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INVESTIGATION OF THE ROLE OF CANDIDATE GENES IN *Drosophila melanogaster* IMMUNITY BY AN *IN VIVO* RNAi SCREEN

by
RAWAN MONZER MERHI

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AN ABSTRACT OF THE THESIS OF

Rawan Monzer Merhi for Master of Science
Major: Biology

Title: Investigation of the role of candidate genes in Drosophila melanogaster Immunity by an in vivo RNAi screen

Drosophila is an excellent model organism to genetically dissect innate immune pathways without interference from the adaptive responses since insects lack adaptive immunity. The abundant genetic tools available for this model are also a major advantage, and the recent expansion of RNAi techniques and the availability of transgenic flies that express hairpin constructs via the UAS/Gal4 system allow for the knockdown of any gene in a spatially and temporally controlled manner. The humoral response is an important component of fly immune defenses; it includes melanization and the synthesis by the fat body of antimicrobial peptides whose expression is controlled by two main pathways: Toll and Imd. Previous studies have identified a number of genes that are upregulated at the transcriptional level after immune challenge. Several of these genes are of unknown function and do not have clear characterized orthologues in other species. On the other hand, among the induced genes that have a putative attributed function, the Serpin family is well represented. Some serpins have been shown to act as serine-protease inhibitors upstream of the Toll pathway and others as negative regulators of melanization cascades. Using an *in vivo* RNAi screen, this proposal aims at investigating the potential involvement of some of the serpins that have not been previously analyzed and of the unknown candidate genes in *Drosophila melanogaster* defenses.

CONTENTS

ILLUSTRATIONS

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

A. *Drosophila* as a Model Organism

Drosophila melanogaster is one of the most studied model organisms for over a century since most biological pathways are conserved in this model. Moreover, 75% of the genes associated with diseases in humans are conserved in *Drosophila* (Pandey et al., 2011). It is also an easy tool to build and combine transgenic lines with limited ethical consideration as compared to mouse. In addition, *Drosophila* is associated with a wide toolbox ranging from gene reporters, Bloomington deficiency kits, transgenic RNAi lines and conditional gain-of-function (GOF) stocks. Interestingly, mammals and *Drosophila* share high molecular and signaling similarities in triggering an innate immune response against microbial pathogens (De Gregorio et al., 2002).

B. Overview on the Immune System

In general, the immune system is divided into two categories: innate and adaptive system. The innate immune system is triggered to induce an immediate immune response against the antigen in a non-specific manner. However, the adaptive immune system is more complex than the innate system. It works in an antigen-specific manner and induces an adaptive immune response which takes few days to get into function. Interestingly, invertebrates like the fruit fly, lack adaptive immunity and rely on the

innate system as a sole system to defend them against pathogens. Both the adaptive and innate systems have cellular and humoral components. The cellular components in *Drosophila* immunity include the immune surveillance cells “hemocytes” which circulate the hemolymph (equivalent to blood in mammals). Hemocytes are divided into three types: Crystal cells which mediate melanization, lamellocytes which mediate pathogen encapsulation, and plasmatocytes which are involved in phagocytosis and production of antimicrobial peptides (AMPs) (De Gregorio et al., 2002). The humoral component is represented by the production of AMPs by the main immune tissue in *Drosophila*, the fat body (equivalent to liver in mammals). AMPs expression is controlled by the nuclear transcription factor Dif and Relish which are downstream targets to the Toll and immune deficiency (Imd) cascades respectively (Valanne et al., 2011).

C. *Drosophila* Toll vs Human Toll

The Toll receptor was first characterized as having a role in the embryonic dorso-ventral patterning. During this process, Spaetzle (Spz) precursor is cleaved through a proteolytic cascade consisting of Gastrulation Defective, Snake and Easter (Fullaondo et al., 2011). In later studies, Toll was then found to be involved in immune response. In general, the Toll pathway is activated by Gram-positive bacteria and fungi while the Imd pathway is activated by Gram-negative bacteria. These pathways are highly conserved in evolution when compared to mammalian Toll-like receptor (TLR) and Tumor necrosis factor receptor (TNF-R) signaling cascades (Fullaondo et al., 2011).

Similarities between human and *Drosophila* signaling cascades are illustrated in figure 1. As a general overview, human TLR (hToll) and *Drosophila* Toll membrane-bound receptors are stimulated by the binding of an extracellular ligand. The receptors extracellular domains consist of multiple copies of Leucine-rich repeats (LRRs). These are composed of 24 amino-acid motifs involved in protein-protein interactions (Dushay& Eldon, 1998). The LRRs domains are flanked by cysteine-rich regions that lead to the receptor constitutive signaling when mutated. Toll intracellular domain shares high similarity to the interleukin-1 receptor (IL-1RI) which holds interleukin-1 (IL-1) as ligand, a potent activator of NF-κB. Stimulation of the receptors lead to the activation of a protein phosphorylation cascade mediated by interleukin-1 receptor-associated kinase in humans and by Pelle in the case of flies. As a result of the phosphorylation cascade, NF-κB inhibitor (I-κB) in humans and Cactus in *Drosophila* are phosphorylated and degraded. This degradation leads to the translocation of a transcription-factor dimer (NF-κB in humans and Dorsal-related immunity factor (Dif), Dorsal or Rel in flies) from the cytoplasm to the nucleus where the expression of downstream genes is initiated (Dushay & Eldon, 1998) (Figure 1).

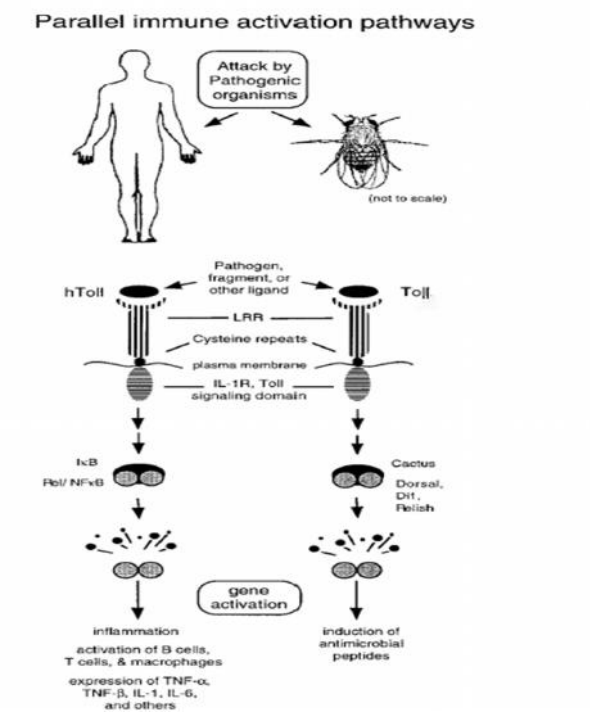

Figure 1: Comparing similarities in signaling between *Homo* and *Drosophila* (Dushay & Eldon, 1998). Attack by pathogenic organisms lead to the activation of hToll and *Drosophila* Toll leading to degradation of I-κB in humans and Cactus in *Drosophila*. Then human Rel/NF-κB and *Drosophila* Dorsal, Dif, or Relish will lead to the activation of several immune responses and to the induction of antimicrobial peptides in humans and *Drosophila* respectively.

D. Detailed Overview of *Drosophila* Toll pathway

As a more detailed overview of the *Drosophila* signaling cascade, Gram-positive bacteria infecting the fly is recognized by the extracellular recognition proteins which are the peptidoglycan recognition protein (PGRP)-SA, PGRP-SD and the Gram-negative bacteria binding protein 1 (GNBP1) (Valanne et al., 2011). After recognition, the Toll pathway is activated in the fat body by a serine protease cascade that processes the Toll receptor soluble ligand cytokine-like protein Spaetzle (Spz) in the hemolymph which in turn binds to its receptor Toll, activates an intracellular signaling cascade, and promotes the degradation of the Iκ-B-like protein Cactus (Valanne et al., 2011). This allows the nuclear translocation of NF-κB-like transcription factors Dif and Dorsal and

the subsequent expression of AMP-encoding genes such as *Drosomycin* (*Drs*) in the case of Gram-positive bacteria or fungi, and *Diptericin* (*Dpt*) in the case of Gram-negative bacteria. These AMPs are secreted in hemolymph to directly kill invading microbes (Valanne et al., 2011). Upon fungal infection, a serine protease called Persephone (psh) is proteolytically matured by the secreted fungal virulence factor PR1 and activates the Toll cascade (Ligoxygakis et al., 2002). Psh activation is negatively regulated by the serine protease inhibitor (serpin) Nec (Green et al., 2000). A schematic summary for the activation of the toll pathway following pathogen recognition and during embryonic patterning is illustrated in figure 2.

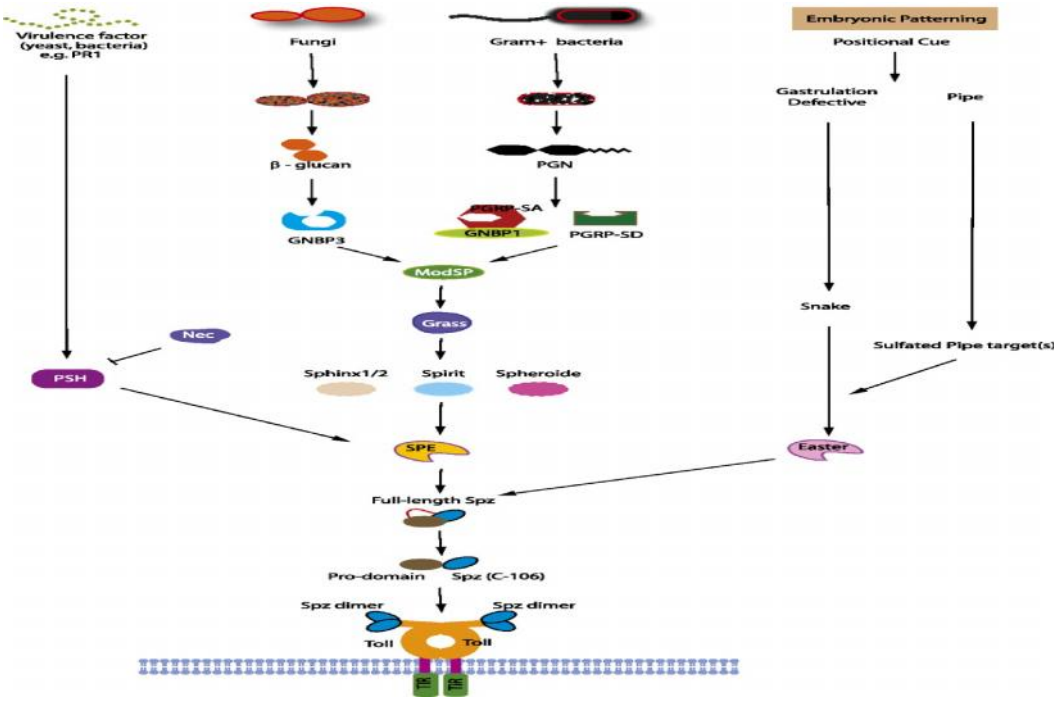

Figure 2: Toll pathway activation following fungal or Gram-positive bacterial infection and during embryonic patterning (Valanne et al., 2011). Extracellular cleavage of Spaetzle (Spz) leads to Toll pathway activation. In early embryogenesis, the protease cascade Gastrulation Defective-Snake activates the Easter protease, which cleaves Spz. In the immune response, three protease cascades lead to the activation of SPE to cleave Spz; Live Gram-positive bacteria and fungi activate the Persephone (PSH) cascade which senses virulence factors and the other two cascades are activated by pattern recognition receptors that bind cell wall components from Gram-positive bacteria and fungi, respectively. All cascades converge at ModSP-Grass to activate SPE downstream. Spz binding to the Toll receptor initiates intracellular signaling.

