutants in any of the challenges, which indicates that removing two of the three components might be sufficient to shut off Toll signalling.

Hayan and *Skanda* are evolutionarily conserved throughout the *Drosophila* genus (*Figure S6*), while *psh* evolved specifically in the Melanogaster group from an ancestral *Hayan* gene (Dudzic *et al.*, 2019). We hypothesize that an ancestral *Skanda* and ancestral *Hayan* gene might have regulated Toll signalling together, with Skanda acting as a co-factor for Hayan. *psh* was later created by a gene duplication event in an ancestor of the Melanogaster group flies, and was probably retained due to evolutionary pressure. This pressure may have been due to the different pathogens encountered by Melanogaster group flies in their ecological niche. It has already been shown that different ecological niches in *Drosophila* can lead to the duplication and rapid evolution of AMPs (Hanson *et al.*, 2023). It is possible that niches can also drive the evolution of SPs.

Psh then developed an extended protease bait region, allowing it to be cleaved by a variety of microbial proteases (Issa *et al.*, 2018; Dudzic *et al.*, 2019). We hypothesize than this might have marked the beginning of the divergence of the Toll and melanisation SP cascades. Skanda might now act as a co-factor for Psh, together regulating the Toll pathway. Hayan might have specialised to play a more critical role in the melanisation cascade, seeing how flies lacking Hayan show major melanisation defects but minor Toll signalling defects. Due to their similar structures, however, Hayan is still able to participate in Toll signalling, as evidenced by the lack of Toll induction in *Hayan-psh* double mutants, and Psh is able to participate in melanisation, as evidenced by the inability of *Hayan-psh* double mutant to melanise even upon septic injury (Dudzic *et al.*, 2019). We hypothesize that Hayan, Psh and Skanda together allow for the differential regulation and precise tuning of the Toll and melanisation cascades.

4.3 A mystery SP involved in Toll response to *S. aureus*

Looking at *Drs* expression in the *Hayan-psh-Skanda* compound mutants upon infection with *S. aureus* revealed something strange. When pricked with *M. luteus*, *Psh-Skanda*^{Def} flies show almost no expression of the AMP. However, when pricked with *S. aureus*, they show wild-type *Drs* expression. Additionally, all compound mutants showed a strange peak in expression at 32h post infection, which was not observed in *spz*^{rm7} mutants. This indicates the presence of another SP induced by *S. aureus* that is capable of activating the Toll pathway. It is possible that this SP might act as a sensor of effector triggered immunity like Psh, specifically for *S. aureus* proteases.

A good candidate for this SP is MP2, also known as Sp7. It has been shown that MP2 is involved in defence against *S. aureus*, as flies lacking MP2 are quite susceptible to *S. aureus* infection (Dudzic *et al.*, 2019). A recent biochemical analysis revealed that MP2 can cleave SPE *in vitro* (Shan *et al.*, 2023). Therefore, it is possible that *S. aureus* infection activates MP2, which cleaves SPE, eventually activating Toll signalling. Hence, even in the absence of Hayan, Psh and Skanda, flies express *Drs* when pricked with *S. aureus*. This could be tested by creating a triple mutant of Psh, Skanda and MP2 and checking whether Toll signalling is completely abolished upon pricking with *S. aureus*.

