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SCREENING FOR NEW SERINE PROTEASES INVOLVED IN *Drosophila melanogaster* IMMUNITY

by
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AN ABSTRACT OF THE THESIS OF

Jeanette EL Hajj for Master of Science
Major: Biology

Title: Screening for New Serine Proteases Involved in *Drosophila melanogaster* Immunity

Drosophila melanogaster is simple model organism used to study the innate immune system. Research focused for the past few decades on the two arms of the immune system: the cellular and the humoral arms. The humoral immune response of *Drosophila melanogaster* relies mostly on activation of melanization cascades and anti-microbial peptides (AMP) production. Two different pathways control the AMP production: the immune deficiency pathway (Imd) and the toll pathway. Considering that most of the intra-cellular players of the toll pathway have been characterized, our focus was on the extracellular players in the toll pathway. Previous studies demonstrated the presence of a serine protease (SP) cascade that results in cleavage of spatzle, the ligand of the toll receptor. However, among the 200 SPs encoded by *Drosophila melanogaster* genome, researchers found that only five were implicated in the activation of the toll pathway. Using an RNA interference screen we searched for candidate SP implicated for the activation of toll pathway, we showed that six SPs are implicated in the extracellular cascade of the toll pathway.

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ABBREVIATIONS

RNAi	RNA interference
%	Percent
IR	Inverted repeat
°C	Degrees Celsius
KD	Knock-down
g	Grams
min	Minutes
LB	Luria-Bertani
nm	Nanometers
OD	Optical density
PDA	Potato dextrose agar
nL	Nanoliters
S.aureus	*Staphylococcus aureus*
E.carotovora	*Erwinia carotovora*
E.coli	*Escherichia coli*
E.faecalis	*Enterococcus faecalis*
B.bassiana	*Beauveria bassiana*
RT-PCR	Real-time Polymerase Chain Reaction
h	Hours
µL	Microliters
V	Volume
RT	Reverse transcription
Rp49	Ribosomal protein 49
Ct	Cycle threshold
mg	Milligrams
mL	Milliliters
Wt	Wild-type
*	Crossed to
Spn	Serpin
SP	Serine protease
F1	First filial generation
PO	Phenoloxidase
N.I	Non-infected
Inf	Infected
AMP	Antimicrobial peptide
GFP	Green fluorescent protein

CHAPTER I

INTRODUCTION

In the past, studies focused on the adaptive immune system. Recently, studying innate immunity gained more interest (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999). The innate immune system is evolutionary conserved throughout the plant and animal kingdoms (Carvalho Ade & Gomes, 2011) (Aerts, Francois, Cammue, & Thevissen, 2008).

A. Life of *Drosophila melanogaster*:

Drosophila melanogaster, commonly known as the fruit fly or vinegar fly, belongs to the order Diptera, family Drosophilidae. Charles W. Woodworth was one of the first scientists to breed it at Harvard University. He then suggested it as a model organism to William E. Castle. Subsequently, several scientists were interested in its use, amongst them Frank Eugene Lutz. The later recommended it to Thomas Hunt Morgan, who at the time was looking for a species to breed in a limited space. As a result of Thomas H. Morgan's work, *Drosophila melanogaster* is now used as a model organism in genetics studies, development, neurobiology, evolution and immunity (Miller, C. 2000. "Drosophila melanogaster").

Drosophila melanogaster lives in a broad range of habitats. Humidity and temperature are necessary for the flies' survival. The females lay eggs on a rotten fruit. The embryo develops into larvae. The latter goes through three larval stages: the first instar larval stage, the second instar larval stage and the third instar larval stage. After

that, the larva undergoes pupation. After pupation the embryo begins metamorphosis and finally emerges as an adult (*Miller, C. 2000. "Drosophila melanogaster").*

Under optimal conditions, the duration of development from egg to larva is five days, followed also by five days of metamorphosis. At lower temperatures (20 °C) the fly's lifecycle takes up to fifteen days. Where the larva development takes eight days followed by seven days of metamorphosis. Cold temperatures (below 12°C) and high altitudes influence the offspring development and the adult flies' physiology. On the other hand, when bred at temperatures above 30° C the flies may become sterile (Miller, C. 2000. "Drosophila melanogaster").

B. *Drosophila melanogaster* model organism:

The genetic approach is important to understand what happens at the molecular and cellular level. Yet, a particular limitation is the requirement of a large number of progeny. That is why geneticist prefer to use: unicellular organisms such as *Escherichia coli* or simple multicellular organisms such as *Caenorhabditis elegans*, *Danio rerio* and *Drosophila melanogaster* (Yamamoto-Hino & Goto, 2013).

Drosophila melanogaster is an easy organism to breed and it has a relatively short lifecycle. It has only four pairs of chromosome, the genome sequence is determined (Adams et al., 2000), it was used as a model organism for genetic screens in the past decade (Boutros et al., 2004) and transgenic fly strains are commercially available for research use. Due to the fact that *Drosophila melanogaster* have a large number of F1 progeny, survival studies are more significant than the ones carried out by mammals. Additionally, being an i*n vivo* model organism *Drosophila melanogaster* is

suitable for the analysis of physiology integrated with immunity and overall the effect of experiments on the whole organism (Buchon, Silverman, & Cherry, 2014). Furthermore, there are a lot of similarities between vertebrates and invertebrates.

The schematic drawing below represents how physiological processes are evolutionary conserved between invertebrates and vertebrates.

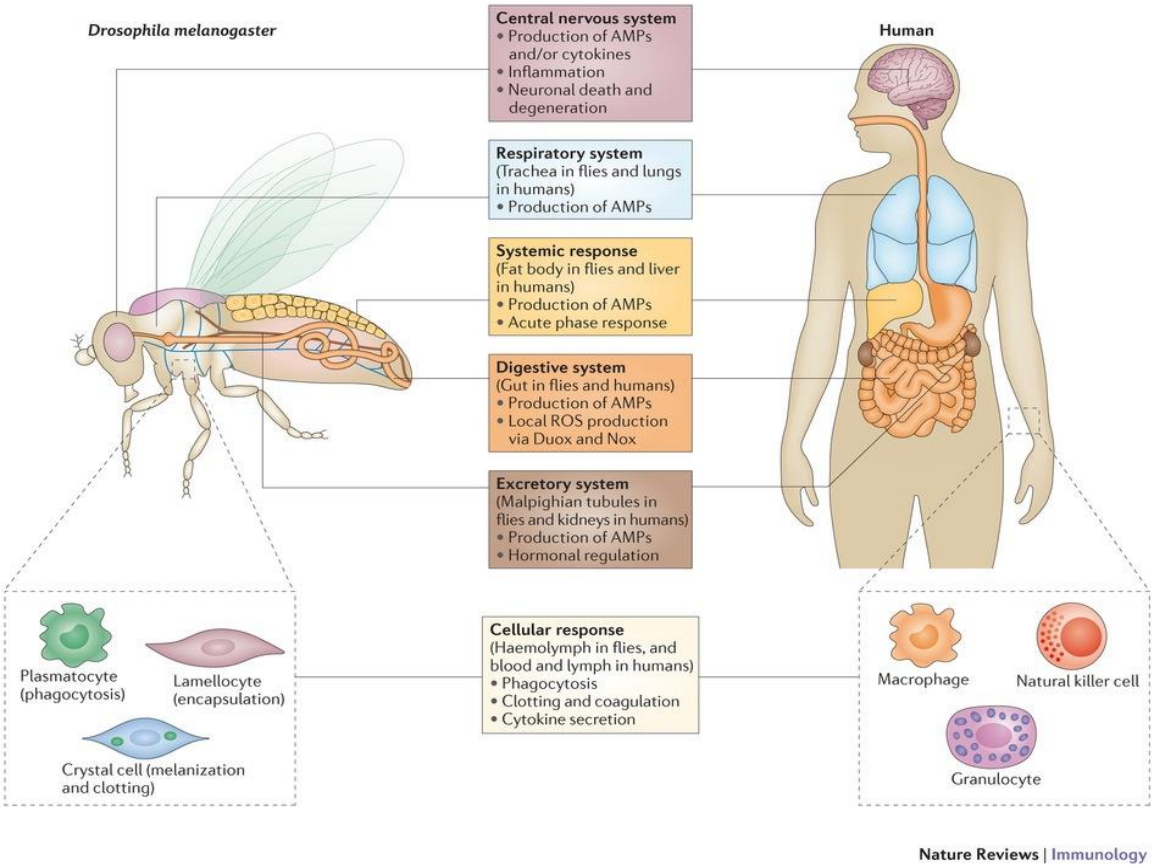
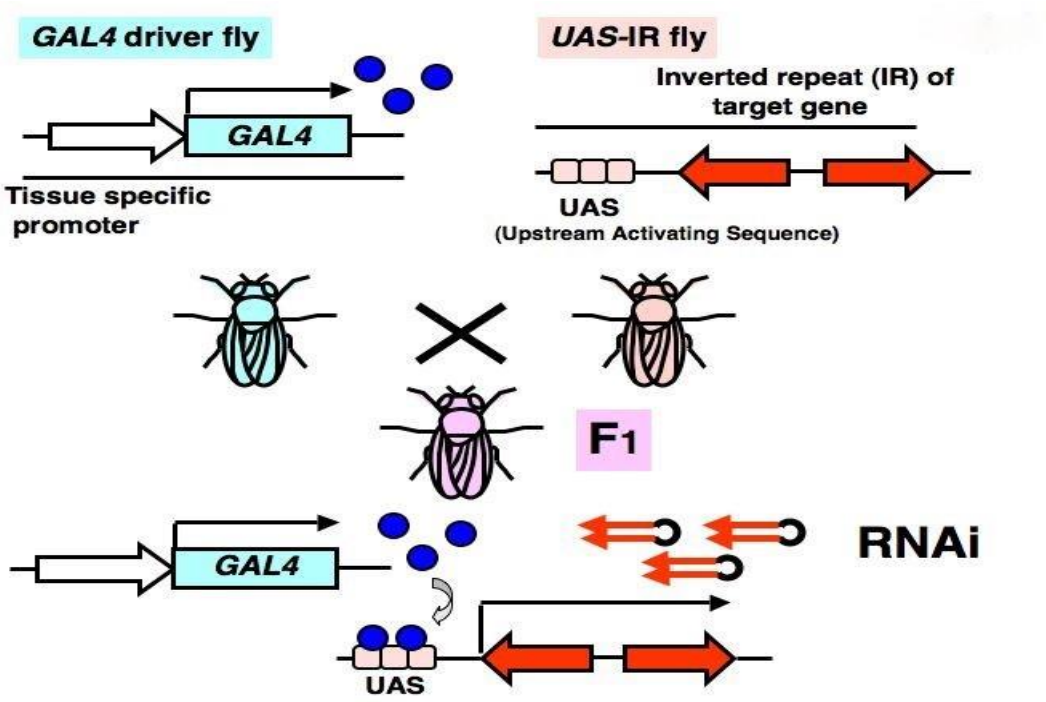


Figure 1: Evolutionary conservation of physiological processes from invertebrates to vertebrates. (Buchon et al., 2014) The simple organ systems of invertebrates carry out an analogous basic function of the complexes organ systems of invertebrates. The gut functions in absorption and is a physical barrier for invading pathogens. The malpighian tubules are analogous to the kidneys. The flies have an open circulation unlike vertebrates who have a closed circulation. Like vertebrates, flies possess different types of immune cells collectively known as hemocytes (lamellocytes, plasmatocytes and crystal cells. The hallmark of the humoral innate immune response in flies is the secretion of AMPs by the gut, the excretory system, the fat body and the respiratory system. AMPs are cationic peptides, very important in defense against microbes, evolutionary conserved from invertebrates to vertebrates.

C. RNA interference (RNAi):

Most eukaryotic cells use RNA interference (RNAi) for regulation of gene expression (Timmons, Tabara, Mello, & Fire, 2003). In addition, plant and animal cells use RNAi as defense mechanism against viruses and other parasitic nucleotide sequences such as transposable elements (Li, Lu, Han, Fan, & Ding, 2013; Saurabh, Vidyarthi, & Prasad, 2014; Wilkins et al., 2005) (Vasselon, Bouttier, Saumet, & Lecellier, 2013). Over the past decade, studies showed the efficacy of RNAi as a genetic tool in cell cultures as well as in vivo organisms. It is precise and specific, presenting many advantages over other technologies such as antisense technology (Katoch & Thakur, 2013; Saurabh et al., 2014).

The schematic drawing below represents how the UAS/Gal-4 system is used to inhibit the expression of a specific gene.