E. *Drosophila* Imd pathway

As stated previously, the Imd pathway is activated after Gram-negative bacterial infection. Bacteria is first recognized through the transmembrane recognition receptor

PGRP-LC assisted by the receptor PGRP-LE (Myllymäki et al., 2014). Following recognition, a signaling complex is recruited and consists of Imd, a death domain protein, the adaptor protein dFadd and the caspase-8 homolog Dredd. Dredd, activated following ubiquitination, cleaves Imd which is in turn ubiquitinated. This results in the recruitment and activation of the Tab2/Tak1 complex which then phosphorylates the IκB kinase (IKK) complex. This complex phosphorylates Rel and leads to its activation resulting in its translocation to the nucleus to induce the activation of AMPs encoding genes such as *Diptericin*. Interestingly, the Imd pathway shares high signaling similarities with the mammalian TNF signaling cascades (Myllymäki et al., 2014).

F. Induced Genes in Microarray Analysis

In previous studies, a DNA microarray was performed on 400 genes in *Drosophila* infected with Gram-positive or Gram-negative bacteria or fungi. Among these genes, 230 were found to be induced after microbial challenge (De Gregorio et al., 2001; Irving et al., 2001). Some were established immune genes such as the two serpins *necrotic* and *TEP2* and others were referred to genes of unknown functions that may have a role in immunity, and the rest were of characterized families with no clear homologs in other species. Of these, 8 serpins were found to have an altered immune response after microbial challenge such as CG7219 and CG16713 (De Gregorio et al., 2001; Irving et al., 2001).

G. Overview on the Serpin Family

Serpins are present in viruses and all living organisms but they are not well categorized in bacteria and fungi. More than 800 serpin genes have been identified to date in these organisms (Irving et al., 2002). The majority acts as serine protease inhibitors of about 400 amino acids in length containing a core of three beta-sheets (A, B and C) connected to alpha-helical linkers (7-9 α-helices). In addition, a reactive center loop (RCL) of about 20 amino acids extends out from the carboxyl terminal of the serpin in its native state in order to act as bait for the protease (suicide substrate) (Figure 3).

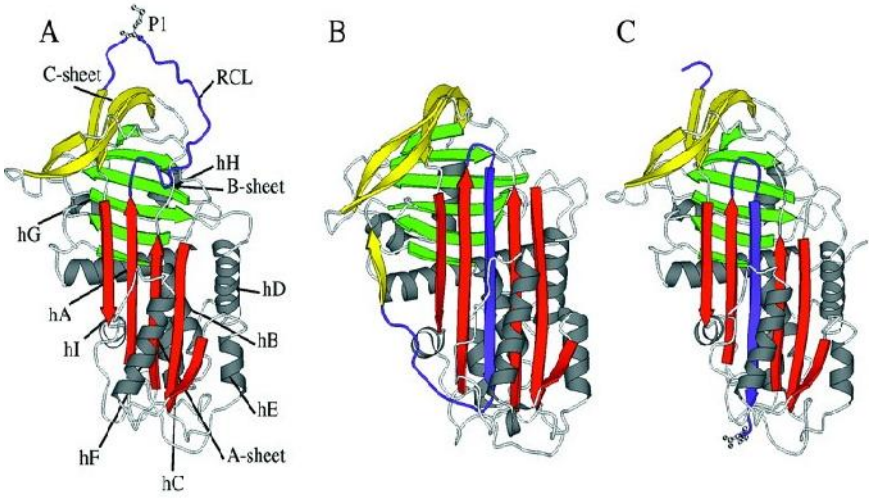

Figure 3: 3-Dimentional structures of an inhibitory serpin in its native (A), latent (B) and cleaved (C) forms (Silverman et al., 2001). In all structures the A-sheet is in red, the B-sheet is *in* green, the C-sheet is in yellow, and the RCL is in purple. The helices are in gray and are labelled on the structure of native serpin (A).

The protease will first form a non-covalent acyl bond with the serpin through interactions with the P1 and P1' residues. The active site serine on the P1-P1' bond will then be attacked by the protease forming a covalent acyl bond between Ser-195 of the protease and the backbone carbonyl of the P1 residue. At this stage, the RCL will insert with the linked protease into β-sheet A and irreversibly inhibits the protease activity by targeting the serpin/protease complex for proteolytic destruction (Reichhart et al., 2011) (Figure 4).

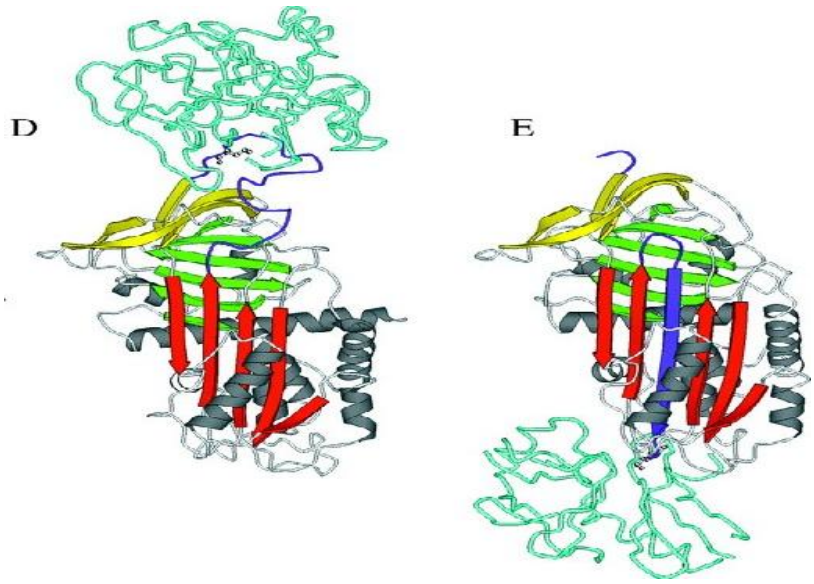

Figure 4: Serpins inhibitory mechanism involving a change from S to R conformation (Silverman et al., 2001). The serpin has first its reactive center loop exposed when binding a protease. The protease cleaves the loop which inserts into the β-sheet A. This conformational deformation inhibits the protease activity. In both structures the A-sheet is in red, the B-sheet is in green, the C-sheet is in yellow, and the RCL is in purple. The protease is shown as a cyan coil and the helices are in gray.

On the other hand, non-inhibitory serpins can perform several roles such as hormone transport (corticosteroid-binding globulin or thyroid-binding globulin), blood pressure regulation (angiotensinogen), and storage protein (ovalbumin) (Silverman et al., 2001).

The conformational flexibility of serpins make them susceptible to mutations leading to misfolding, disruption by heat, spontaneous conformational change and formation of inactive serpin polymers leading to human diseases such as cirrhosis (through accumulation of serpins that contain inclusion bodies), emphysema (through depletion of serpin), dementia (aggregation of neuroserpin in neurons) and blood coagulation disorders (Law et al., 2006). Many human serpins are well documented today, in particular those playing a role in fibrinolytic and clotting cascades (Silverman et al., 2001).

H. Overview on *Drosophila* Serpins

In *Drosophila*, 29 serpin genes are identified. Compared to the 36 serpin genes in humans, this is a high number but it is most likely correlated to the high number of 211 serine proteases in *Drosophila* (Ross et al., 2003; Law et al. 2006). Among the 17 inhibitory hinge region encoding genes, Spn42Da encodes 8 different protein isoforms that have 4 different reactive center loops (RCL) regions. This adds to a total of 24 serpins with inhibitory functions (Reichhart et al., 2011). Previous studies have identified five serpins (Spn27A, Spn28Dc, Spn43Ac, Spn77Ba and Spn88Ea) that are involved in *Drosophila* immunity (Reichhart et al., 2011).

For instance, Spn43Ac (476 a.a, *necrotic*, *nec*, CG1857) and Spn27A (447 a.a) are thoroughly studied among these serpins. It has been shown that Nec inhibits a serine protease that acts upstream of Spz in the Toll pathway after fungal infection. It has also been shown that Nec mutations cause necrosis in cells and suppress melanotic tumors (Green et al., 2000; Ligoxygakis et al., 2002). Adult flies with Nec mutation develop necrotic black spots on their leg joints and/or on their body and they die briefly after hatching. Furthermore, the gene encoding Spn43Ac is found in a cluster of four serpin genes 43A (Spn43Aa, Spn43Ab, Spn43Ac and Spn43Ad). Deletion of Spn43Ac transcript results in a lethal phenotype (Green et al., 2000). On the other hand, serpin Spn27A was found to be implicated in melanization regulation in *Drosophila*. It mainly works by inhibiting the terminal protease prophenoloxidase-activating enzyme (PPAE) (De Gregorio et al.2002; Ligoxygakis et al., 2002).

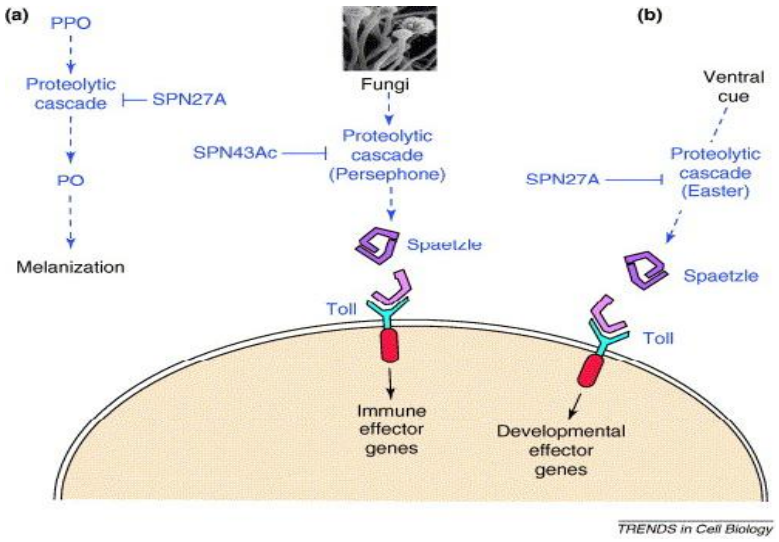

Figure 5: Controlled proteolytic cascades by SPN27Aand SPN43Ac in immunity (a) and development (b) (Reichhart, 2005). The proteolytic cascade is negatively controlled by SPN27A and by SPN43Ac in melanization and in Toll activation following fungal infection respectively (a). SPN27A works also as a negative regulator of the proteolytic cascade involved in development (b).