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Supplementary Figures

Figure S1

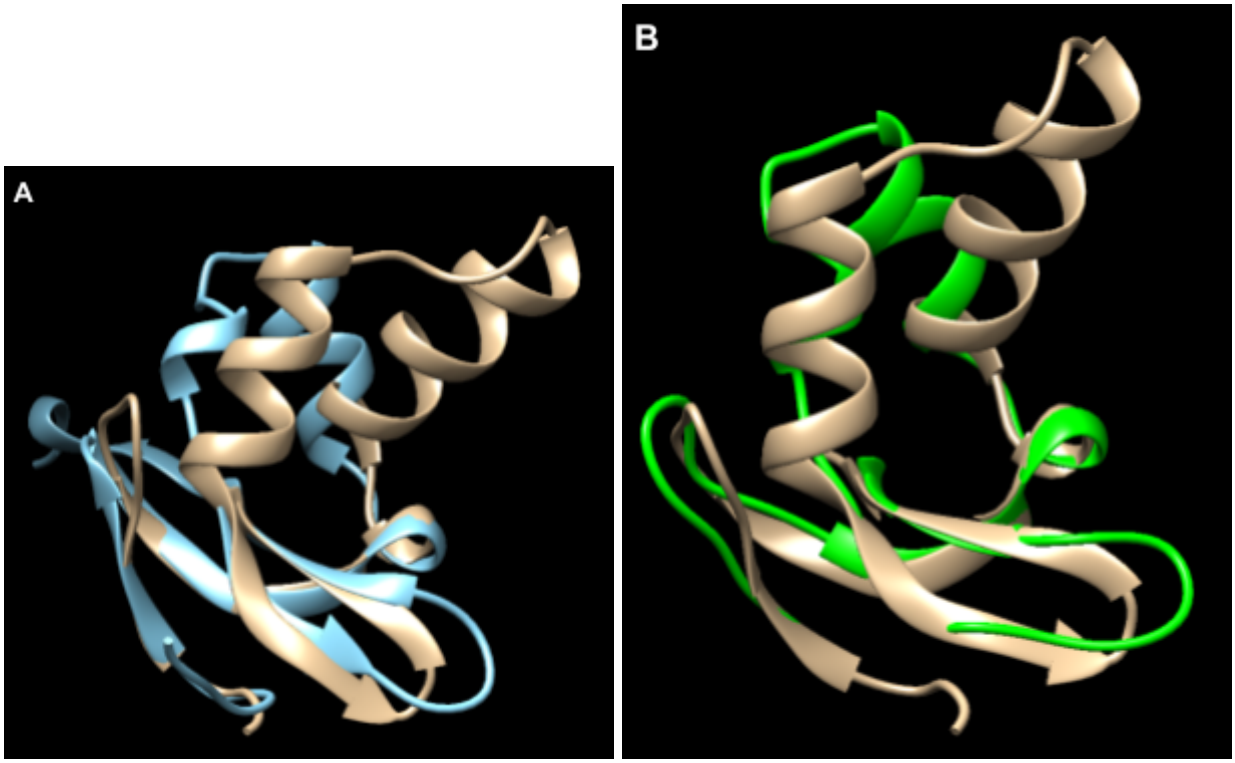

Figure S1. ***Comparison of Skanda and Grass CLIP domains****: Overlap of the Grass CLIP domain (brown) with (A) the Skanda CLIP domain closer to the N-terminus (blue) and (B) the Skanda CLIP domain further from the N-terminus (green) visualised using Chimera v1.17.3.*