Figures 2: UAS/Gal-4 system:(Mohr, 2014) A fly carrying the Gal-4 driver is crossed to a fly carrying the inverted repeat of a specific gene under the control of Upstream activating sequence (UAS-IR). This results in the expression of the Gal-4 protein in the F1 progeny in a spatiotemporal manner. The expression of the dsRNA leads to gene knockdown.

RNAi relies on the delivery of a double stranded RNA to the cell. The dsRNA can be delivered by several methods: bidirectional transcription of an inverted repeat (Clemens et al., 2000; Lam & Thummel, 2000), by soaking or direct injection into cells (Fire et al., 1998; Tabara, Grishok, & Mello, 1998). Specifically in *Drosophila melanogaster*, the Gal-4-UAS system is used for gene knockdown (Martinek & Young, 2000; Tavernarakis, Wang, Dorovkov, Ryazanov, & Driscoll, 2000): A cross is carried out between a fly carrying the Gal-4 sequence (under the control of a tissue specific or ubiquitous heat inducible promoter (Lam & Thummel, 2000)) and a fly carrying the Upstrem Activating Sequence- Inverted repeat (UAS –IR) (UAS is the binding site of Gal-4). In the F1 progeny when the Gal-4 protein is expressed and binds the UAS sequence; thereafter an inverted repeat of the gene is transcribed and a double stranded hairpin RNA (dsRNA) is produced. The latter is fragmented by the RNAase III, Dicer, into 21 base pair small interfering RNA (siRNA) (Svobodova, Kubikova, & Svoboda, 2016). The siRNA unwinds into small single stranded RNAs (ssRNA). The ssRNA are used as templates by the RNA-Induced Silencing Complex (Tavernarakis et al.) to recognize the complementary mRNA sequence and cleave it. It is worthy to note that using RNAi for gene silencing in mammalian cells is not applicable due to the rapid and global degradation of all mRNAs.

D. Immunity of *Drosophila melanogaster*:

Mammals rely on both the inherited innate immunity and the adaptive immunity. *Drosophila melanogaster*, like other insects rely solely on the innate immune system for defense. The cuticle (a physical barrier) and the chemical barrier of the gut are a passive defense against pathogens. In addition, *Drosophila melanogaster* has three

major lines of defense: first, the humoral response that results in the production of antimicrobial peptides (AMP) by the fat body (functionally analogous to the mammalian liver). AMPs are cationic peptides potent against a wide array or microorganisms. Second, circulating blood cells engulf microbes and destroy it.

Third, parasites that are too large to be destroyed by phagocytosis, like parasitoid wasps, are encapsulated by hemocytes and melanin (Cherry & Silverman, 2006).

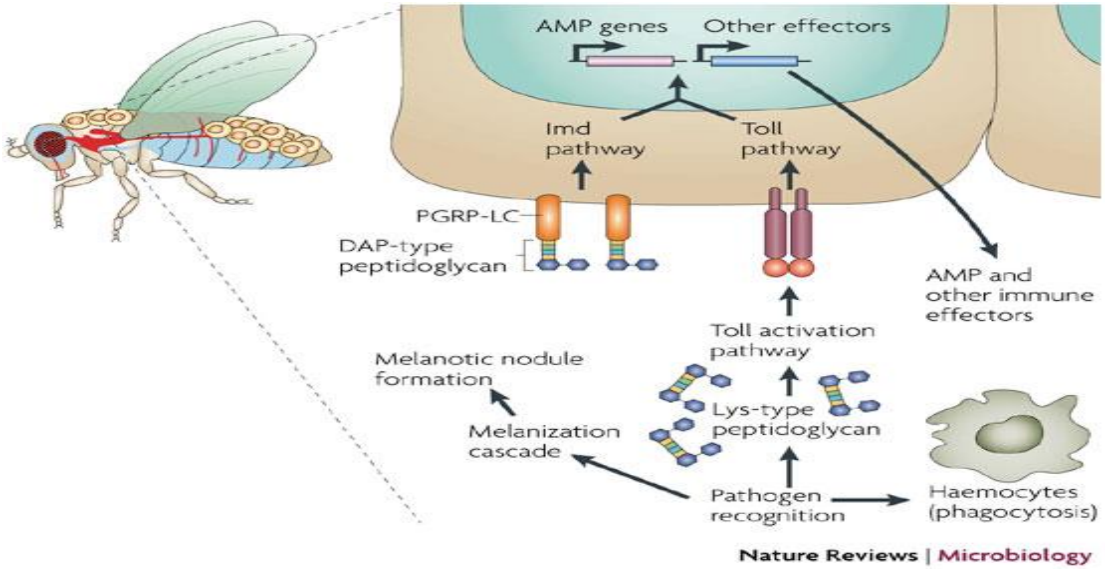


Figure 3: Immune response of *Drosophila melanogaster* (compendium of fly methods) The immune response of *Drosophila melanogaster* is comprised of two main branches: the cellular immune response, the humoral immune response that is subsequently divided into melanization cascades and AMP production cascades.

E. Phagocytosis:

Phagocytosis in flies is similar to that in mammals but simpler with much less redundancy. There are many receptors expressed at the surface of hemocytes to allow the efficient engulfment and destruction of many microbes (Stuart & Ezekowitz, 2005). One example is "eater" required for the successful phagocytosis of gram-negative and gram-positive bacteria (Kocks et al., 2005). In invertebrates, like vertebrates, there is diversity of receptors present on the surface of immune-competent cells. Down syndrome cell adhesion molecule (Dscam) is another type of surface receptor on hemocytes. It belongs to the immunoglobin (Ig) superfamily receptor. The loss of Dscam inhibits phagocytosis of many bacteria (Watson et al., 2005).

F. Toll and IMD pathways:

The humoral immune arm is divided into two major branches: the melanization cascade and the cascade that leads to the production of antimicrobial peptides. The latter is divided into two distinct pathways: the toll pathway and the IMD pathway. The toll pathway recognizes gram- positive bacteria and fungi. This results in the translocation of the NF-kB transcription factor Dif into the nucleus and the production of AMPs mainly Drosomycin. On the other hand, the IMD pathway is activated by gram- negative bacteria. This involves the translocation of Relish to the nucleus (another NF-kB transcription factor). Eventually, many AMPs are produced the major one being Diptericin. Even though, there is no evidence of crosstalk between the two pathways. At some point they may induce the same antimicrobial peptides (Cherry & Silverman, 2006; Ferrandon, Imler, Hetru, & Hoffmann, 2007). Antimicrobial

peptides are playing key roles in innate immunity, targeting a wide variety of pathogens such as bacteria, fungi, parasites and viruses.

(Lehrer & Ganz, 1999). These are potent cationic amphipathic molecules that kill the bacteria directly (Hancock & Sahl, 2006) or modify the innate immune response (Niyonsaba et al., 2007). The toll pathway is activated in when triggered by: a developmental signal or an immune signal. In development, the extracellular serine proteases cascade includes: Nudel, Gastrulation defective, Snake and Easter. This results in the cleavage of Spatzle (Spz), the toll ligand. Upon cleavage, Spz binds the extracellular domain of toll. Signaling results in the translocation of the NF- KB transcription factor Dorsal into the nucleus and gene transcription begins (Chasan, Jin, & Anderson, 1992; Cho, Stevens, & Stein, 2010; Hong & Hashimoto, 1995). When infected by a microbe, different players are involved in the extracellular signaling cascades. The current model suggests that three different cascades lead to the cleavage of Spz (Jang et al., 2006). The β- glucan of fungi binds the receptor GNBP3 and the lysine type peptidoglycan carried by gram-positive bacteria binds PGRP-SA (El Chamy, Leclerc, Caldelari, & Reichhart, 2008). In both cases, the serine protease Grass is activated. There is still controversy in the field about whether Grass is only a gram-positive specific serine protease (Kambris et al., 2006). A study showed that it could also be involved in the defense against fungi (El Chamy et al., 2008). Three other serine proteases were identified as required for defense against both gram-positive bacteria and fungi: Spirit, Spheroid and Sphinx 1/2 (Kambris et al., 2006). The proteolytic cascade activates Spz cleaving enzyme (SPE), another SP that cleaves Spz (Jang et al., 2006). Upstream of Grass a modular serine protease (modSP) integrates signals form the two receptors PGRP-SA and GNBP3 (Buchon et al., 2009).

Persephone mediates a third proteolytic cascade (Ligoxygakis, Pelte, Hoffmann, & Reichhart, 2002) It is activated when it is cleaved by secreted virulence factors form fungi (Gottar et al., 2006) or triggered by the presence of gram-positive bacteria (El Chamy et al., 2008). Intracellular, Myd88- tube- pelle cassette leads to the phosphorylation and the degradation of I-kb homologue cactus thus the transcription factor Dif translocate into the nucleus (Wu & Anderson, 1998). This leads to the production of antimicrobial peptides by the fat body, the gut and the respiratory tract (De Gregorio et al., 2002).

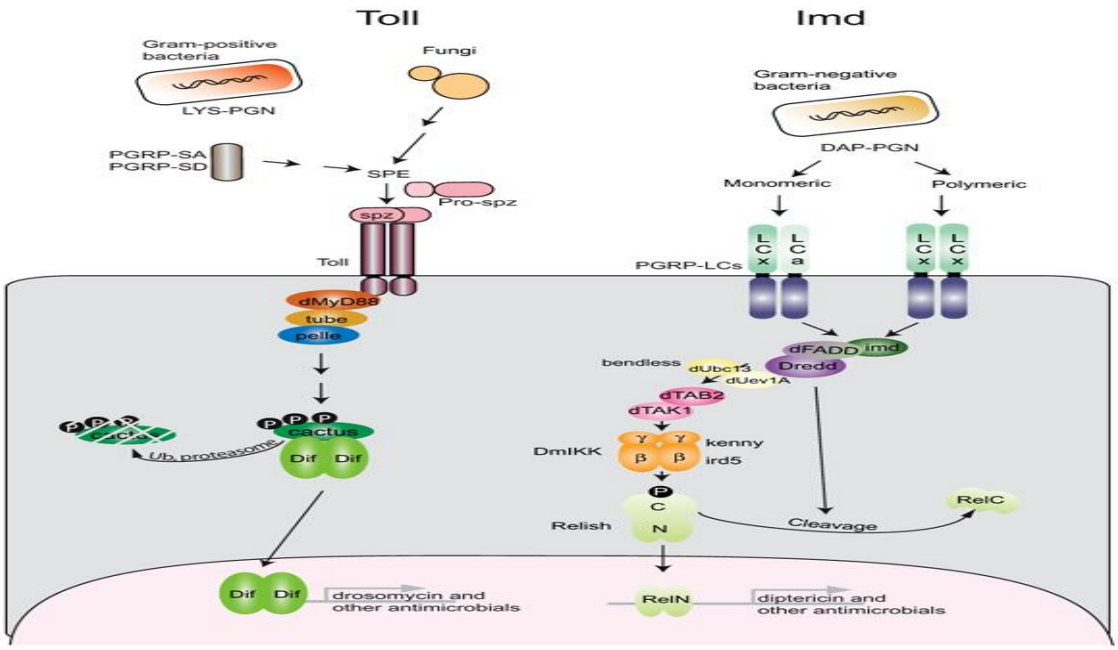


Figure 4: Schematic representation of the toll and the IMD pathways (Cherry & Silverman, 2006)

G. Melanization:

Melanization is one of the most rapid pathways in arthropods' immune response (Tang, 2009). In melanization quinon is cross-linked into melanin by the enzyme phenol oxidase (PO) at a site of injury or invading microbe (Benelli, Lo Iacono, Canale, & Mehlhorn, 2016; Cerenius & Soderhall, 2004). Quinone is involved also in the production of hydroxyl radicals and superoxide that kill the encapsulated microorganism (Zhao, Lu, Strand, & Jiang, 2011). The zymogen pro-Phenoloxidase (PPO) is activated by serine proteases after infection or wound injury. The *Drosophila melanogaster* genome encodes for three PPOs. In adult flies, PPO1 and PPO2 are found in the hemolymph (Honti, Csordas, Kurucz, Markus, & Ando, 2014). Pro-PO3 is predominantly active in the lamellocytes (Nam, Jang, Asano, & Lee, 2008).