I. Melanization in *Drosophila*: Mechanism, Regulation and Function

Melanization is an immediate immune reaction upon infection or injury in arthropods. It is characterized by the production of melanin involved in encapsulation processes and is visible as dark spots at the sites of wound or infection (Tang, 2009). Similar to the Toll pathway, melanization involves serine proteases cascade (inhibited by serpins) that activates the phenoloxidase (PO), a crucial enzyme that promotes the production of melanin by catalyzing the oxidation reaction of phenols to quinones. This reaction is followed by polymerization of quinones to form melanin. PO is present in the hemolymph and cuticle of insects in its inactive form prophenoloxidase (PPO) which is in turn cleaved into PO by the action of prophenoloxidase-activating enzyme (PPAE) (Tang, 2009). It has been shown that serpins such as Spn28D (De Gregorio et al., 2002) and Spn77Ba (Tang et al., 2008) regulate the PO cascade in *Drosophila* at the level of wounds and trachea respectively. A detailed illustration of the melanization cascade is shown in figure 6.

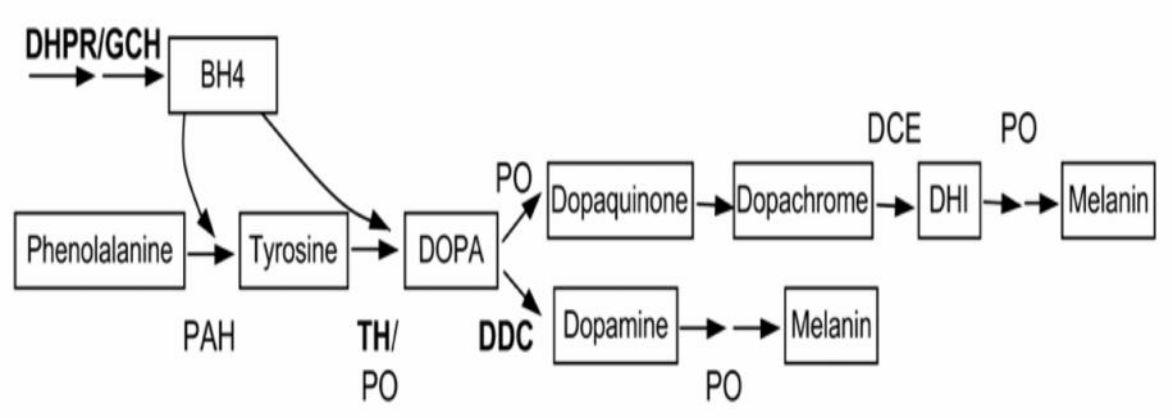

Figure 6: Schematic representation of melanin biosynthesis in *Drosophila* (Tang, 2009). Phenylalanine is hydroxylated to tyrosine by phenylalanine hydroxylase (PAH) in the presence of tetrahydrobiopterin (BH4) which is synthesized by enzymes including dihydropteridine reductase (DHPR) and GTP cyclohydrolase (GCH). Then, Tyrosine is converted to DOPA by phenol oxidase (PO) or tyrosine hydroxylase (TH). DOPA is in turn converted to dopaquinone by PO or to dopamine by dopa decarboxylase (DDC). Non-enzymatically, Dopaquinone can convert to dopachrome, which leads to 5,6-dihydroxyindole (DHI) by dopachrome conversion enzyme (DCE) that decarboxylases DHI. Dopamine and DHI are eventually converted to melanin following some reactions that involve PO.

A previous study has identified two crucial proteases named melanization protease MP1 and MP2 which are involved in the melanization cascade activated after microbial infection in *Drosophila* (Tang et al., 2006). This study suggests that MP2 is involved in a melanization cascade activated by fungal infection and that another protease is activated after bacterial infection. This protease in addition to MP2 converge on MP1 that commonly activates the phenoloxidase (Figure 7).

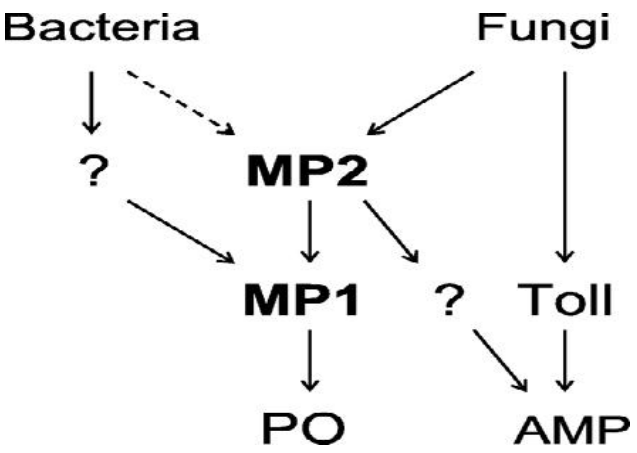

Figure 7: MP1 and MP2 proteases depiction in several immune pathways (Tang et al., 2006). In the melanization cascade, MP1 and MP2 activate PO, which is the crucial enzyme in melanin synthesis. This cascade is triggered primarily by fungi and to a lesser extent by bacteria. Another melanization cascade that involves an unidentified protease and MP1 may be more specifically induced during bacterial infection. An unknown mechanism involving MP2 may also lead to the induction of antimicrobial peptide expression by a cross-talk with the Toll pathway. Arrows represent genetic relationships and not necessarily direct biochemical interactions.

In addition to melanin, the process of melanization leads to the production of reactive oxygen species (ROS) in order to optimize microbe clearance (De Gregorio et al.2002; Ligoxygakis et al., 2002). Intriguingly, vertebrates do not possess a PO system. However, they possess a similar cascade represented by blood clotting reactions and complement activation (De Gregorio et al.2002). The common features between these reactions in vertebrates and invertebrates are the serine protease cascades that must be regulated by serpins in order to avoid any systemic response which could be fatal (De Gregorio et al.2002).

J. UAS/Gal4 System: a Targeted Gene Expression Strategy

Many techniques have been employed to study gene function in model organisms. In the last decade, genetic toolbox has enormously expanded in model organisms, in particular in *Drosophila* (Duffy, 2002). In 1993, scientists have developed the UAS/Gal4 system which is a biochemical method that was proven to be the most effective strategy for *in vivo* targeted gene expression in a temporal and spatial fashion in *Drosophila* (Brand & Perrimon, 1993). Gal4 encoding a protein of 881 amino acids is first identified in the yeast *Saccharomyces cerevisiae* in which it controls the transcription and expression of genes (Gal10 and Gal1) induced by galactose. This regulation is achieved by Gal4 protein binding to an Upstream Activating Sequences (UAS) of four 17 basepair (bp) sites located between Gal10 and Gal1 loci (Duffy, 2002). UAS is similar to an enhancer element in eukaryotes and is crucial for the transcriptional activation of the previously stated Gal4-regulated genes. Geneticists have designed many varieties of Gal4 lines in *Drosophila* each expressing Gal4 in subsets of their tissues. For instance, some lines may express Gal4 in neurons or in antennae only and so on. Importantly, Gal4 expression in *Drosophila* does not show any phenotypic deleterious effects (Duffy, 2002). The UAS/Gal4 system is illustrated in figure 8. It consists of a responder parental line in which the target gene is located downstream of the UAS element and of a driver parental line expressing the driver. The responder line remains in a transcriptionally inactive state because the presence of Gal4 is essential for the transcription of the target gene. In order to activate transcription, the responder line is mated to the parental driver line which consists of flies that express the Gal4 driver in

a specific pattern. Thus in the progeny, Gal4 proteins will directly bind to UAS which will drive the expression of the target gene (Brand & Perrimon, 1993).

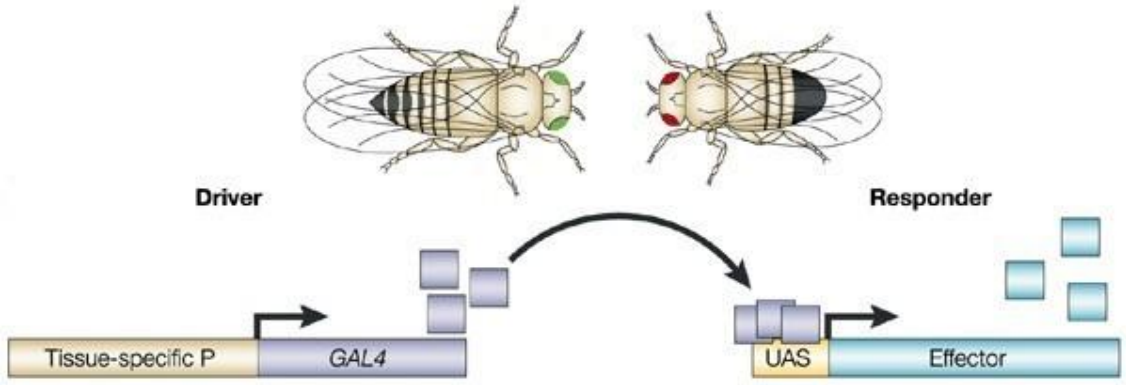

Figure 8: The UAS/Gal4 system in *Drosophila* (Wimmer, 2003). In the progeny resulting from driver and responder mating, Gal4 is expressed and binds to UAS leading to the expression of the effector, which is the target gene.

Interestingly, Gal4 activity in *Drosophila* was found to be temperature dependent. Minimal activity is acquired at a temperature of 16 degrees Celsius (°C) and maximal activity at 29°C with little effects on viability and fertility at high temperatures. Thus UAS/Gal4 is a flexible system in which changing the temperature results in a range of variable expression levels achieved by any responder (Duffy, 2002).

K. RNAi Mechanism for Gene Silencing

One effective strategy to characterize gene function *in vivo* is by gene knock-down (KD) and then analyzing the effect of this KD which leads to assessing gene function. Gene KD can be achieved using RNA interference (RNAi) strategy which causes the degradation of the specific mRNA. For *in vivo* studies, four types of RNAi reagents are used: small hairpin RNAs (shRNAs), synthetic siRNAs, long dsRNAs and small hairpin microRNAs (shmiRNAs) (Perrimon et al., 2010). In our work, the target gene of the parental responder fly is a small hairpin RNA inverted repeat. The concept of the UAS/Gal4 system used in our laboratory is illustrated in figure 9 in which inverted repeats of the target gene are translated and bind to the specific mRNA to induce its degradation thus gene silencing.

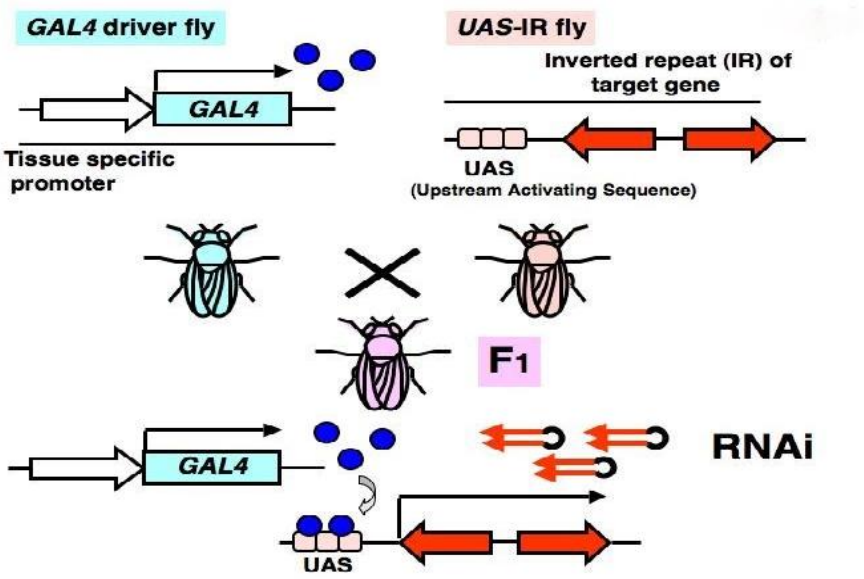

Figure 9: UAS/Gal 4 system in *Drosophila* used for gene knockdown (Mohr, 2014). Gal4 driver fly mating with UAS-IR (upstream activating sequence-inverted repeat) fly lead to the expression of Gal4 transcription factor in the progeny which will bind to UAS leading to the synthesis of dsRNAs.