Figure S2

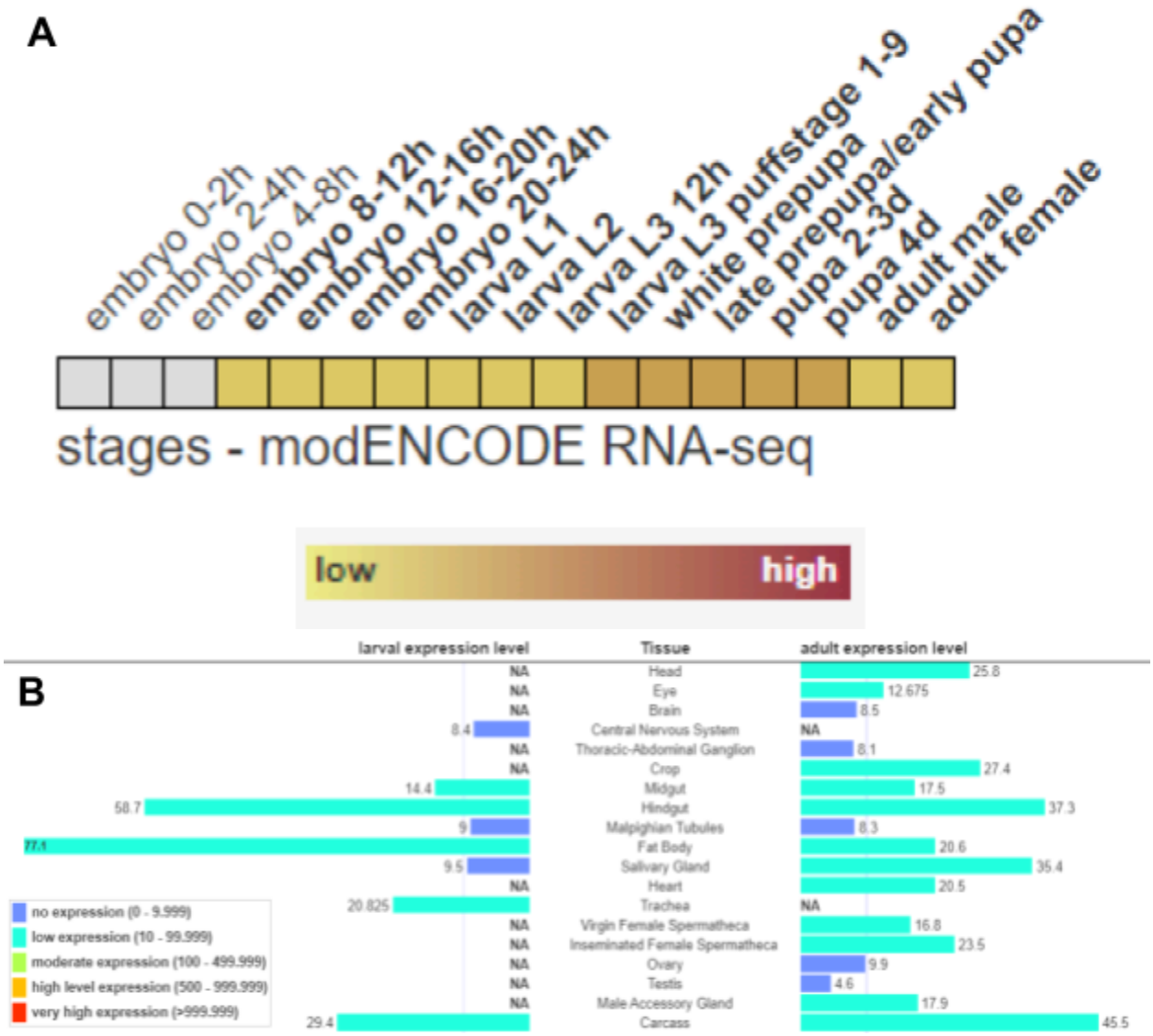

Figure S2. ***Skanda expression in* Drosophila***: (A)* Skanda *expression across Drosophila development (B) Tissue specific expression profile of Skanda in larvae and adults. Adapted from flybase.org*