Mutants' *ppo1* and *ppo2* show a reduced viability after microbial infection. This demonstrates their importance in defense against pathogens (Binggeli, Neyen, Poidevin, & Lemaitre, 2014). In *Drosophila melanogaster*, there are three clip-domain serine proteases that activate PPO: MP1, MP2, and hayan (Castillejo-Lopez & Hacker, 2005; Tang, Kambris, Lemaitre, & Hashimoto, 2006). The knockdown of MP1 or MP2 does not severely affect the viability of the flies after microbial infection (Castillejo-Lopez & Hacker, 2005; Tang et al., 2006). This implies that other pathways complementary to the MP1/MP2 melanization cascade exist (Ayres & Schneider, 2008; Nam, Jang, You, Lee, & Lee, 2012).

H. Serpins:

Serpins (Spn) are serine protease inhibitors. Their knockdown leads to the excessive and spontaneous formation of melanotic tumors (De Gregorio et al., 2002). Hence, they prevent excessive melanization at the site of injury or bacterial infection (De Gregorio et al., 2002; Tang et al., 2006). There are 29 serpins encoded by the *Drosophila melanogaster* genome. Amongst those the serpins identified to be involved in the immune response are: Spn 43Ac, Spn 27A, Spn 28Dc, Spn 77Ba and Spn 88Ea. For instance, Spn 27A is a clear link between the toll pathway and melanization pathway in *Drosophila melanogaster* immunity (Ligoxygakis, Pelte, Ji, et al., 2002).

I. Serine proteases:

Serine proteases (SP) constitute one of the largest families in *Drosophila melanogaster* genome (Veillard, Troxler, & Reichhart, 2016). There are 200 identified SPs, amongst which there are 84 small size SPs that comprise of about 300 residues of amino acids. Scientist suggests that they might me mainly involved in digestion.

While other larger multi-domain SPs are 24 and the serine protease homologues are 13. Those contain at least one clip regulatory domain (Ross, Jiang, Kanost, & Wang, 2003). SPs function in a wide range of physiological processes: immunity, coagulation, and digestion. Secreted in form of zymogens the enzymes are activated in a sequential cascade where one activated serine protease cleaves the inactive zymogen of the subsequent enzyme (Veillard et al., 2016). This is key for the insertion of multiple controls sites and for the amplification of the response (physiological or pathological). Different SPs with different functions possess different structures. The SPs involved in digestion such as trypsin usually have a small

N- terminal domain. While, the SPs involved in more complexes pathways such as immune pathways have many domains. At the N-terminus a pro- domain enables the interaction and the relative orientation of different of sequential SPs in extracellular cascades (Veillard et al., 2016). So far the identified serine proteases in embryonic development have a clip domain linked with the catalytic end are Easter and snake. Evidence suggests that clip –SPs are involved in the melanization cascade and antimicrobial peptide production (Kellenberger et al., 2011). The clip-SPs found to work in the toll pathway are: ModSP, Grass and SPE. The later was identified in homology with Easter (Jang et al., 2006).

Additionally Kambris et al. identified three serine protease homologues: Spirit, Spheroid and Sphinx1/2 to be essential for the activation of the toll pathway. Epistasis and genetic analysis revealed that serine protease homologues Spirit, Spheroid and Sphinx1/2 are downstream of Grass and upstream of SPE. Even though in epistasis analysis Grass was shown to be downstream of ModSP (Buchon et al., 2009). *In vitro* evidence shows that ModSP does not directly cleave Grass.

Hence, the extracellular pathway is still incomplete. Persephone also involved in fugal detection is also a clip SP (Kellenberger et al., 2011).

J. Significance of the project:

Drosophila melanogaster is a powerful model to manipulate genetically. RNAi was shown in past studies to be very important in identifying serine proteases involved in physiological processes such as immunity (Kambris et al., 2006).

In our study, we used RNAi to screen 90 genes (112 lines). To determine whether there are other serine proteases involved in the toll pathway that remains unidentified to date. For this purpose, we first checked for lethality with the Actin-Gal-4 ubiquitous driver. Since a high percentage of the KD genes were lethal at the whole organism level we used a more restricted knockdown: The C564-Gal-4 driver enables knockdown in the fat body was chosen. After knockdown the flies were infected and survival carried out. We chose positive Serine proteases that were sensitive to microbial infection. Real time PCR was performed to quantify AMP levels for positive candidates. Our work plan was based on two specific aims:

1. Specific aim 1:

We hypothesize that the knock down of certain SP genes results in a weak ability to eliminate specific invaders.

- ✓ Knock down of serine proteases (SP) gene using an RNAi strategy.
- ✓ Check for lethality, if lethal we will knock down the gene specifically in the fat body
- ✓ Infect the F1 progeny of knocked down flies with different microorganisms.
- ✓ Carry out survival assays of the infected flies.

2. Specific aim 2:

We hypothesize the KD of serine proteases that led to compromised survival was due to the lack of anti-microbial peptide production. To assess that we chose several SPs and:

- ✓ Repeated the knockdown assays and the infection with different pathogens.
- ✓ Quantified antimicrobial peptides via RT-PCR at different time points after the microbial infection.

CHAPTER II

MATERIALS AND METHODS

A. Microbial strains

Gram-positive bacteria:

Staphylococcus aureus (Sa), *Enterococcus fecalis* (Ef)

Gram-negative bacteria:

Erwinia carotovora carotovora strain 15 (Ecc15)

Fungus:

Beauveria bassiana (Bb)

B. *Drosophila melanogaster* stocks and maintenance

All *Drosophila melanogaster* IR lines are purchased from the Vienna *Drosophila melanogaster* Resource Center (VDRC). Feeding medium is a mixture of soy flour, polenta (cornmeal), molasses, agar and propionic acid. It is poured in 50 ml *Drosophila melanogaster* vials. The main stocks are stored at 18°C, while the crosses were kept in an incubator at 25°C or 29°C depending on the need. To maintain the fitness of the flies: humidity is set at more than 50%. The lights are turned on/off automatically every 12 hours for a healthy circadian rhythm.

C. *Drosophila melanogaster* crosses

Virgin females carrying the Gal-4 driver are collected from amplified Actin- Gal-4/Cyo or C564-Gal-4 stocks, stored at 18°C.

Approximately, ten virgin females are crossed to five males carrying the IR constructs in vials with fresh medium. The crosses are maintained at 25°C until progeny reaches the third instar larval stage. By then, crosses are transferred to a temperature of 29°C for maximum efficiency of the UAS-Gal-4 system.

D. Microbe preparation

Liquid bacterial cultures were incubated overnight at a temperature ranging from 30 to 37 in shaking motion. The culture were then spun at 4000g for 10 min, cells were re-suspended in LB. By spectroscopy we measured the bactreial OD at 595nm. The resuspended bacteria were stored at 4°C. Before the injections the desired OD was adjusted by serial dilutions.

The fungus used in the experiment is *Beauveria bassiana.* After it is grown on PDA plates for 3 weeks, spores were filtered using glass wool and distilled water. After preparation, the spores were counted and diluted to the desired concentration of spores/ηl by dilution (Approx 100 spores were injected per fly).

E. Infection of *Drosophila melanogaster*

Fifteen to twenty female or male flies from the F1 progeny were collected at an age ranging between three to six days. Flies were injected with 32.2 nL of the microbe (Sa OD= 0.05, Ecc15OD=0.1, *E. coli* OD =0.1, Ef OD=0.05 or 4 spores/nL of *B. bassiana*) using a nano-injector (NanojectII, Drummond Scientific). The injected flies are returned into the vials and maintained at 25 degrees. For survival assays, dead flies were counted at regular intervals. Survival graphs were then plotted as percentage of surviving flies in function of time. Flies that were infected for RNA extraction and Real-Time PCR were frozen at -20°C 24 hours after bacterial infection and 48h after fungal infection.

F. RNA Extraction, Reverse Transcription and quantitative Real-Time PCR

For RNA extraction, approximately 15 flies were homogenized in 500 µL TRizol reagent (Invitrogen) and RNA was then separated from cellular constituents by spinning at 15 000g for 10 minutes at 4°C. 100 µL of Chloroform were added to the supernatant, the mix was vigorously vortexed for 2 minutes, then spun at 20 000g to separate the phases. The aqueous phase was transferred to a clean eppendorf and RNA was precipitated by the addition of 0.7 V of isopropanol and spinning at 20 000g for 20min at 4^{o}C. The pellet was washed in 70% ethanol and re-suspended in 50 µL Nanopure double-distilled water. Extracted RNA was then diluted to a concentration of 500 ng/µL and then 1 µL were reverse transcribed into cDNA at 42°C for 45 min using the iScript cDNA Synthesis Kit (Biorad). The reaction was stopped by a five minute incubation at 85°C. Then via Real-Time PCR, the gene transcription level was

quantified using a 1:20 dilution of the RT product. A mixture of *Drosophila melanogaster*cDNA (5µL), reverse and forward primers specific to the gene of interest (0.5µL each) and QuantiFAST SYBR green PCR mix (10µL, Qiagen) were subjected to 40 cycles of denaturation (at 95°C), annealing of the primers (at 60°C), elongation (at 72°C) and quantification at the end of each cycle. The expression levels of *Drosomycin* and *Diptericin* were used as read-outs for the Toll and Imd pathways respectively. The gene that encodes for the ribosomal protein Rp49 was considered as a reference gene for normalization and the Delta Ct method was used for calculations.

CHAPTER III

RESULTS

A. Lethality of SP genes KD

In our study, we analyzed 90 transgenic strains (total of 112 lines) of flies allowing to KD different SP genes. Some of these SPs were previously shown in a genome-wide analysis of the fly's immune response to be up regulated after immune challenge (De Gregorio, Spellman, Rubin, & Lemaitre, 2001). We checked for lethality, after ubiquitously knocking down the gene using the Actin-Gal-4 driver. We have performed at least two independent repeats. Twenty three genes out of ninety were found to be lethal after ubiquitous knockdown: CG 17571 SP, CG 11911 SP, CG 14892 SP, CG 1304 SP, CG 11529 SP, CG 9676 SP, CG 11037 SP, CG 13744 SP, CG 13318 SP, CG 9249 SP, CG 12256 SP, CG 1773 SP, CG 3355 SP, CG 9897 SP, CG 14760 SP, CG 12388 SP, CG 11668 SP, CG 4927 SP, CG 10882 SP, CG 4386 SP, CG 15873 SP, CG 9733 SP, CG 9737 SP. Due to high lethality rate when using the Actin-Gal-4 driver we decided to use the less ubiquitous driver C564-Gal-4 which drives high Gal-4 expression in the fat body. With the C564 driver no lethality was observed after SP KD.

B. Survival studies

After tissue specific gene knockdown, we collected the F1 progeny three to seven days post emergence from pupae. We sorted the flies into males and females and made sure they do not contain balancer chromosomes. The tubes contained from 15 to 20 flies. We infected each batch of the flies with one of the four different microbes: Sa and Ef (gram-positive), Ecc15 (gram-negative), and the entomopathogenic fungus Bb. After infection the flies were counted at regular time intervals. Survival graphs were realized by plotting the percentage of surviving flies in function of time. Males and females were infected separately. Females were chosen for infection with Sa because they are bigger and slightly more resistant than males. On the other hand males were chosen for fungal infection because these infections do not kill the flies rapidly and females lay eggs on the medium, which makes it more liquid, and probably falsify the results.

1. Survival graphs of SP KD flies after infection with a gram-negative bacterium

For gram-negative infection the bacteria used is Ecc15 (OD adjusted to 0.1). After tissue specific knockdown males and females were infected with Ecc15. Survival graphs were plotted. Note that the OD chosen for any bacteria was previously determined by trial and error. All results were confirmed in three independent repeats.

In all survival graphs the vertical axis represents the percentage of surviving flies. The horizontal axis represents the time (Hours). The controls used for infections with gram-positive bacteria or fungus are: Dif KD flies (C564* 100537 KK CG 6794 Dif) and wild type flies (C564* W^{1118} and C564* Org^{E20}). For infection with the gram-negative bacteria Ecc15 we used the following controls: Relish mutant flies

(Rel E20), wild type flies (C564* W^{1118} and C564* Org^{E20}), and Dif KD flies (C564* 100537 KK CG 6794 Dif).