The mechanism of gene knockdown by RNAi is shown in figure 10. Basically, after the dsRNA is taken into cells, it is cut by an RNAse III called DICER into siRNAs. Then in an ATP-dependent process, both strands of siRNAs are unwound and link themselves to the RNA-induced silencing complex (RISC) where binding to the complementary sequence of the mRNA occurs. The latter is thus degraded and its translation inhibited leading to gene silencing (Buckingham et al., 2004).

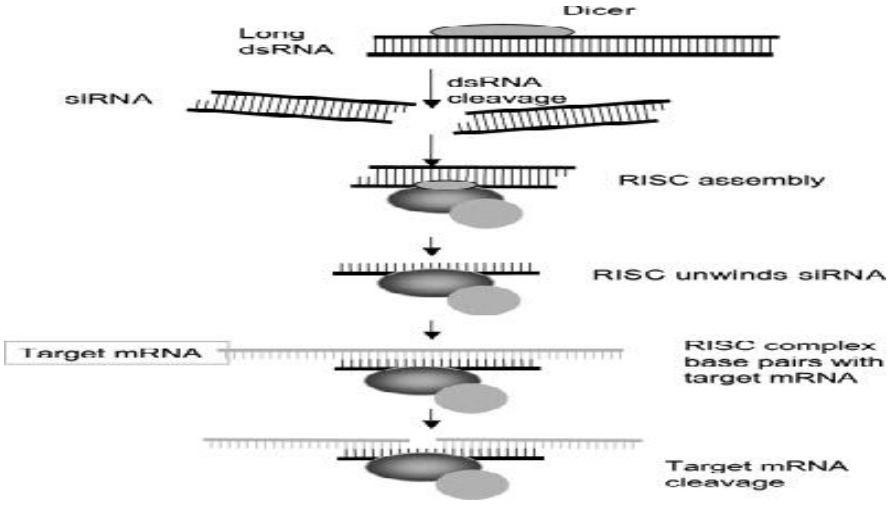

Figure 10: Gene silencing mechanism via RNAi strategy (Karpala et al., 2005). The dsRNA (double-stranded RNA) or shRNA (short-hairpin RNA) is cut by DICER into siRNAs (small-interfering RNA). Both strands of siRNAs are unwound and link themselves to the RNA-induced silencing complex (RISC) where binding to the complementary sequence of the mRNA occurs leading to mRNA slicing and gene silencing.

L. Significance and aims of our project

Nowadays, RNAi is considered a valuable tool to effectively silence gene expression and it is commonly applied today in genome-scale functional screens in *Drosophila* and other organisms. For example, several *in vivo* RNAi screens were performed to identify new genes involved in *Drosophila* immunity. One of these screens led to the identification of five serine proteases involved in Toll activation (Kambris et al., 2006).

In our work, we will be using a similar approach to investigate the potential involvement in *Drosophila melanogaster* defenses of some uncharacterized serpins and of the unknown candidate genes whose expression profile was upregulated after microbial challenge.

To achieve our aims, we will check first lethality of the KD flies crossed to the ubiquitous driver Actin-Gal4/Cyo. We will then investigate whether the induced genes of unknown function play a role in the Toll pathway. Furthermore, we will check whether serpins KD flies lead to the constitutive activation of the Toll pathway which may lead to a better flies' survival after microbial infection. Then, we will assess lifespan of serpins KD flies and check whether the KD leads to a shorter lifespan. Finally, we will investigate whether certain serpins work as negative regulators of melanization by checking whether the KD of these serpins lead to the activation of melanization in absence of infection by examining the formation of melanotic spots and then checking the PO activity in absence of microbial infection.

1. Specific aim 1

We hypothesize that the immune-upregulated unknown genes play a role in *Drosophila* immunity and that they are required for the survival and activation of AMPs following microbial infection. To test our hypothesis we will perform the following experiments:

- Knock-down the candidate genes by crossing available IR lines to ubiquitous driver (Actin-Gal4) or fat body restricted driver (C564-Gal4) if the KD with ubiquitous driver leads to lethality.
- Check whether the survival of KD *Drosophila* is compromised after infection with different microbes as compared to the wild-type counterparts.
- Quantify AMPs in KD *Drosophila* via quantitative real-time PCR (RT-PCR) and compare the expression of AMPs to that observed in wild-type flies.

2. Specific aim 2

We hypothesize that the knockdown (KD) of certain Spn genes may lead to lethality or reduced life-span and may induce high AMPs production via the Toll pathway. To test our hypothesis we will perform the following experiments:

- Knock-down each serpin candidate gene by crossing available IR lines to ubiquitous driver (Actin-Gal4) or fat body restricted driver (C564-Gal4) if the KD with ubiquitous driver leads to lethality.
- Investigate the lifespan of Spn KD *Drosophila.*

- Check whether Spn KD leads to a better survival after Gram-positive bacterial or fungal infections.
- Quantify AMP levels in Spn KD *Drosophila* using quantitative real-time PCR (RT-PCR).

3. Specific aim 3

We hypothesize that the KD of certain Spn genes may lead to the activation of melanization in absence of infection. To test this hypothesis we will:

- Examine the formation of melanotic tumors using the stereomicroscope after gene KD.
- Compare the Phenoloxidase (PO) activity in Spn KD *Drosophila* to that of the wild-type.

CHAPTER II

MATERIALS AND METHODS

A. *Drosophila* stocks, rearing and stock maintenance

All *Drosophila* IR lines used were obtained from Vienna *Drosophila* RNAi Center (VDRC). 50 mL vials were used to rear stocks. Medium in these vials contained a mixture of soy flour, polenta (cornmeal), molasses, agar and propionic acid. Flies were stored either at 18°C or at 25°C depending on need. A 12 hour light: dark cycle was used to maintain the stocks.

B. *Drosophila* crosses

Virgin females containing the driver were collected from vials stored at 18°C less than 16 hours post-eclosion from pupae. Approximately, ten virgin females were crossed to five males carrying the IR constructs in vials with fresh medium and the crosses were 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 Gal4 system and gene KD.

C. Microbe preparation

Bacterial cultures were incubated overnight with shaking at a temperature of 37°C. The culture were then spun at 4000g for 10 min, cells were re-suspended in LB,

OD measured with the spectrophotometer (595nm) and adjusted to the desired concentration. *Beauveria bassiana* fungus was grown on PDA plates for 3 weeks then spores were collected by filtration and adjusted to the desired number of spores/nL.

D. Infection of *Drosophila*

Fifteen to twenty flies (males and/or females) of each *Drosophila* cross progeny were collected at an age ranging between three to six days. Flies were injected with 32.2 nL of the microbe (*S. aureus* OD 0.05, *E. carotovora* OD 0.1, *E. coli* OD 0.1 or 4 spores/nL of *B. bassiana*) using a nano-injector armed with a capillary needle. They were then returned to their vials and maintained at 25°C. For survival assays, dead flies were counted at regular intervals. Survival graphs were then plotted as a percent survival as a 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.

E. 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 20

min at 4°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* 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.

F. Melanization Spot Test

Third instar larvae were pricked at their posterior with a pointed needle dipped in a concentrated bacterial suspension of *E. coli* (OD 20 approximately). Larvae were then maintained in *Drosophila* vials at 25°C. Two hours post-infection, two larvae from each cross were torn apart using forceps and their hemolymph was dipped on a filter paper soaked with L-DOPA (2 mg/mL) which is light-sensitive, so the filter paper was covered using Aluminum foil to avoid light exposure. Non-infected larvae hemolymph were also tested. The darkening of the spots was monitored every ten minutes for a one

hour period at room temperature and compared to spots for infected and non-infected wild-type larvae (control).

CHAPTER III

RESULTS

A. Lethality of unknown gene KD

In our study, we analyzed IR transgenic strains targeting different immunity-upregulated genes: twelve genes of unknown function and three genes previously shown to be involved in biological processes other than immunity. For two of the unknown genes (CG2217 and CG5729), three different transgenic lines (different insertions) were available; and for one gene encoding a Dopa decarboxylase (CG10697) two different IR lines were available. Seven out of these fifteen genes were found to be lethal when crossed with the ubiquitous driver (Actin-Gal4/CyO), thus we will have to use a more restricted driver (C564-Gal4) to achieve gene KD. These genes correspond to: CG2217, CG6394, CG5150, CG3829, CG6822, CG14938 and CG10697. One out of the fifteen genes (CG14938) was even found to be lethal when crossed with the more restricted driver C564-Gal4 (fat body specific driver) and was not further analyzed.

Due to the high lethality observed when crossing IR transgenic flies with Actin-Gal4/Cyo, we decided to use the C564-Gal4 driver to achieve gene KD for all these genes in our study.

B. Survival of unknown gene KD flies after microbial infection

First generation (F1) progeny was collected three to seven days post-eclosion from pupae and fifteen flies infected with the corresponding microbe. After counting

dead flies at frequent intervals, survival graphs were plotted. This procedure was repeated at least three times (different biological replicates) and a representative graph is shown for each microbial infection. Female KD flies are preferred for infection experiments for practical reasons (due to their bigger body size as compared to males) and to their slightly higher resistance.

After infection with the Gram-positive bacterium *E. faecalis* (OD 0.05), flies with CG2217, CG9186 and CG18067 gene KD show compromised survival as compared to the wild-type flies (C564*W1118 and C564*Or) and to the other KD genes. This result was confirmed in 3 independent repeats of the experiment (Figure 11), except for CG18607 KD that did not lead to increased sensitivity to *E. fecalis* infections in all the experiment repeats (not shown).

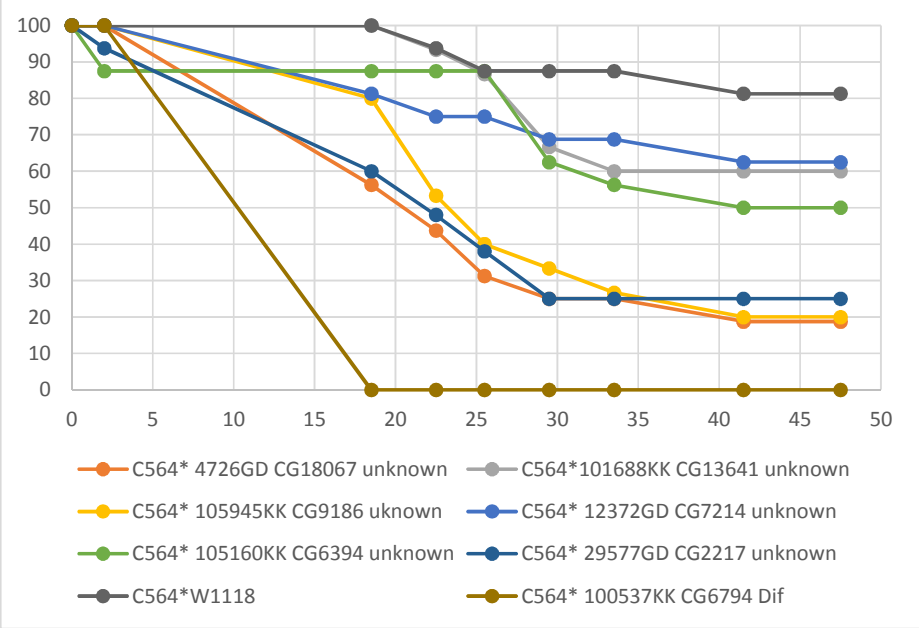

Figure 11: C564*Unknowns KD survival after *E. Faecalis* infection (OD 0.05). The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118). Dif KD flies represent the positive control.