Figure S3

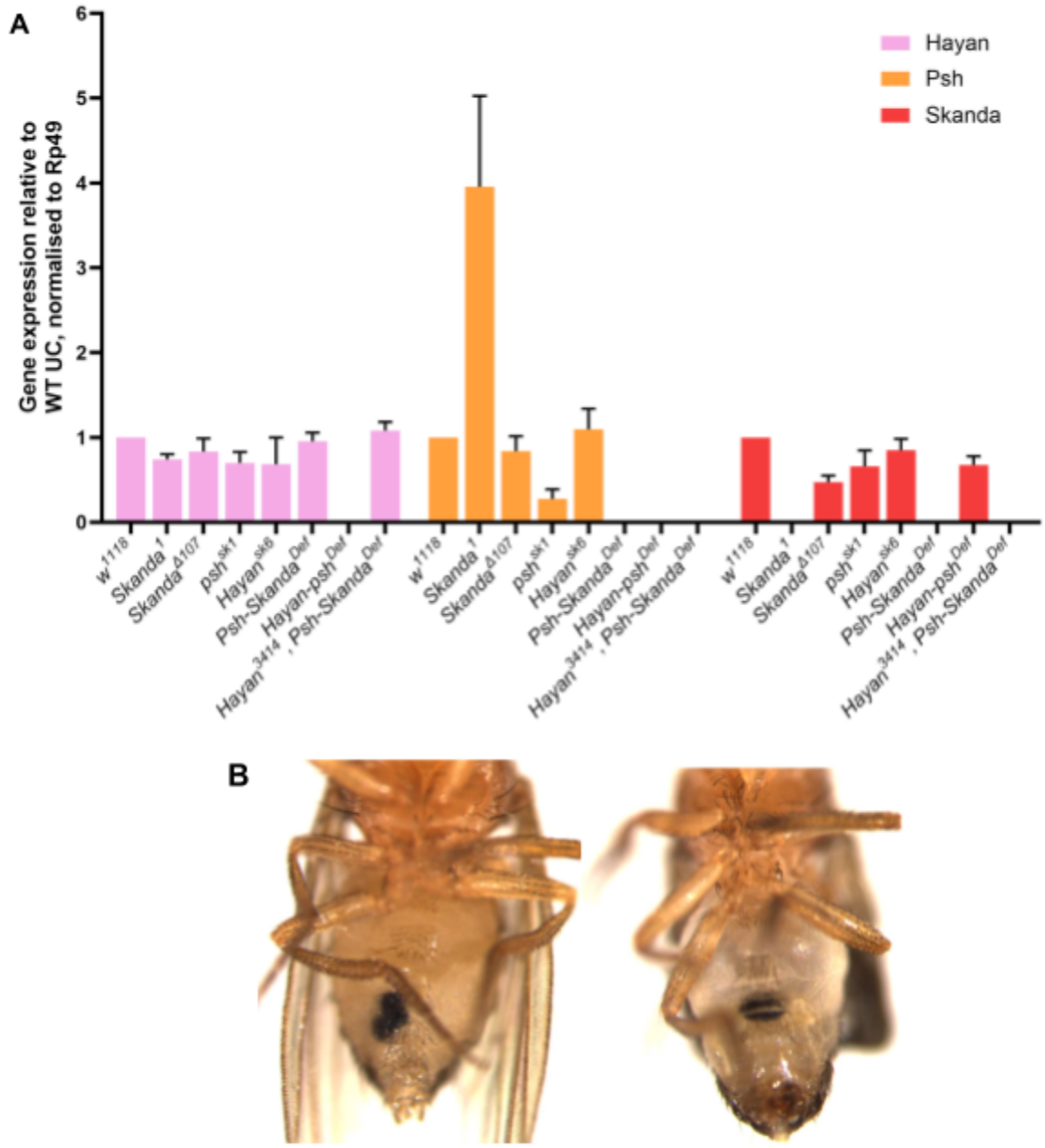

Figure S3. **Hayan, psh *and* Skanda *expression in mutant lines***: *(A)* Hayan, psh *and* Skanda *expression in the mutant lines used in this study. (B)* Skanda[1] *mutant flies have melanotic tumors, characteristic of over-active Toll signalling.*