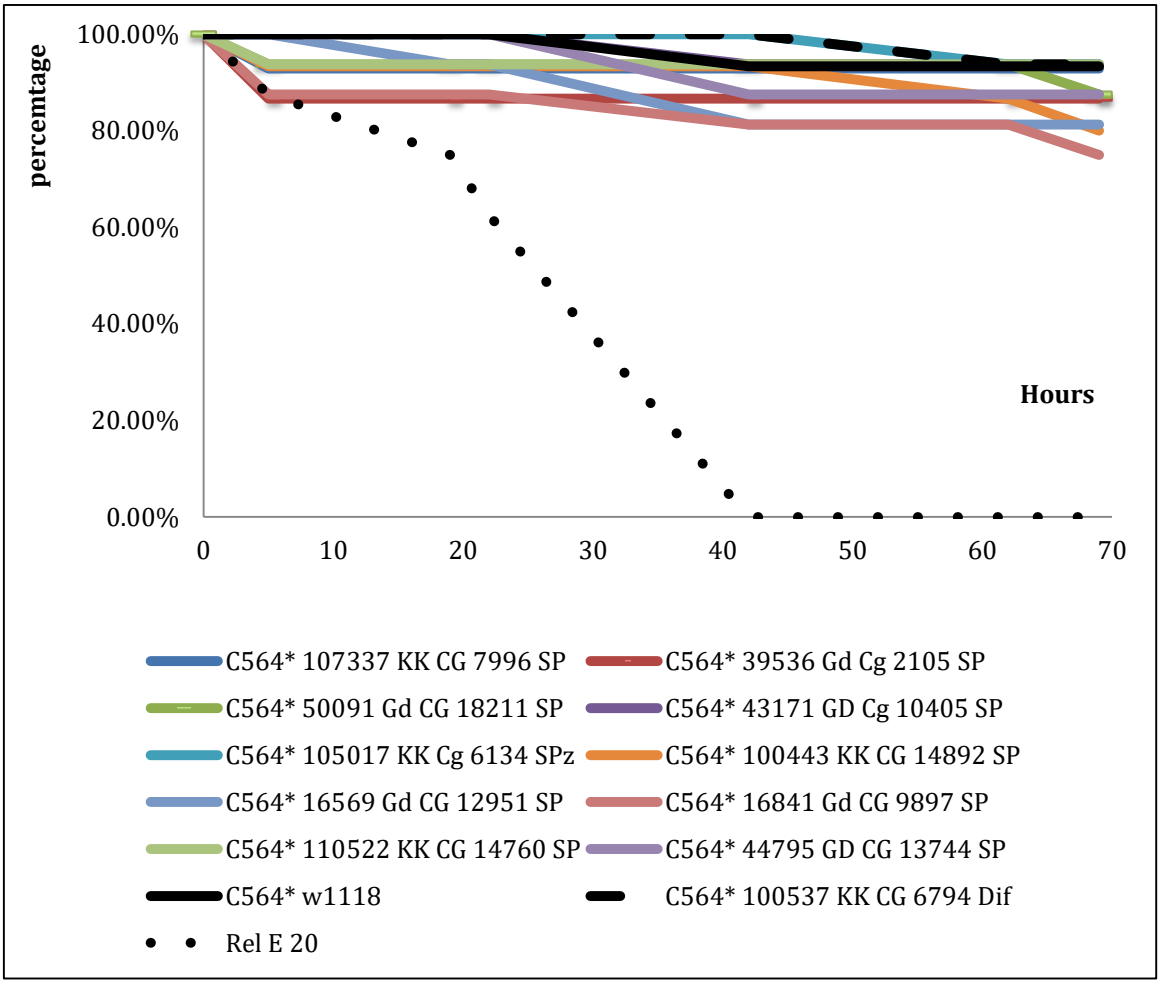


Figure 5: SP KD flies survival graph post- infection with Ecc15 (OD=0.1)

After gene knockdown and infection with the gram-negative bacteria Ecc15, the SP KD flies had no compromised survival, like wild type flies, unlike Rel^{E20} mutant that succumbed fast after microbial infection.

2. Survival studies with gram-positive bacteria:

a. Survival graphs of SP KD flies after infection with Sa

For gram-positive infection the bacteria used is Sa (OD adjusted to 0.05). After tissue specific knockdown males and females was infected with Sa. Survival graphs were plotted.

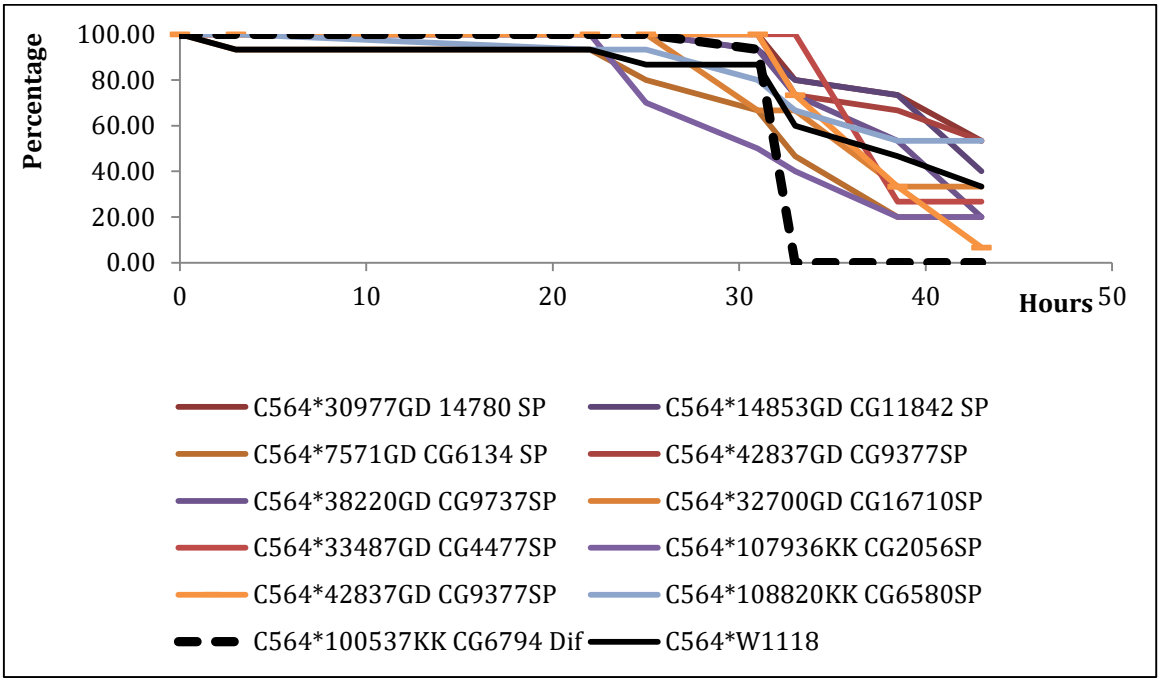


Figure 6: SP KD flies survival graph post infection with Sa (OD=0.05)

After gene knockdown and infection with Sa a high number of SP KD showed intermediate phenotypes and died at relatively fast rate. We were not able to

differentiate between the positive SP KD and the intermediate SP KD. We repeated the study and continued it using another gram-positive bacteria Ef.

b. Survival graphs of SP KD flies after infection with Ef

Survival assays with Ef were carried out with an OD =0.05. After tissue specific knockdown males and females flies were infected. Survival graphs were plotted. All results were confirmed at least with three independent repeats.

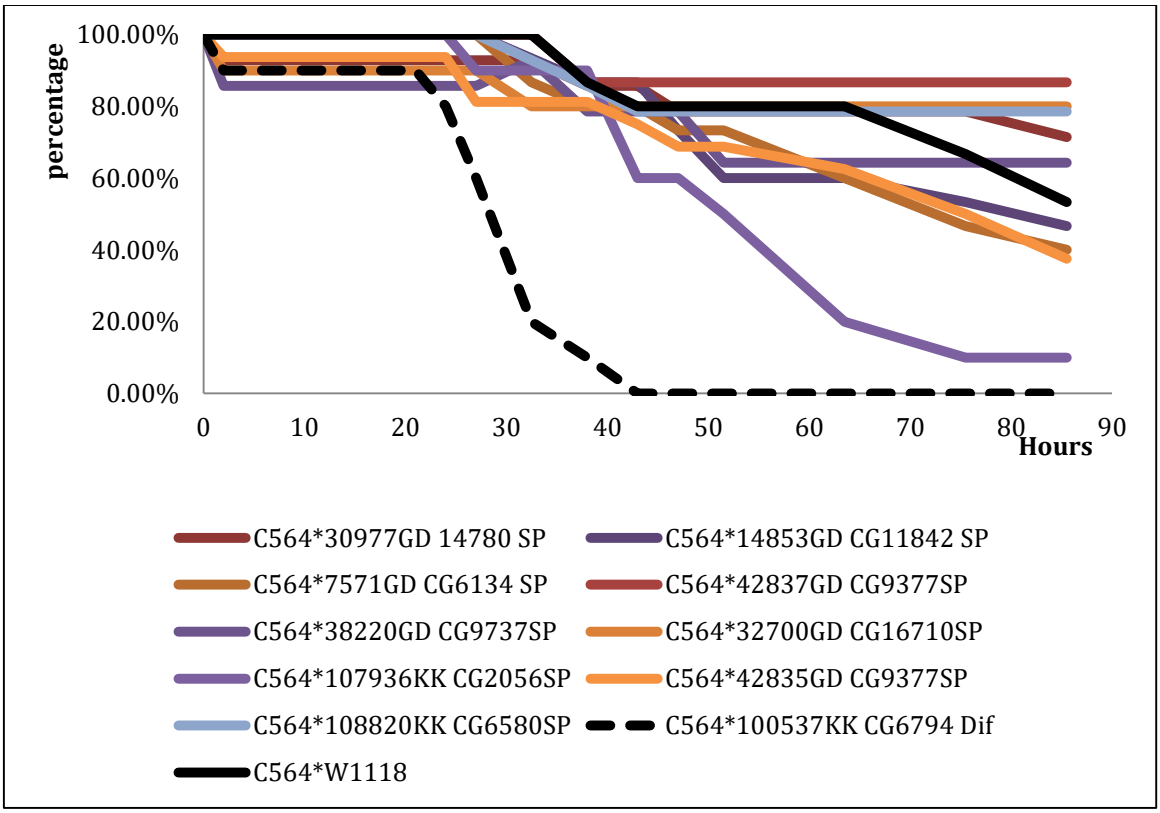


Figure 7: SP KD flies survival graph post infection with Ef (OD=0.05)

This representative graph infers that after CG 2056 SP KD the flies showed compromised survival when infected with Ef.

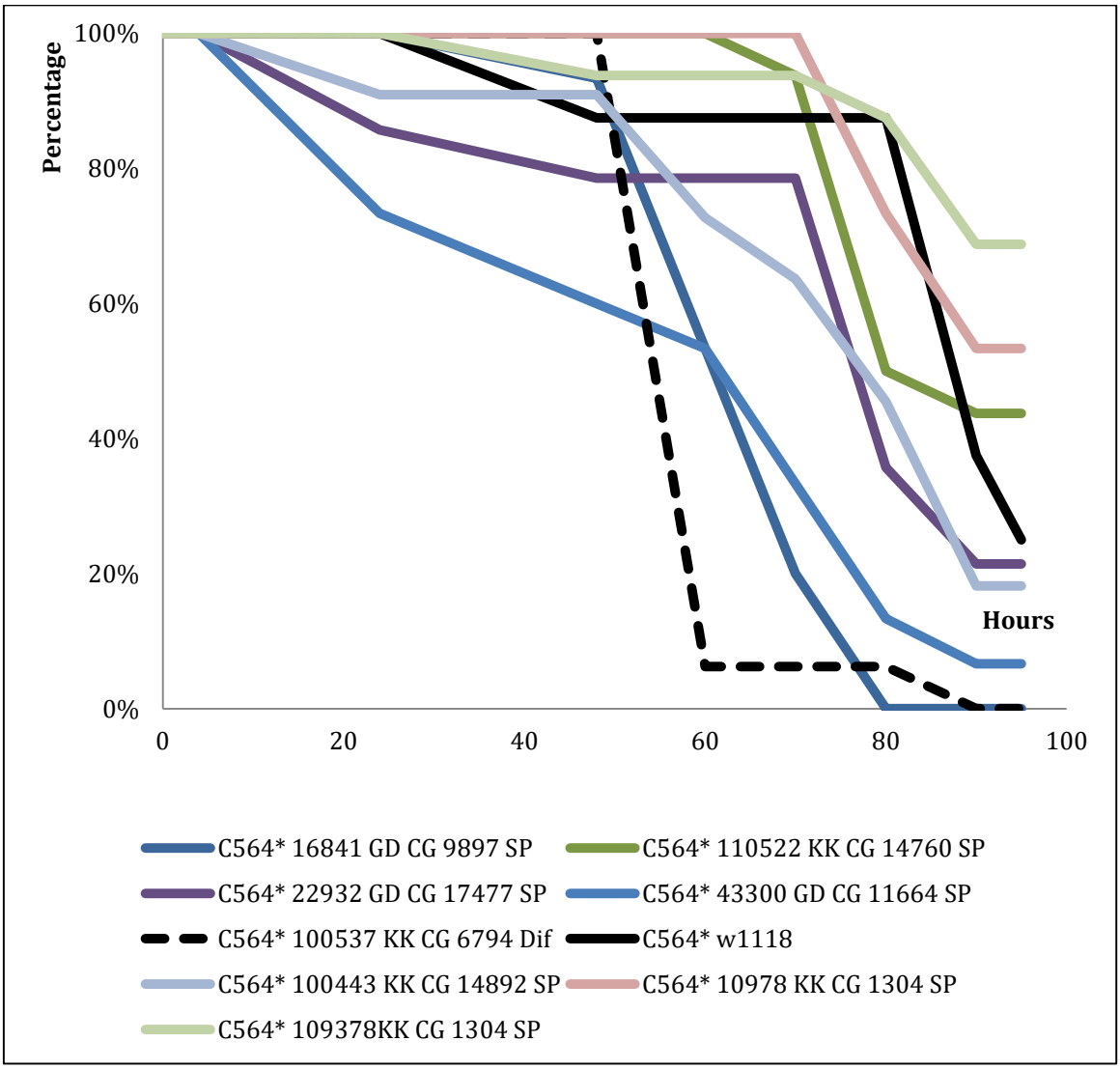


Figure 8: SP KD flies survival graph post infection with Bb

The survival graph with Bb shows CG 11664 SP KD, CG 9897 SP KD results in a compromised survival after infection with BB.