Although the susceptibility of CG18067 flies to *E. faecalis* needs to be further investigated, these flies show severely compromised survival after fungal infection with *B. Bassiana* as compared to the wild-type flies and to the rest of KD genes (Figure 12). For all infections with Gram-positive bacteria or fungi, Dif KD flies constitute the positive control and indeed show the lowest survival rates among all infected flies.

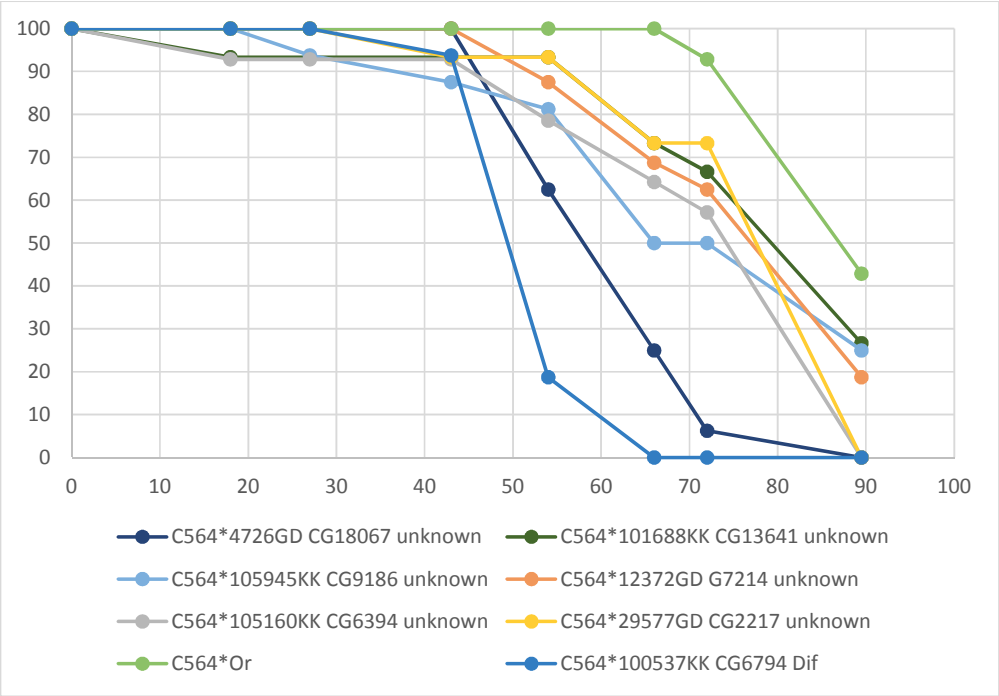

Figure 12: C564*Unknowns survival after *B. bassiana* infection. The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by Oregon (Or). Dif KD flies represent the positive control.

On the other hand, none of the flies infected with the Gram-negative bacteria *E. carotovora* (OD 0.1) show compromised survival as compared to the wild-type flies and to the positive control Rel KD flies which show the lowest survival rate (Figure 13).

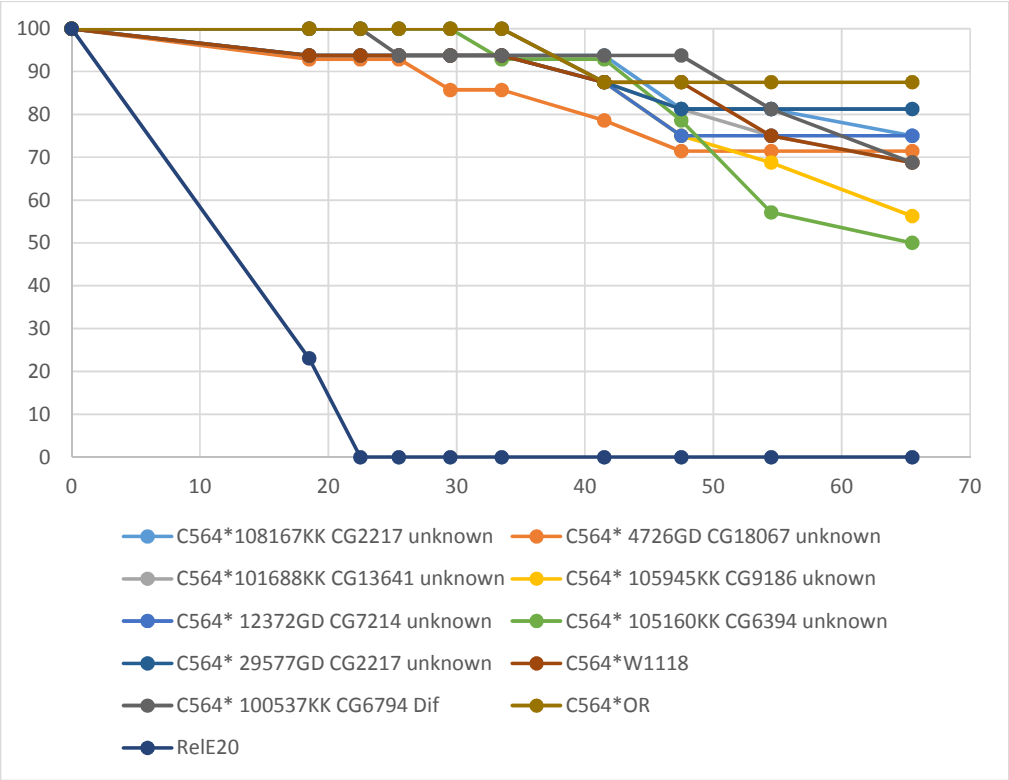

Figure 13: C564*Unknowns survival after *E. carotovora* infection (OD 0.1). The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118) and Oregon (Or). Rel KD flies represent the positive control.

C. AMP levels after *B. bassiana* infection

Three to five days old flies were freezed after 48h of fungal infection. Following *B. bassiana* infection, CG18067, CG6394 and CG2217 KD flies failed to induce *Drosomycin* expression (less than 50%) as compared to the infected wild-type flies (figure 14).

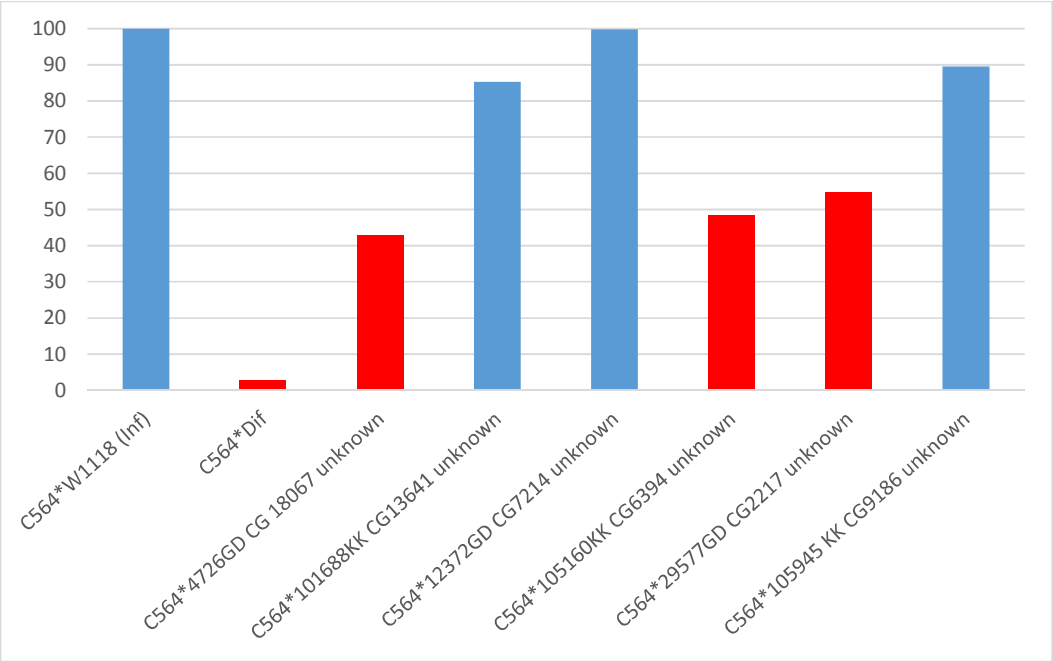

Figure 14: *Drosomycin* quantification after fungal infection with *B. bassiana*. The vertical axis represents the percentage of *Drosomycin* normalized to *Rp49* and the horizontal axis represents the genotypes of the studied gene KD. The lowest *Drosomycin* induction levels are represented in red, the rest in blue.

D. Lethality of serpin gene KD

We obtained IR lines targeting seven different serpin genes. For one of the serpins (CG12172 Spn43Aa), three different IR lines were available, for five serpin genes (CG16713, CG7219, CG9460, CG18525 and CG1857 Nec) two different IR lines were available and for Spn27A (CG11331) only one IR line was in our possession.

Five out of seven serpin KD genes studied were found to be lethal when crossed with the ubiquitous driver (Actin-Gal4/CyO). The corresponding lethal genes are: CG16713, CG12172 Spn43Aa, CG7219, CG1857 (Nec) and CG11331 Spn27A.

Due to the high lethality observed with the ubiquitous driver, we decided to use the fat body restricted driver C564-Gal4 to achieve serpin KD.

E. Survival of Spn KD flies after microbial infection

Three to seven days old serpin KD females were infected with each microbe and dead flies were counted at frequent intervals. This was repeated at least three times and survival graphs were plotted. A representative graph is shown.

Following infection of flies having serpin KD with the Gram-positive bacterium *S. aureus* (OD 0.05), flies with CG12172 SPN43Aa KD show compromised survival as compared to the wild-type flies (C564*W1118) and to the other serpin KD flies. Intriguingly, one line corresponding to this serpin (30783GD) shows very similar death rate as compared to the Dif KD flies (figure 15).