Figure S4

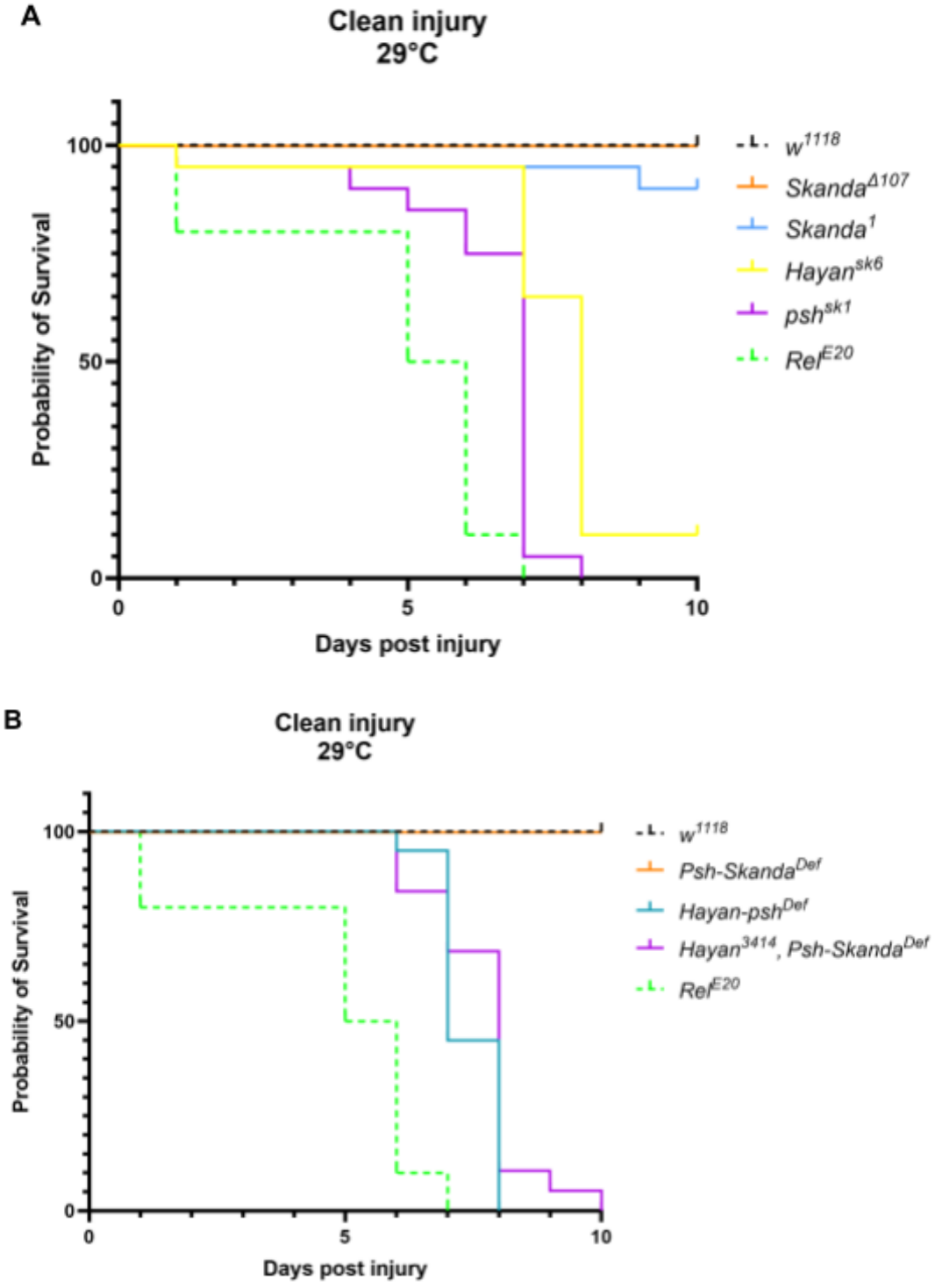

Figure S4. ***Survival of Hayan-psh-Skanda mutants to clean injury****: Survival of Hayan-psh-Skanda (A) single and (B) compound mutants to clean injury. Flies were maintained at 29°C post injury.*

Figure S5

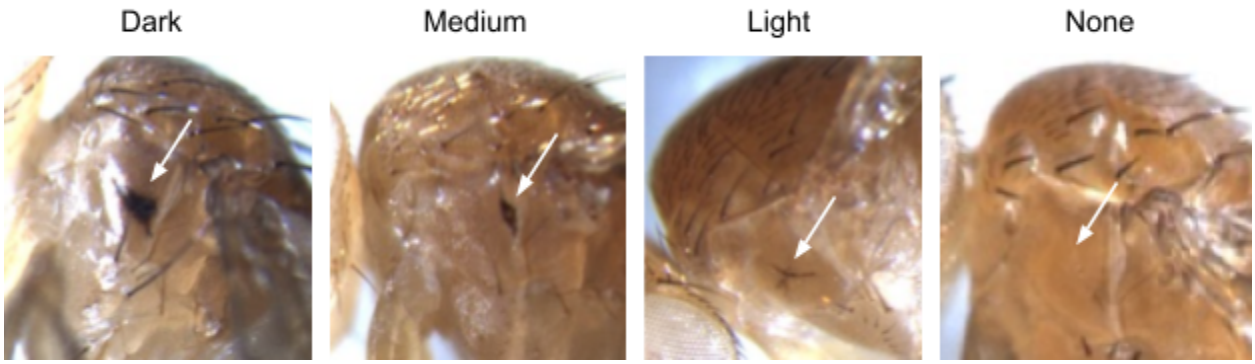

Figure S5. ***Classification of melanisation intensity in flies****: Flies were pricked with an ethanol-sterilised needle, and melanisation was looked at 16h post injury. Intensities were classified as dark, medium, light or no melanisation.*

Figure S6

Figure S6. ***Evolution of*** **Hayan, psh** ***and*** **Skanda***: (A) Phylogenetic tree showing the evolution of* Hayan *and* psh*. The two genes share the same common ancestor.* Hayan *is conserved throughout the* Drosophila *genus , while* psh *evolved specifically in the Melanogaster group. (B) Phylogenetic tree showing the evolution of* Skanda*.* Skanda *is conserved throughout the* Drosophila *genus. Images from* [*http://eggnog5.embl.de/*](http://eggnog5.embl.de/)