3. Survival graphs of SP KD flies after infection with Bb

Bb spores were filtrated from a plate. The spores were injected in flies with the SP KD (approximately 100 each). Survival graphs were plotted. All results were confirmed at least three times.

C. Screen II

After the initial screen the SPs that showed compromised survival after KD and infection with a microbe were chosen. In the second screen the chosen SPs were KD again and survival graphs plotted for Ef and Bb.

1. Survival graphs of SP KD flies after infection with Bb

Bb spores were injected in flies. Survivals were carried out. And survival graphs were plotted.

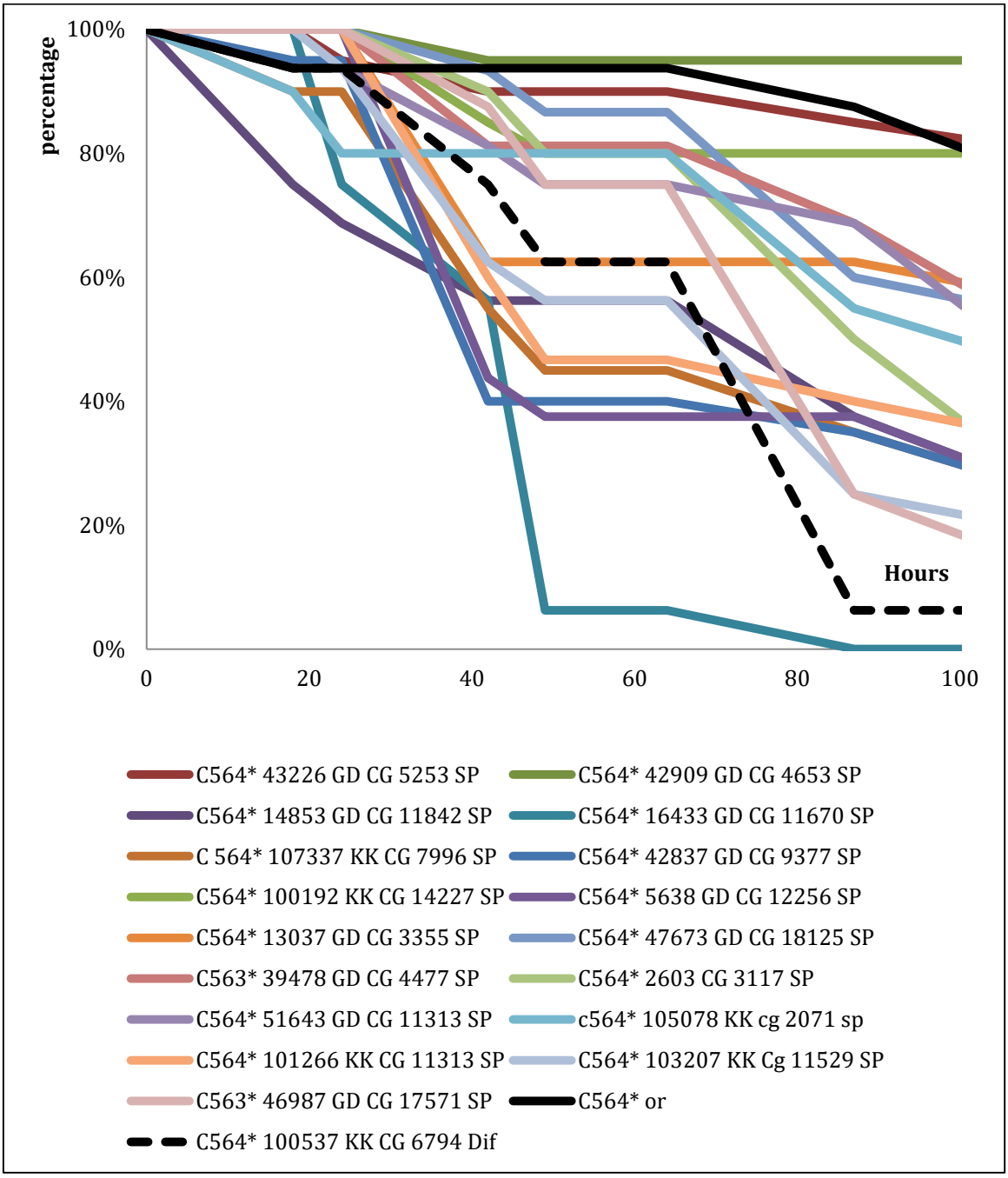


Figure 9: SP KD flies survival graph post infection with Bb

In the survival graph the vertical axis represents the percentage of survived SP KD flies post infection. The horizontal axis represents the time in hours. The graph implies that CG 11670 SP KD showed compromised survival after infection with Bb.

2. Survival graphs of SP KD flies after infection with Ef

After SP KD the flies were infected with Ef. Survival assays were carried out and survival graphs were plotted.

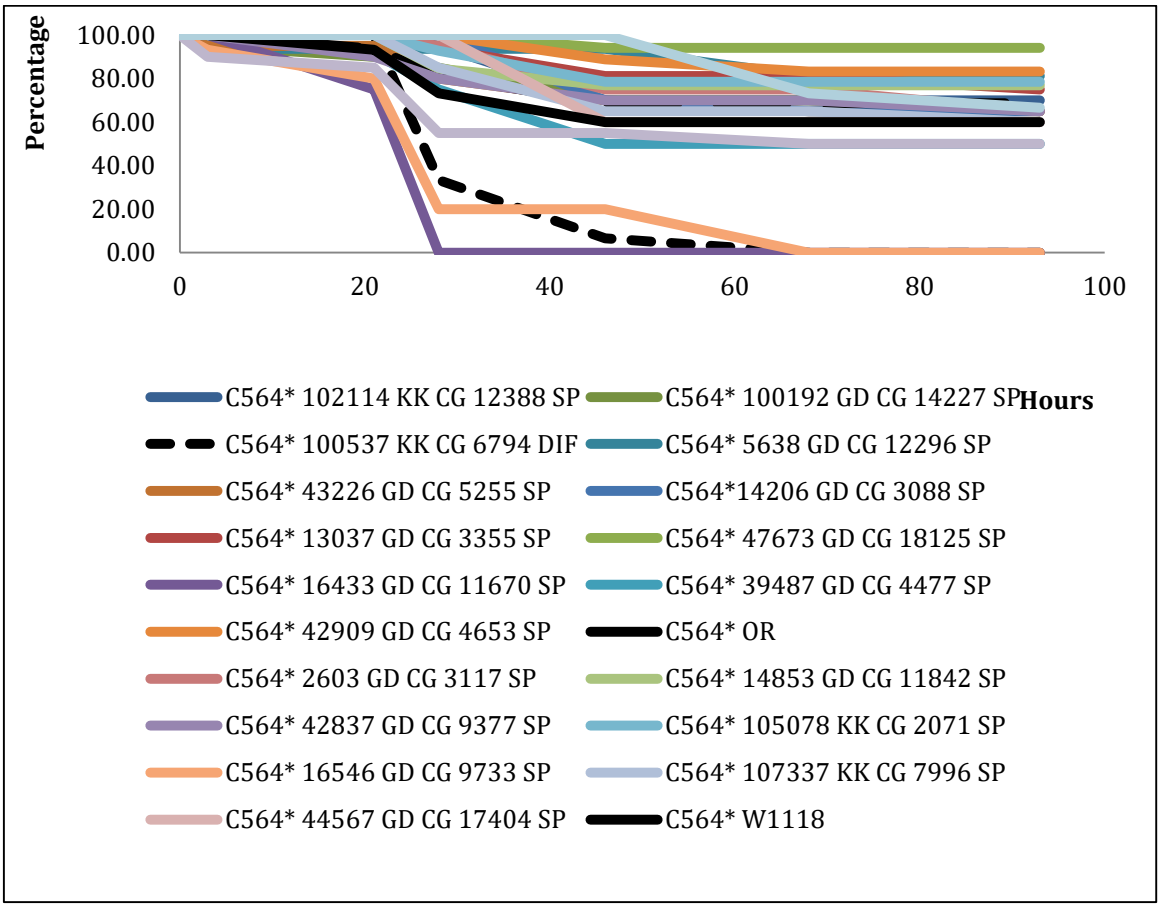


Figure 10: SP KD flies survival graph post infection with Ef

In the survival graph two SPs showed compromised survival after KD: CG 11670 SP and CG 9733 SP.

Genotype	Bb	Ef	Ecc15
CG11670 SP	+++	+++	---
CG10232 SP	+++	+++	---
CG 12256 SP	+++	+++	---
CG 9897 SP	+++	+++	---
CG 9733 SP	+++	++	---
CG 11664 SP	---	+++	---
CG 12951 SP	+++	---	---

Table 1: Summary of CG numbers of SPs that showed compromise survival after KD.

Four SPs had compromised survival with fungus and with gram-positive bacteria after KD: CG 11670 SP, CG 10232 SP CG 12256 SP and CG 9897 SP. CG 11664 SP KD flies had compromised survival after infection with gram positive bacteria but not with fungus. CG 12951 SP KD flies had compromised survival after fungal infection but not after gram-positive bacterial infection. Each result was confirmed at least in three independent survivals. None of SP KD died upon infection with the gram-negative bacteria Ecc15.