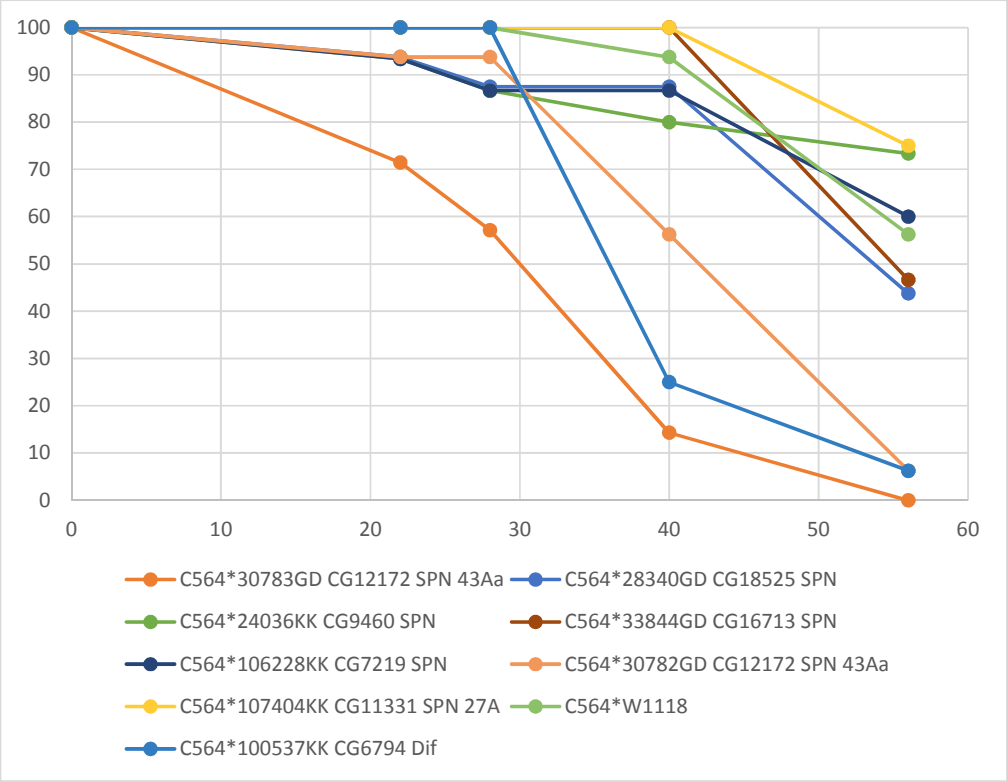

Figure 15: C564*Spn KD survival after *S. aureus* infection (OD 0.05). The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118). Dif KD flies represent the positive control.

Similarly, after infecting serpin KD flies with the other Gram-positive bacterium *E. faecalis* (OD 0.05) or with *B. bassiana*, flies with CG12172 Spn43Aa KD show compromised survival as compared to the wild type flies (C564*W1118). This is apparent for both Spn43Aa lines (30782GD and 30783GD) (Figures 16 and 17).

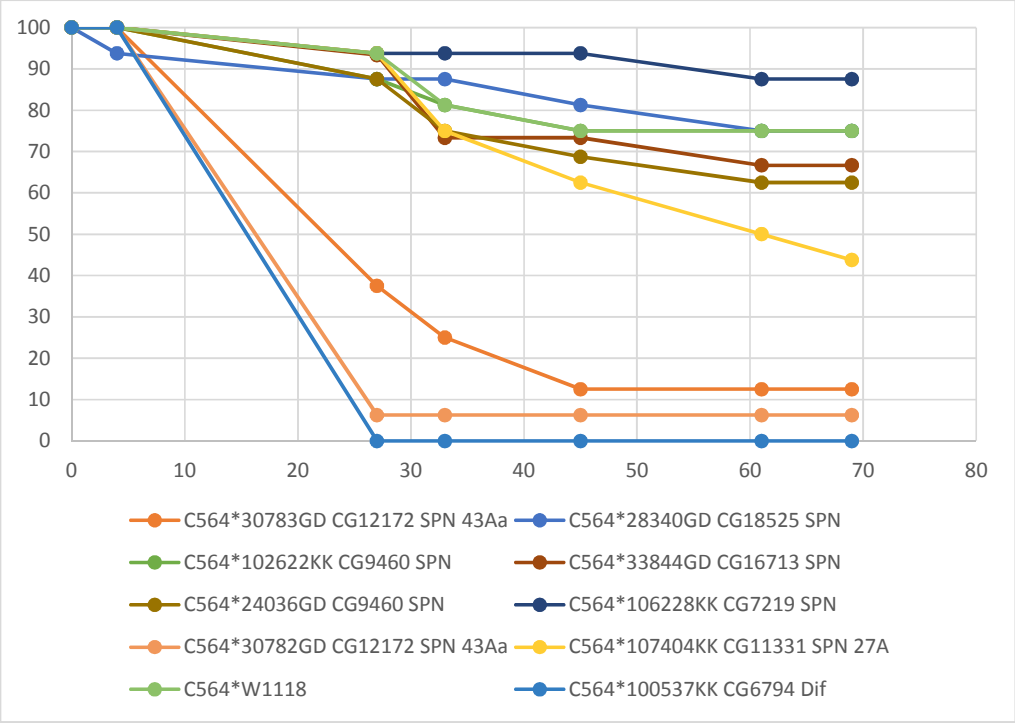

Figure 16: C564*Spn KD survival after *E. faecalis* infection (0D 0.05). The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118). Dif KD flies represent the positive control.

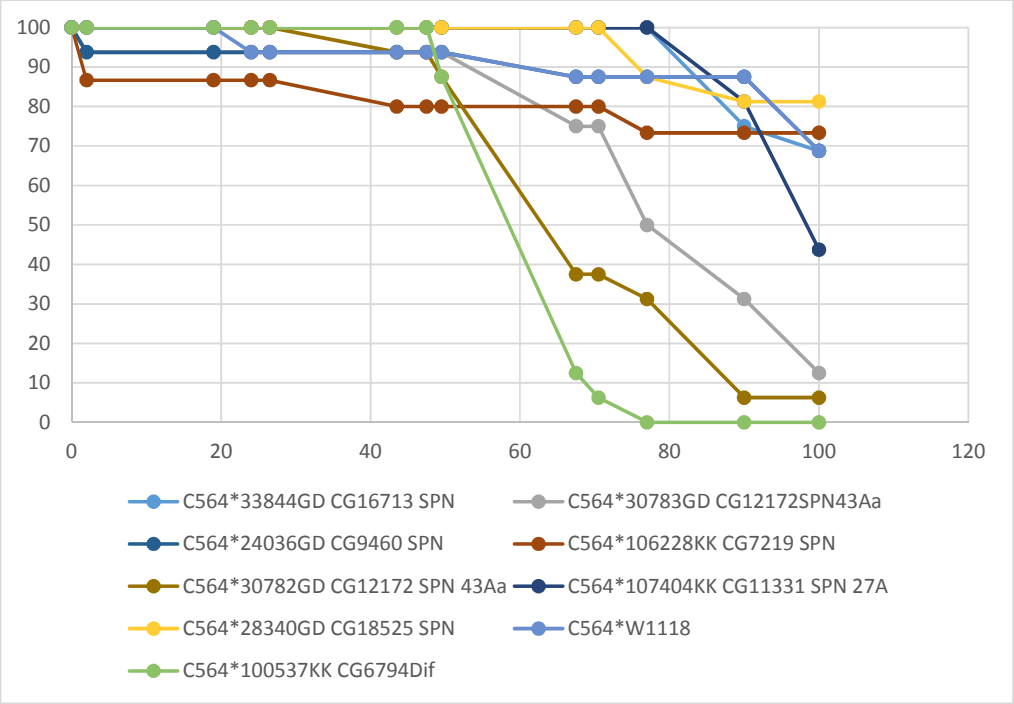

Figure 17: C564*Spn KD survival after *B. bassiana* infection. The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118). Dif KD flies represent the positive control.

However, none of the Spn KD flies including Spn43Aa, show compromised survival when infected with *E. carotovora* (OD 0.1) as compared to the wild type flies and to the positive control Rel KD flies (Figure 18).

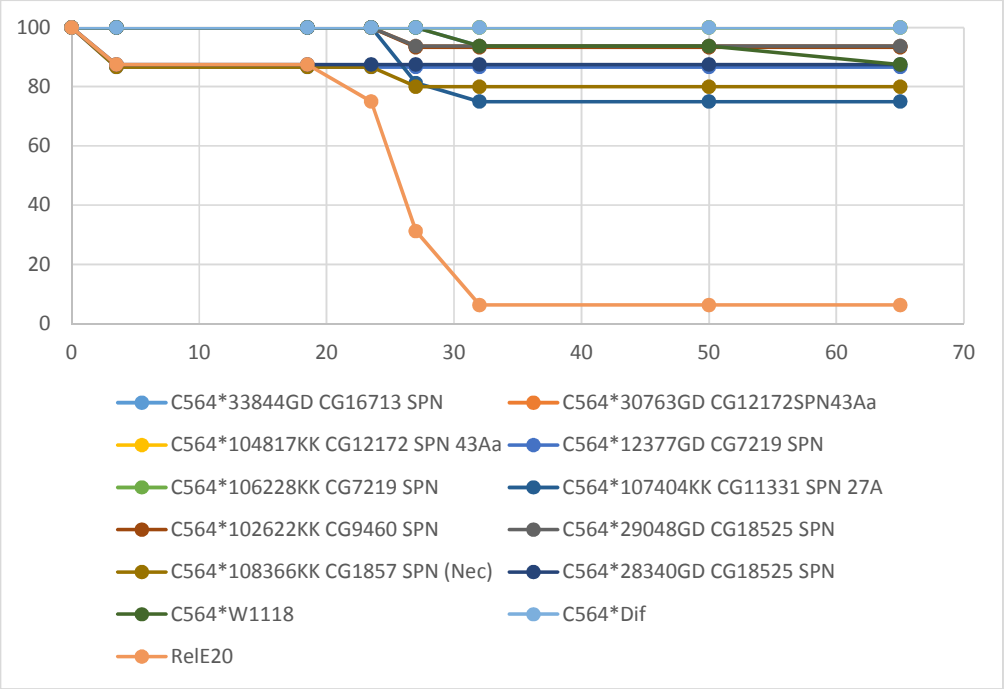

Figure 18: C564*Spn KD survival infected with *E. carotovora* (OD 0.1). The vertical axis represents the percentage of survived flies and the horizontal axis represents time (in hours). Wild-type flies are represented by White1118 (W1118). Rel KD flies represent the positive control.

F. Lifespan of Spn KD flies

Three to seven days old F1 generation flies having serpin KD were maintained at a temperature of 29°C. Lifespan was assessed everyday approximately by counting the number of dead flies and survival graphs were plotted. Apparently in females, CG12172 Spn43Aa KD flies possess the shortest lifespan as compared to the wild-type flies (C564*W1118 and C564*Or) and die rapidly after two weeks approximately (Figure 19).