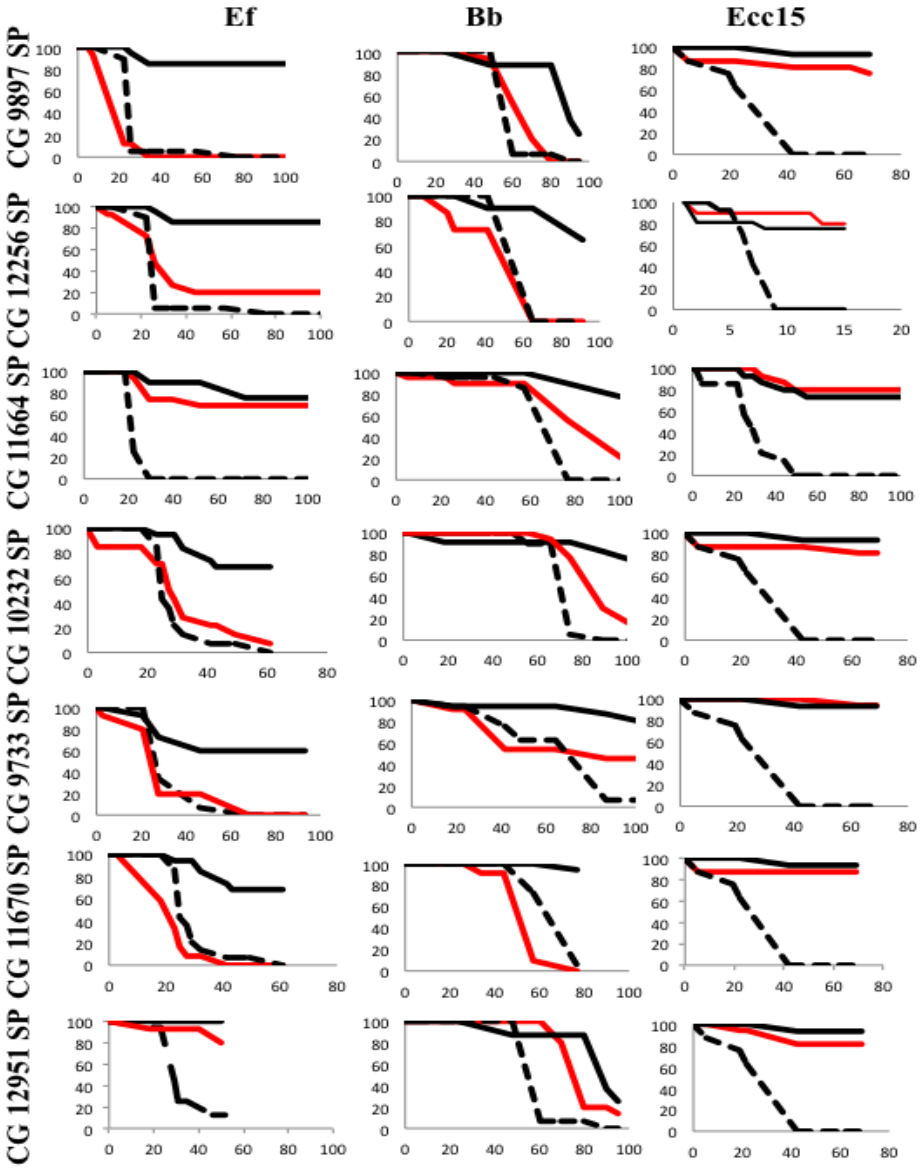


Figure 11: A selection of survival graphs of seven SP KD genes after different microbial challenge.

The first column represents the survival graphs after infection with the gram-positive bacteria Ef. The second column represents the survival graphs after infection with the entomopathogenic fungus Bb. The third column represents the

graphs after infection with the gram-negative bacteria Ecc15. The survival assays have at least three independent repeats.

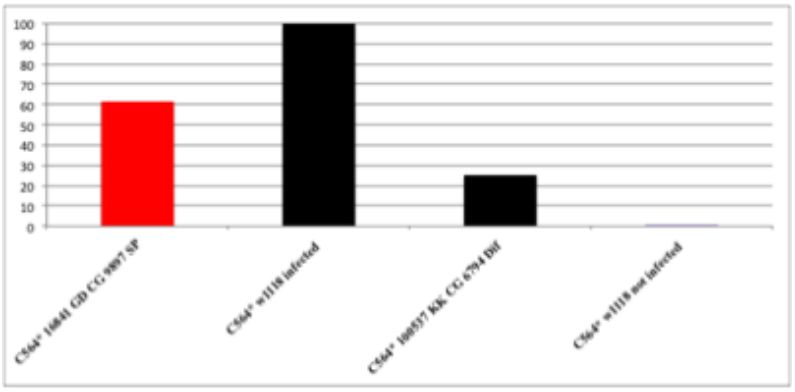
D. Antimicrobial peptide quantification (AMP)

For antimicrobial peptide quantification a batch of males was infected with either Ef or Bb. When infected with Ef males were frozen after 24 hours. When infected with Bb males were frozen after 48 hours. RNA extraction was performed followed by Real time PCR. Drosomycin is used as a read out of the activation of the toll pathway. The reference gene used for normalization was rp49.

The controls used in AMP are: infected Dif KD flies (C564* 100567 KK Cg 6794 Dif), infected wild type flies C564* W^{1118}, non-infected wild type flies C564* W^{1118}.

CG 9897 SP

Drosomycin quantification after Ef infection



Drosomycin quantification after Bb infection

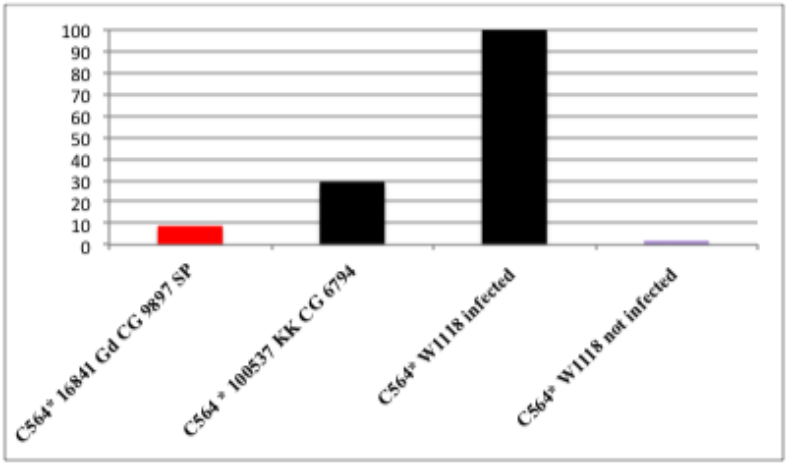


Figure 12: AMP quantification in CG 9897 SP KD flies after infection

After CG 9897 SP KD there is failure to induce Drosomycin to similar level as compared to the wild type infected flies.

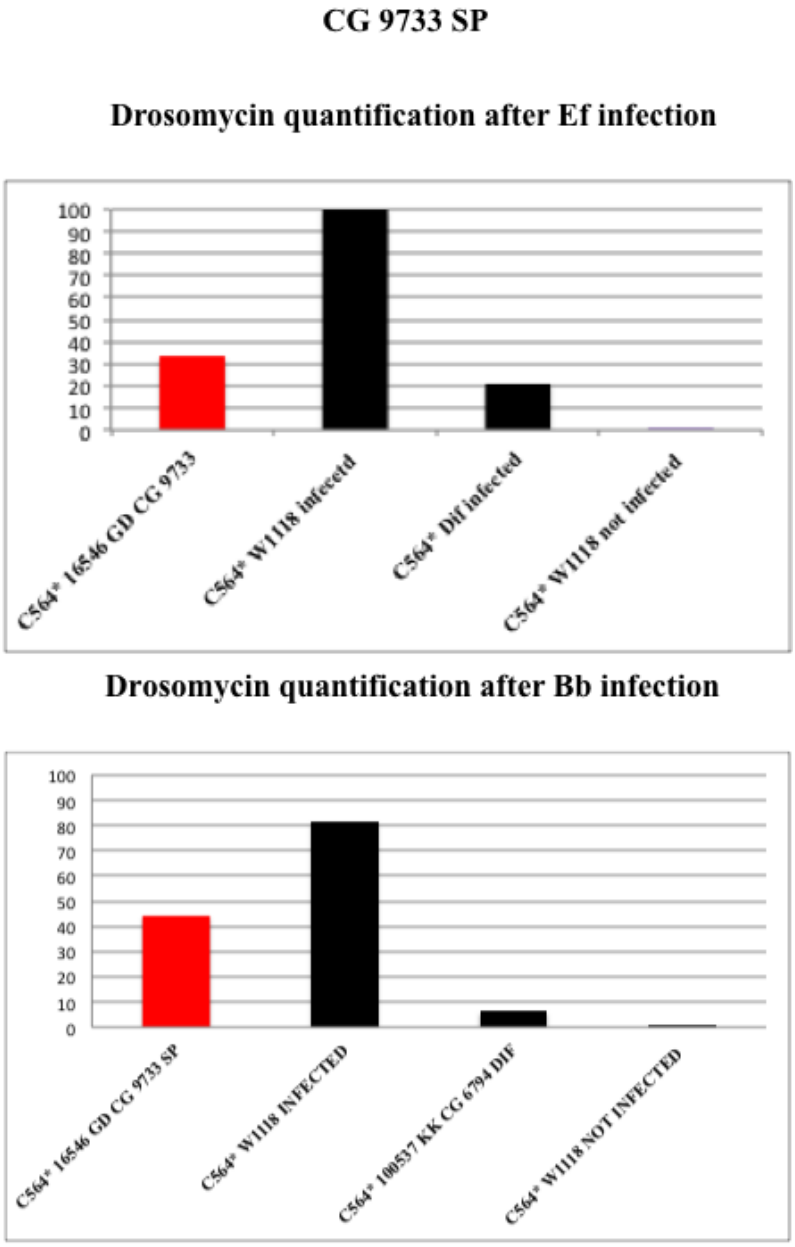
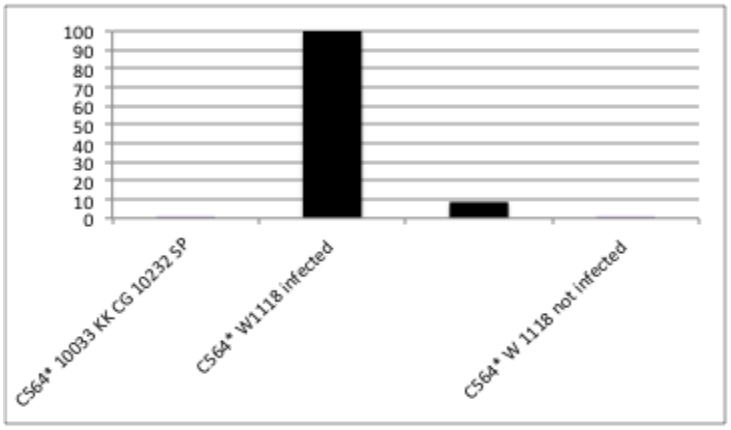


Figure 13: Drosomycin quantification in CG 9733 SP KD flies after infection

After the knockdown of CG 9733 SP there is failure to induce Drosomycin to similar level as compared to the wild type infected flies.

CG 10232 SP

Drosomycin quantification after Ef infection



Drosomycin quantification after Bb infection

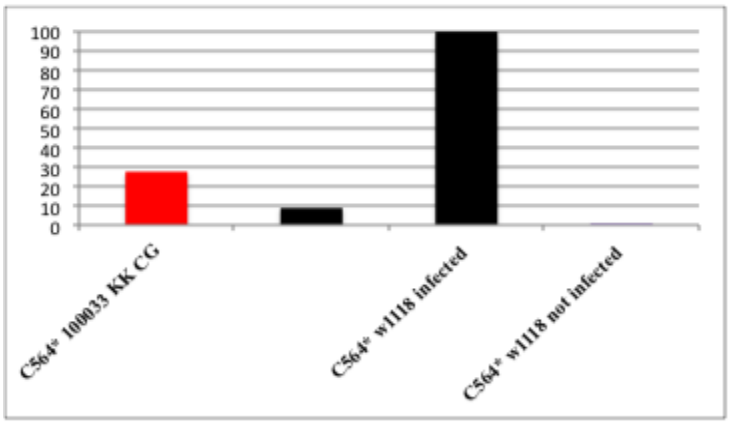


Figure 14: Drosomycin quantification in CG 10232 SP KD flies after infection.

CG 10232 SP KD showed to be positive in survival assays (figure 11) failed to induce Drosomycin to similar levels to that of the wild type flies after infection.

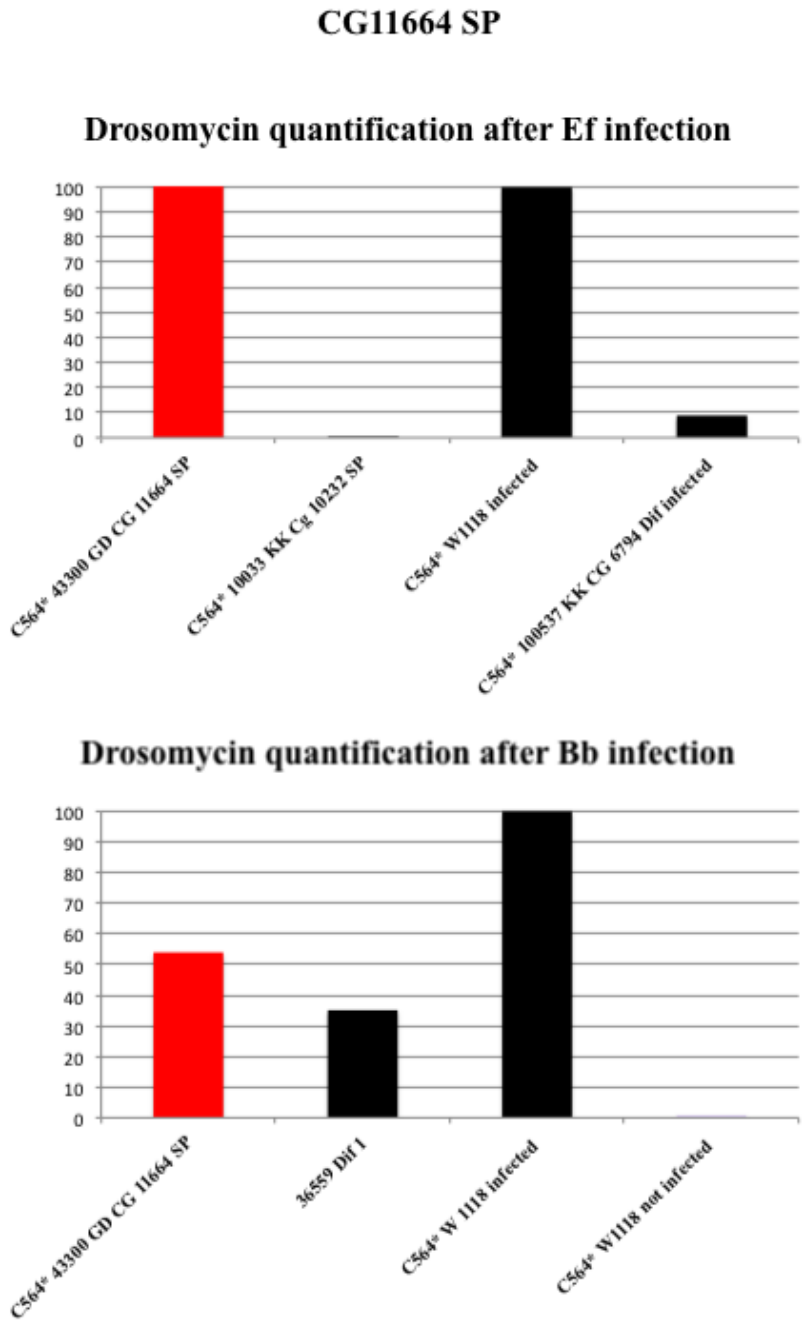


Figure 15: Drosomycin quantification in CG 11664 SP KD flies after infection.

After CG 11664 SP KD flies failed to induce drosomycin to similar levels as compared to the wild flies after infection with Bb. While the SP KD flies successfully produced Drosomycin after Ef infection.

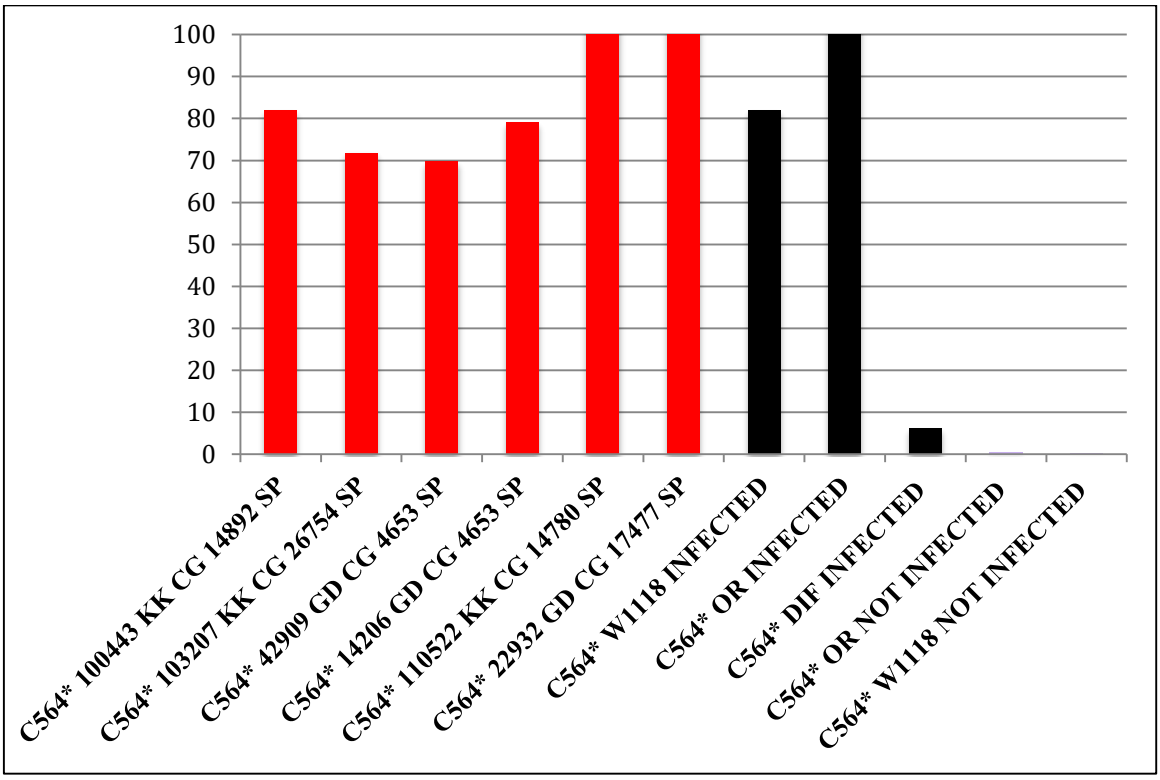


Figure 16: Drosomycin quantification in CG SP KD flies shown to be negative in survival assays after BB infection.

Some of the SP KD shown to be negative in survival assays were also tested and showed normal Drosomycin levels after the infection.

CHAPTER IV

DISCUSSION

SP are involved in a wide array of functions such as coagulation, digestion and immunity. Among 200 SPs encoded by the drosophila genome, 84 are small proteins, about 300 amino acid residues; those are suggested to be involved in digestion. There are 24 large multi-domain proteins SPs and SPHs that are mostly involved in immunity. Those contain at least one clip regulatory domain (Ross et al., 2003).

First, we used a ubiquitous driver Actin-Gal-4/cyo for gene KD. Due the fact that ubiquitously knocking down genes led to a high number of lethality amongst the SPs tested. We used a fat body restricted driver: C564-Gal-4. Since the fat body is the main site of production of antimicrobial peptides. Using this driver allowed us to assess which SPs are involved in immunity.

RNAi was the technique used to KD specific SPs. RNAi has many advantages over other KD techniques: it is fast, efficient and specific. As previously mentioned a large library of IR strains is available in VDRC center. In parallel, it is good to keep in mind the disadvantages of this technique. For instance, due to some similarities in different gene sequences off target effects (OTE) that can lead to false positive results. Sometimes, there is a KD of other genes such as the genes necessary for the transcription machinery in the cell.

However, we checked for the selected SPs with an immune phenotype in our screen and they are not leading to any off target effect (http://dscheck.rnai.jp).

False negatives may be obtained when the efficacy of the knock down is low. This may lead to missing some candidate SP. For instance in the case of control one can notice that Dif RNAi dies at a slightly slower rate than Dif real mutant.

After screening 90 SP genes (a total of 112 lines), we narrowed it down to seven serine proteases possibly involved in the extracellular cascade that activates the toll pathway.

As a validation to our results in survival experiments: CG 2056 SP KD flies had compromised survival upon infection with gram-positive bacteria Ef or the entomopathogenic fungus Bb (Figure 7). This gene was previously identified as the SPH *Spirit* and was shown to act between Grass and SPE (Kambris et al., 2006) . CG 16705 SP KD flies also had compromised survival upon infection with gram-positive bacteria or fungus. This SP was previously identified as SPE in homology with Easter (Jang et al., 2006).

CG 12256 SP KD flies had compromised survival with Ef and Bb. However, this SP KD gave contradictory results in survival assays. We did not investigate it further.

CG 11670 SP KD flies showed compromised survival after infection with Ef and BB. CG 11670 SP is also called SP27. It is a trypsin like serine protease. There is no evidence for a regulatory clip domain in the corresponding protein. Evidence suggests that it has an endopeptidase activity. Furthermore, it is up regulated after immune challenge with fungus or septic injury (De Gregorio et al., 2001). CG 10232 SP KD flies had compromised survival after infection with Bb and with Ef (Figure 11). Drosomycin quantification suggests that there is a reduction in the amp

production after immune challenge of SP KD flies (figure 15). It has two clip domains (Ross et al., 2003; Veillard et al., 2016)

CG 9733 SP KD flies showed compromised survival after infection with Ef and Bb. In previous studies showed that it is up regulated (8 folds) after septic injury only induced two folds after fungal challenge. It has a clip domain (Ross et al., 2003; Veillard et al., 2016)

CG 9897 also known as SPH195 is still an unknown gene. There are still no phenotypical traits characterized with this gene. In our screen it was proved several times by the survival assays that CG 9897 SP KD flies die after infection with Bb and with Ef (figure 11). The results are in agreement with the AMP results (figure 13).

CG 11664 SP is also known as SPH193. No phenotypic data is available. In our study, SP KD flies had compromised survival after infection with fungus but not gram-positive bacteria. Real time PCR showed reduced levels of Drosomycin after fungal infection as compared to the wild type infected controls. On the other hand, AMP levels were similar to those observed to wild type after Ef infection.

CG 12951 SP is another serine protease. It does not have a clip domain. Evidence suggests that it possesses an endopeptidase activity and may be involved in proteolytic cascades. In our study, Survival assays CG 12951 SP KD flies showed compromised survival upon infection with BB but not Ef (figure 11). AMP results suggest that in CG 9897 SP, CG 9733 SP and CG 10232 SP failed to induce Drosomycin after infection with BB and Ef. CG 11664 SP failed to induce drosomycin after fungal infection, while after Ef infection SP KD flies have shown drosomycin induction similar to wild type infected flies. However, there is still a quantity of

Drosomycin present in the hemolymph. The secretion of AMPs is mainly by the fat body of the fly. besides that, there are multiple pathways that may lead to the activation of the toll pathway. So If the knockdown of serine protease is in the same pathway of Grass and modSP the microbes may be activating a PRR- independent pathway such as: Persephone and this may lead to the partial activation of the toll pathway and the partial secretion of antimicrobial peptides. To find out if it fails to produce AMPs, null mutants must be purchased from VDRC. Mutants should be infected and Drosomycin should be quantified.

No SP KD flies showed compromised survival after infection with gram-negative bacteria Ecc15.To confirm the results with Ecc15 we can to quantify Diptericin (Read- out of the IMD pathway). To Asses if the SP KD flies were dying from injury, we injected flies with a sterile needle. The flies had no compromised survival after sterile injection (data not shown). This further confirmed after injection with a gram negative bacteria. After SP KD the flies did not develop melanotic tumors. This suggests the SP KD may be in a pathway that is different from the melanotic cascade.

Flies that have a KD gene may be weaker than wild type flies. Amongst the chosen SPs CG 9897 SP and CG 9733 SP showed lethality when ubiquitously knocked down. To make sure that the SP KD flies are dying from bacterial amplification and not from a low fitness level, bacterial colony quantification can be performed. CG 9897 and CG 11664 are respectively SPH 195 and SPH193. SPH have no proteolytic activity. They may be regulators of the toll pathway.

To date the proteolytic cascade upstream of the toll pathway is still not clear. The serine proteases shown to be involved in the extracellular cascades are Grass, persephone, SPE, modSP and Spirit. The SPH identified by Kambris et al. in 2006 are Sphinx, Spheroid1/2. Epistatical analysis suggested that Sphinx, Spheroid and Spirit are located in between Grass and SPE and modSP is above Grass. However, *in vitro* evidence suggests that modSP does not directly cleave Grass. This implies that other undetermined SPs are involved in the toll pathway activation. Moreover, Grass was first identified to be susceptible to only gram-positive bacteria after KD. Recent evidence suggests that Grass is not only gram-positive specific but it is also activated by fungal infections (El Chamy et al., 2008). Furthermore, Persephone was known for its function to detect only fungal infection. However, more recent evidence suggests that Persephone is activated by fungal proteases and by gram-positive bacteria (El Chamy et al., 2008). There is a need to investigate more the SPs and SPH involved in *Drosophila melanogaster* immunity to complete the picture of the extracellular pathway involved in the cleavage of SPZ.

To determine the hierarchy of the SPs in the pathway upstream of toll, epistatical analysis remains to be done. Crosses will be performed to obtain a strain of *Drosophila melanogaster* with a genotype UAS:Psh/Cyo; da/da. These flies have a constitutively (even in absence of infection) activated toll pathway, hence the Drosomycin level will be higher than the non-infected wild type flies. The strain will be crossed to the RNAi to see if the KD of different SPs suppresses this constitutive activation. This way we will be able to locate the position of the SP (in the same pathway below Persephone or in another pathway). Constitutive form of toll receptor

UAS- toll 10b can be used as a negative control (it activates the pathway and should not be suppressed by crosses with RNAi).