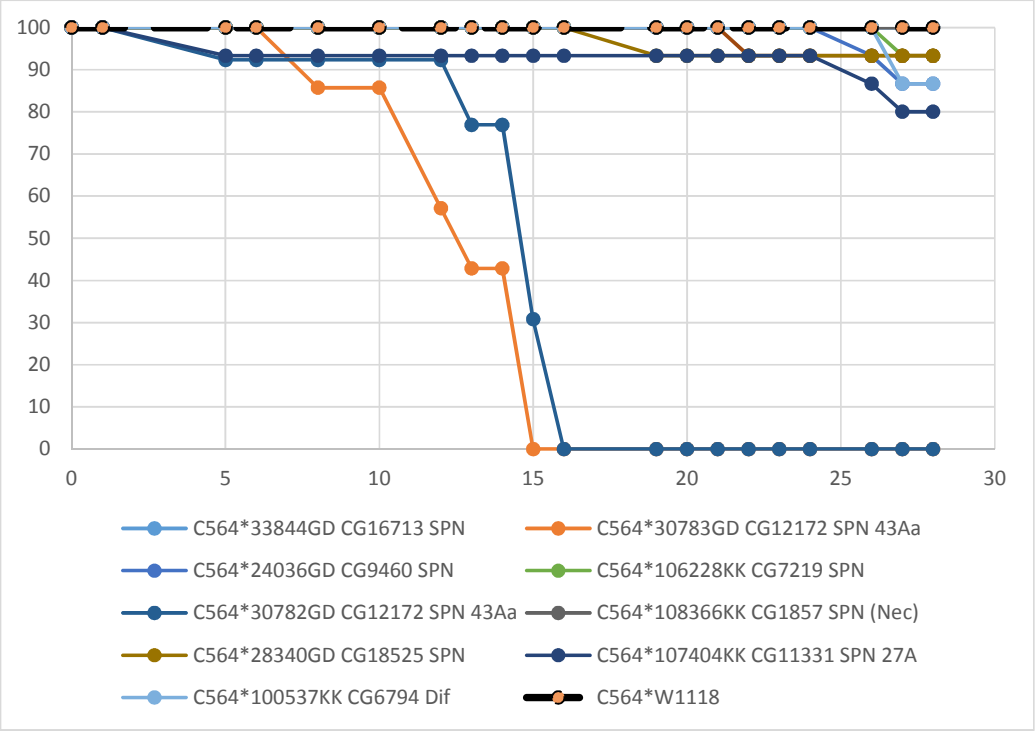

Figure 19: C564*Spn KD females' lifespan. The vertical axis represents the percentage of flies and the horizontal axis represents time (in days). Wild-type flies W1118 are represented by black dashed lines.

Similarly, in males, CG12172 Spn43Aa KD flies have the shortest lifespan but die even before females after almost 10 days (Figure 20).

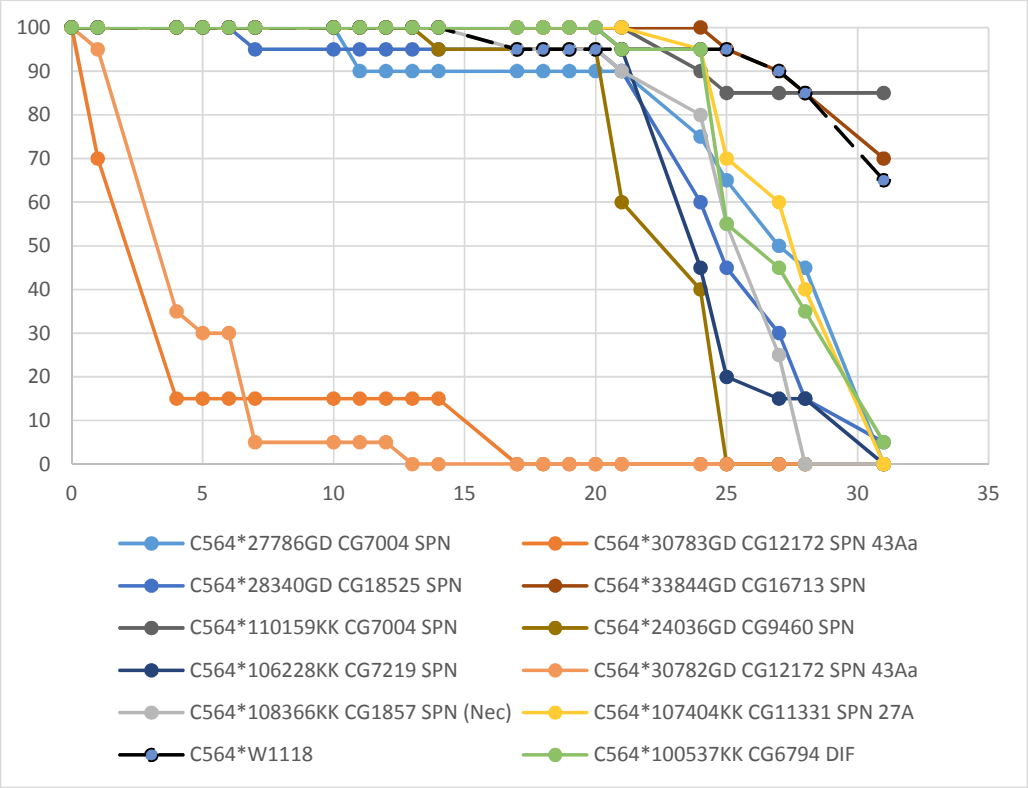

Figure 20: C564*Spn KD males' lifespan. The vertical axis represents the percentage of flies and the horizontal axis represents time (in days). Wild-type flies W1118 and Or are represented by black dashed lines.

G. AMP levels of Spn KD flies

Non-infected flies with Spn43Aa KD and CG9460 KD show the highest *Drosomycin* induction as compared to the non-infected Spn KD flies and to the wild-type flies (C564*W1118) (Figure 21).

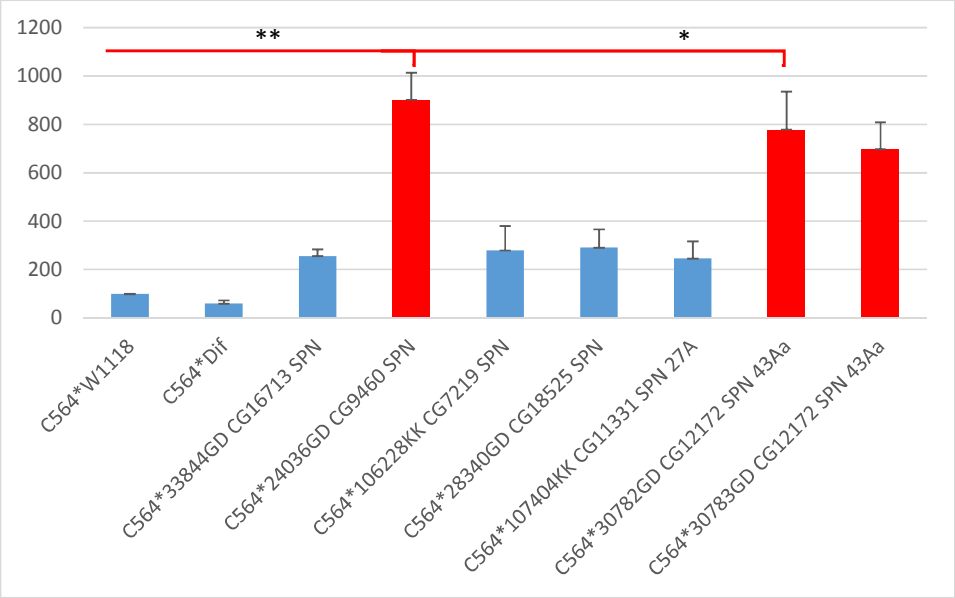

Figure 21: *Drosomycin* quantification in non-infected Spn KD flies. The vertical axis represents the percentage of *Drosomycin* normalized to *Rp49* and the horizontal axis represents the genotypes of the studied Spn KD. The highest Drosomycin induction levels are represented in red, the rest in blue. Values are averages of three independent experiments and bars shown represent the standard error. (* represents $p<0.05$ and ** represents $p<0.01$).

However, when infected with *E. faecalis,* Spn43Aa KD flies fail to induce *Drosomycin* to reach the high levels observed in wild-type control flies (Figure 22).

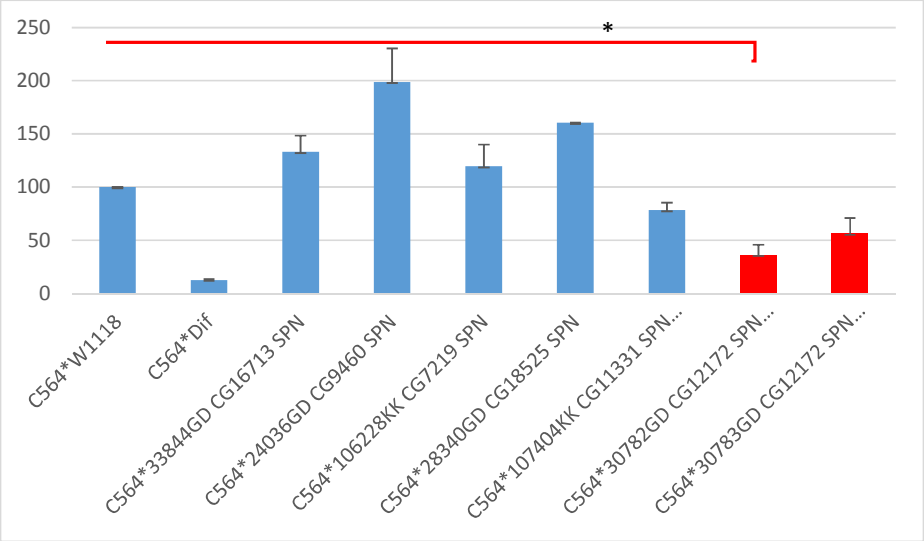

Figure 22: *Drosomycin* quantification in *E. faecalis* infected Spn KD flies. The vertical axis represents the percentage of *Drosomycin* normalized to *Rp49* and the horizontal axis represents the genotypes of the studied Spn KD. The lowest Drosomycin induction levels are represented in red, the rest in blue. Values are averages of independent experiments and bars shown represent the standard error. Stars represent significance compared to the positive control (* represents $p<0.05$).

H. Melanotic spots formation in some of the Spn KD

Spn43Aa KD obtained by crossing C564-Gal4 driver with either of the CG12172-IR lines leads to the formation of large melanotic spots in the thorax of most of the F1 progeny flies (aged 3 to 5 days).

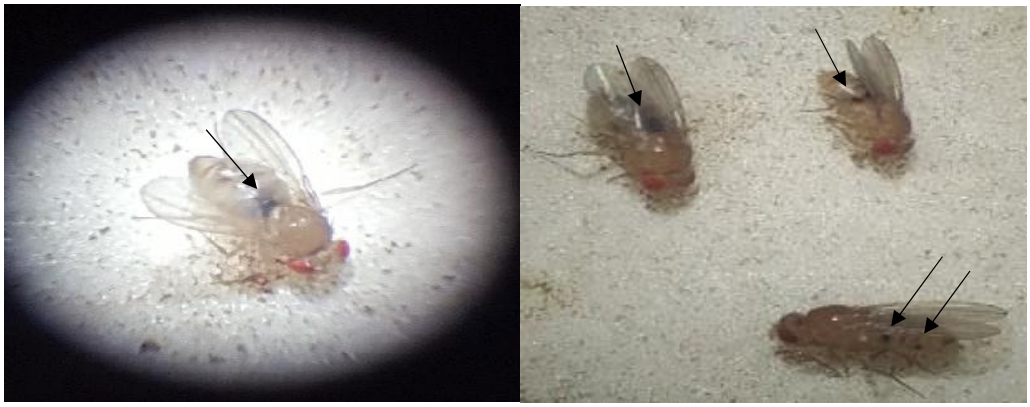

Figure 23: Melanotic spots formation in C564*Spn43Aa KD flies. Arrows indicate the position where melanotic spots are found.