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

Until today, little is known about the extracellular cascade upstream of the toll pathway. Past studies identified several players in this extracellular pathway: Grass, SPE, modSP and Persephone. But a lot remains to be determined. This study adds information about possible candidates in the extracellular proteolytic cascade. There are approximately 200 SPs with redundant functions. A lot has not been identified. The involvement of SPs in probably more than one physiological response makes the task more difficult to deal with. Much more need to be done, the project is still ongoing. We will perform more repetitions of AMP quantification to confirm the results. We will perform an epistatical analysis so that we will be able to locate the SP relative to Persephone.

transgenic flies overexpressing SPs can be generated to see if the over expression of any of theses SPs leads to the constitutive activation of the toll pathway.

References:

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the Drosophila melanogasterimmune response by using oligonucleotide microarrays. *Proc Natl Acad Sci U S A, 98*(22), 12590-12595. doi: 10.1073/pnas.221458698

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., . . . Venter, J. C. (2000). The genome sequence of Drosophila melanogaster. *Science, 287*(5461), 2185-2195.

Aerts, A. M., Francois, I. E., Cammue, B. P., & Thevissen, K. (2008). The mode of antifungal action of plant, insect and human defensins. *Cell Mol Life Sci, 65*(13), 2069-2079. doi: 10.1007/s00018-008-8035-0

Ayres, J. S., & Schneider, D. S. (2008). A signaling protease required for melanization in Drosophila affects resistance and tolerance of infections. *PLoS Biol, 6*(12), 2764-2773. doi: 10.1371/journal.pbio.0060305

Benelli, G., Lo Iacono, A., Canale, A., & Mehlhorn, H. (2016). Mosquito vectors and the spread of cancer: an overlooked connection? *Parasitol Res, 115*(6), 2131-2137. doi: 10.1007/s00436-016-5037-y

Binggeli, O., Neyen, C., Poidevin, M., & Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in Drosophila. *PLoS Pathog, 10*(5), e1004067. doi: 10.1371/journal.ppat.1004067

Boutros, M., Kiger, A. A., Armknecht, S., Kerr, K., Hild, M., Koch, B., . . . Heidelberg Fly Array, C. (2004). Genome-wide RNAi analysis of growth and viability in Drosophila cells. *Science, 303*(5659), 832-835. doi: 10.1126/science.1091266

Buchon, N., Poidevin, M., Kwon, H. M., Guillou, A., Sottas, V., Lee, B. L., & Lemaitre, B. (2009). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. *Proc Natl Acad Sci U S A, 106*(30), 12442-12447. doi: 10.1073/pnas.0901924106

Buchon, N., Silverman, N., & Cherry, S. (2014). Immunity in Drosophila melanogaster--from microbial recognition to whole-organism physiology. *Nat Rev Immunol, 14*(12), 796-810. doi: 10.1038/nri3763

Carvalho Ade, O., & Gomes, V. M. (2011). Plant defensins and defensin-like peptides - biological activities and biotechnological applications. *Curr Pharm Des, 17*(38), 4270-4293.

Castillejo-Lopez, C., & Hacker, U. (2005). The serine protease Sp7 is expressed in blood cells and regulates the melanization reaction in Drosophila. *Biochem Biophys Res Commun, 338*(2), 1075-1082. doi: 10.1016/j.Eccrc.2005.10.042

Cerenius, L., & Soderhall, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunol Rev, 198*, 116-126.

Chasan, R., Jin, Y., & Anderson, K. V. (1992). Activation of the easter zymogen is regulated by five other genes to define dorsal-ventral polarity in the Drosophila embryo. *Development, 115*(2), 607-616.

Cherry, S., & Silverman, N. (2006). Host-pathogen interactions in drosophila: new tricks from an old friend. *Nat Immunol, 7*(9), 911-917. doi: 10.1038/ni1388

Cho, Y. S., Stevens, L. M., & Stein, D. (2010). Pipe-dependent ventral processing of Easter by Snake is the defining step in Drosophila embryo DV axis formation. *Curr Biol, 20*(12), 1133-1137. doi: 10.1016/j.cub.2010.04.056

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A., & Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A, 97*(12), 6499-6503. doi: 10.1073/pnas.110149597

De Gregorio, E., Han, S. J., Lee, W. J., Baek, M. J., Osaki, T., Kawabata, S., . . . Brey, P. T. (2002). An immune-responsive Serpin regulates the melanization cascade in Drosophila. *Dev Cell, 3*(4), 581-592.

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. *Proc Natl Acad Sci U S A, 98*(22), 12590-12595. doi: 10.1073/pnas.221458698

El Chamy, L., Leclerc, V., Caldelari, I., & Reichhart, J. M. (2008). Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll. *Nat Immunol, 9*(10), 1165-1170. doi: 10.1038/ni.1643

Ferrandon, D., Imler, J. L., Hetru, C., & Hoffmann, J. A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol, 7*(11), 862-874. doi: 10.1038/nri2194

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *nature, 391*(6669), 806-811. doi: 10.1038/35888

Gottar, M., Gobert, V., Matskevich, A. A., Reichhart, J. M., Wang, C., Butt, T. M., . . . Ferrandon, D. (2006). Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *cell, 127*(7), 1425-1437. doi: 10.1016/j.cell.2006.10.046

Hancock, R. E., & Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol, 24*(12), 1551-1557. doi: 10.1038/nbt1267

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., & Ezekowitz, R. A. (1999). Phylogenetic perspectives in innate immunity. *Science, 284*(5418), 1313-1318.

Hong, C. C., & Hashimoto, C. (1995). An unusual mosaic protein with a protease domain, encoded by the nudel gene, is involved in defining embryonic dorsoventral polarity in Drosophila. *cell, 82*(5), 785-794.

Honti, V., Csordas, G., Kurucz, E., Markus, R., & Ando, I. (2014). The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomy and regulation. *Dev Comp Immunol, 42*(1), 47-56. doi: 10.1016/j.dci.2013.06.005

Jang, I. H., Chosa, N., Kim, S. H., Nam, H. J., Lemaitre, B., Ochiai, M., . . . Lee, W. J. (2006). A Spatzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. *Dev Cell, 10*(1), 45-55. doi: 10.1016/j.devcel.2005.11.013

Kambris, Z., Brun, S., Jang, I. H., Nam, H. J., Romeo, Y., Takahashi, K., . . . Lemaitre, B. (2006). Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. *Curr Biol, 16*(8), 808-813. doi: 10.1016/j.cub.2006.03.020

Katoch, R., & Thakur, N. (2013). Advances in RNA interference technology and its impact on nutritional improvement, disease and insect control in plants. *Appl Biochem Biotechnol, 169*(5), 1579-1605. doi: 10.1007/s12010-012-0046-5

Kellenberger, C., Leone, P., Coquet, L., Jouenne, T., Reichhart, J. M., & Roussel, A. (2011). Structure-function analysis of grass clip serine protease involved in Drosophila Toll pathway activation. *J Biol Chem, 286*(14), 12300-12307. doi: 10.1074/jbc.M110.182741

Kocks, C., Cho, J. H., Nehme, N., Ulvila, J., Pearson, A. M., Meister, M., . . . Ezekowitz, R. A. (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. *cell, 123*(2), 335-346. doi: 10.1016/j.cell.2005.08.034

Lam, G., & Thummel, C. S. (2000). Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. *Curr Biol, 10*(16), 957-963.

Lehrer, R. I., & Ganz, T. (1999). Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol, 11*(1), 23-27.

Li, Y., Lu, J., Han, Y., Fan, X., & Ding, S. W. (2013). RNA interference functions as an antiviral immunity mechanism in mammals. *Science, 342*(6155), 231-234. doi: 10.1126/science.1241911

Ligoxygakis, P., Pelte, N., Hoffmann, J. A., & Reichhart, J. M. (2002). Activation of Drosophila Toll during fungal infection by a blood serine protease. *Science, 297*(5578), 114-116. doi: 10.1126/science.1072391

Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., Belvin, M., . . . Reichhart, J. M. (2002). A serpin mutant links Toll activation to melanization in the host defence of Drosophila. *EMBO J, 21*(23), 6330-6337.

Martinek, S., & Young, M. W. (2000). Specific genetic interference with behavioral rhythms in Drosophila by expression of inverted repeats. *Genetics, 156*(4), 1717-1725.

Mohr, S. E. (2014). RNAi screening in Drosophila cells and in vivo. *Methods, 68*(1), 82-88. doi: 10.1016/j.ymeth.2014.02.018

Nam, H. J., Jang, I. H., Asano, T., & Lee, W. J. (2008). Involvement of pro-phenoloxidase 3 in lamellocyte-mediated spontaneous melanization in Drosophila. *Mol Cells, 26*(6), 606-610.

Nam, H. J., Jang, I. H., You, H., Lee, K. A., & Lee, W. J. (2012). Genetic evidence of a redox-dependent systemic wound response via Hayan protease-phenoloxidase system in Drosophila. *EMBO J, 31*(5), 1253-1265. doi: 10.1038/emboj.2011.476

Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., . . . Ogawa, H. (2007). Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory

cytokines and chemokines. *J Invest Dermatol, 127*(3), 594-604. doi: 10.1038/sj.jid.5700599
Ross, J., Jiang, H., Kanost, M. R., & Wang, Y. (2003). Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships. *Gene, 304*, 117-131.
Saurabh, S., Vidyarthi, A. S., & Prasad, D. (2014). RNA interference: concept to reality in crop improvement. *Planta, 239*(3), 543-564. doi: 10.1007/s00425-013-2019-5
Stuart, L. M., & Ezekowitz, R. A. (2005). Phagocytosis: elegant complexity. *immunity, 22*(5), 539-550. doi: 10.1016/j.immuni.2005.05.002
Svobodova, E., Kubikova, J., & Svoboda, P. (2016). Production of small RNAs by mammalian Dicer. *Pflugers Arch, 468*(6), 1089-1102. doi: 10.1007/s00424-016-1817-6
Tabara, H., Grishok, A., & Mello, C. C. (1998). RNAi in C. elegans: soaking in the genome sequence. *Science, 282*(5388), 430-431.
Tang, H. (2009). Regulation and function of the melanization reaction in Drosophila. *Fly (Austin), 3*(1), 105-111.
Tang, H., Kambris, Z., Lemaitre, B., & Hashimoto, C. (2006). Two proteases defining a melanization cascade in the immune system of Drosophila. *J Biol Chem, 281*(38), 28097-28104. doi: 10.1074/jbc.M601642200
Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A., & Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat Genet, 24*(2), 180-183. doi: 10.1038/72850
Timmons, L., Tabara, H., Mello, C. C., & Fire, A. Z. (2003). Inducible systemic RNA silencing in Caenorhabditis elegans. *Mol Biol Cell, 14*(7), 2972-2983. doi: 10.1091/mbc.E03-01-0858
Vasselon, T., Bouttier, M., Saumet, A., & Lecellier, C. H. (2013). RNAi and retroviruses: are they in RISC? *Biomol Concepts, 4*(1), 43-52. doi: 10.1515/bmc-2012-0041
Veillard, F., Troxler, L., & Reichhart, J. M. (2016). Drosophila melanogaster clip-domain serine proteases: Structure, function and regulation. *Biochimie, 122*, 255-269. doi: 10.1016/j.biochi.2015.10.007
Watson, F. L., Puttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., . . . Schmucker, D. (2005). Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science, 309*(5742), 1874-1878. doi: 10.1126/science.1116887
Wilkins, C., Dishongh, R., Moore, S. C., Whitt, M. A., Chow, M., & Machaca, K. (2005). RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. *nature, 436*(7053), 1044-1047. doi: 10.1038/nature03957
Wu, L. P., & Anderson, K. V. (1998). Regulated nuclear import of Rel proteins in the Drosophila immune response. *nature, 392*(6671), 93-97. doi: 10.1038/32195
Yamamoto-Hino, M., & Goto, S. (2013). In Vivo RNAi-Based Screens: Studies in Model Organisms. *Genes (Basel), 4*(4), 646-665. doi: 10.3390/genes4040646
Zhao, P., Lu, Z., Strand, M. R., & Jiang, H. (2011). Antiviral, anti-parasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound

generated by phenoloxidase during insect immune response. *Insect Biochem Mol Biol, 41*(9), 645-652. doi: 10.1016/j.ibmb.2011.04.006