Figure 24: Melanotic spots formation in CG7219 KD flies. Arrows indicate the position where melanotic spots are found.

Similarly, CG7219 KD which are three to seven days old flies of the F1 progeny show melanotic spots on their body (figure 24). Interestingly, CG9460 KD male flies show one melanotic spot on the base of each of their wings (figure25).

Figure 25: Melanotic spots formation in CG9460 KD flies. Arrows indicate the position where melanotic spots are found.

I. Spn KD flies spot test

The Phenoloxidase (PO) activity was assessed semi-quantitatively in the hemolymph of non-infected Spn KD flies. These flies show higher PO activity reflected by the large and dark melanization spots as compared to the non-infected wild-type flies which show very faint spot on the filter paper. Flies with CG18525 KD show the largest dark spot among all other serpin KD. Dif KD flies show the least PO activity as compared to the wild-type flies and to the other serpin KD (Figure 24).

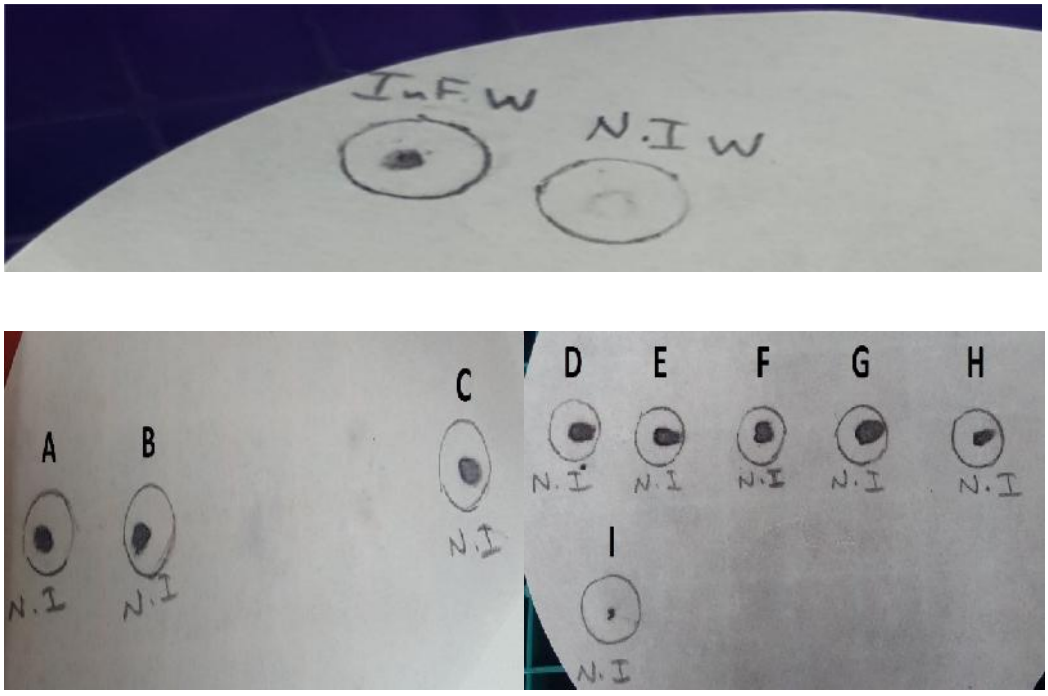

Figure 26: Melanotic spots of non-infected Spn KD flies' hemolymph. Inf W refers to the infected wild-type White (W1118) flies and N.I W refers to the non-infected wild-type W1118 flies. Flies were all crossed to C564 to achieve Spn KD (A: CG16713; B: CG12172 SPN43Aa; C: CG9460; D: CG7219; E: CG12172; F: CG1857; G: CG18525; H: CG11331; I: CG6794 Dif).

CHAPTER IV
DISCUSSION

In this work, the rationale was to investigate the involvement of candidate genes in the immune responses of *Drosophila*. These candidates were either genes whose transcription is upregulated after immune challenge or genes belonging to families that include previously confirmed immunity genes such as the serpin family. We have used an *in vivo* genetic RNAi screen to target some induced genes of unknown function and some genes belonging to the serpin family. Approximately, 50% of the analyzed genes led to fly lethality when crossed to the ubiquitous driver Actin-Gal4/Cyo which may be explained by the importance of these genes for the vital functions of *Drosophila* (seven out of the fifteen for the unknown genes KD and five out of the ten for the serpin KD).

Flies knocked-down for different candidate genes were infected with Gram-positive, Gram-negative or fungi, and their survival was assessed. Interestingly, CG18607 KD flies showed severely compromised survival after fungal infection with *B. bassiana* in three independent experiments. They also showed compromised survival when infected with the Gram-positive bacteria *E. faecalis* in one experiment, but normal survival was observed in two other experiments. In addition, *Drosomycin* levels were poorly induced in this gene KD flies as compared to the wild-type flies after fungal infection with *B. bassiana* and they were induced to normal levels after *E. faecalis* infection in one preliminary experiment (not shown). Thus, if the normal survival to Gram-positive bacteria is confirmed, this gene may be specifically required to fight

fungal infection. Further experimental repeats should be performed to validate this interesting result.

As for the infection with the Gram-negative bacterium *E. carotovora*, all of the unknown genes KD flies have shown normal survival. This suggests that none of these genes plays a role in the Imd pathway. For further validation, RT-PCR could be performed to check for *Diptericin* expression levels after Gram-negative bacterial infection, but we expect to observe normal levels of *Diptericin* expression in KD flies.

Concerning the serpin genes, Spn43Aa KD flies clearly had a distinctive phenotype among the serpin genes we have analyzed. First, following infection with the Gram-positive bacteria, Spn43Aa KD flies have revealed compromised survival as compared to the other Spn KD and to the wild type flies. In particular, following *S. aureus* infection, one of the two Spn43Aa KD line had even higher death rate than the Dif KD flies. This observation was not seen after *E. faecalis* infection probably for the sole reason that *S. aureus* is more virulent than *E. faecalis*, or might be due to some differences in the levels of responses triggered by each bacterium (more melanization reactions versus AMP activation for instance). It is noteworthy that we have at first used *E. faecalis* as the Gram-positive bacterium in the survivals for which results were further confirmed by using the Gram-positive bacterium *S. aureus*.

Spn43Aa KD flies fail to induce *Drosomycin* expression following *E. faecalis* infection which corroborates the compromised survival seen after Gram-positive bacterial infection. Intriguingly, non-infected Spn43Aa KD flies have shown seven times higher basal *Drosomycin* expression levels along with CG9460 KD flies that have approximately ten times higher basal *Drosomycin* levels as compared to the

non-infected wild-type flies. These results indicate that the Toll pathway is somehow deregulated with higher basal activity in absence of infection and failure to reach high activation following infection. However, in both CG9460 and Spn43Aa KD flies, the slightly higher than normal *Drosomycin* levels observed in absence of infections were much lower than those seen in infected wild-type. This suggests that, unlike Nec, these two serpins are not involved in the negative regulation of AMPs production via the Toll pathway. Moreover, the slightly higher basal levels of AMPs in Spn43Aa and CG9460 KD flies may be associated with local melanization that was revealed by the melanotic spots seen under the stereomicroscope on the flies' body. Further investigations must be conducted to fully understand the basis underlying this deregulation. We can check whether the high basal levels of *Drosomycin* observed in absence of infection are due to a low systemic production of this AMP or to a local synthesis (at the melanization sites) by combining the RNAi of these two serpins with a *Drosomycin-GFP* reporter that will allow to visualize fluorescence where *Drosomycin* is expressed.

Spn43Aa KD flies were characterized by a reduced lifespan (in absence of infection) when maintained at 29°C. Interestingly, although the two sexes have similar lifespan in wild-type *Drosophila*, Spn43Aa KD males died within 10 days after emergence and their lifespan was shorter than that of females which died within 2 weeks approximately. This result can be explained by the fragility of males as compared to female *Drosophila* which have bigger body size and more fat tissues or by a better gene KD efficiency in males. Spn43Aa KD flies showed susceptibility to fungal and Gram-positive bacterial infections, but we believe that the compromised survival observed was not a consequence of the reduced lifespan seen at 29°C because infections were done with young flies (before the onset of the premature death). Moreover,

E. carotovora infection did not lead to compromised survival in any of the Spn KD flies including Spn43Aa KD.

Furthermore, observing KD flies under the stereomicroscope have shown melanotic spots on CG7219 KD flies' body. This observation is in accordance with a previous study in which CG7219 called Spn28D was shown to be implicated in melanization reaction regulation in hemolymph and trachea in *Drosophila* (Scherfer et al., 2008). Also, KD of another gene, Spn27A which has been previously shown to be a negative regulator of the melanization cascade (De Gregorio et al., 2002), Spn27A KD showed a clear phenotype of 100% lethality with the Actin-Gal4/CyO driver and abundant melanotic spots with the C564-Gal4 driver. Thus, these two previously characterized Spn KD can be considered a positive control or validation to our work. However, in RNAi screens, one cannot exclude that different lines targeting the same gene lead to different levels of gene silencing. This is partially due to position effect of the transposon with insertions in actively transcribed areas of the chromosomes leading to higher level of expression of the dsRNA and better gene KD. Therefore, we should further check whether the RNAi is sufficiently depleting the target gene mRNA in the progeny by performing RT-PCR before completely ruling out the involvement of a given candidate gene in immunity.

Moreover, non-infected Spn43Aa and CG9460 have shown melanotic tumors on their thorax and on their wings respectively when visualized under the stereomicroscope. This observation may be correlated to the higher *Drosomycin* levels induction in these KD flies. Previous studies have revealed that melanization and the Toll pathway are two dependent pathways. For example, removal of Spn27A from the hemolymph in *Drosophila* after immune challenge requires Toll pathway activation

probably because a newly synthesized putative protease leads to Spn27A elimination (Ligoxygakis et al., 2002). However, most of the non-infected Spn KD show higher melanization levels as compared to the wild-type flies which infers that melanization and Toll pathway activation are not always linked as the non-infected KD flies having low *Drosomycin* levels have shown larger and darker melanization spots as compared to the wild-type flies in the "spot test". Nevertheless, a more precise quantitative method for assessing melanization levels in flies' hemolymph must be used to validate the "spot test" result. This method, called the DOPA assay, consists of measuring the optical density of Spn KD flies' hemolymph to assess the phenoloxidase activity.

The gene encoding Spn43Ac is found in a cluster of four serpin genes 43A (Spn43Aa, Spn43Ab, Spn43Ac and Spn43Ad) (Green et al., 2000). Deletion of Spn43Ac transcript results in a lethal phenotype. In RNAi studies, validation of the results by analyzing null genetic mutant (deletion or deficiency) is often required, but in our case this is not possible because of the expected lethal phenotype of genetics null. Another important concern for RNAi screens, is the problem of off-targets. We believe our results are not due to off target targeting of other serpins, especially that Nec, which is one of the closest serpins to Spn43Aa in term of sequence gives a distinct phenotype when mutated. RT-PCR can be used to rule out off-target silencing of other serpins.

CHAPTER V

CONCLUSION AND PERSPECTIVES

This work adds some new information about some serpins (in particular Spn43Aa) but a lot remain to be discovered about the mechanisms of function of serine proteases and serpins. The large size of these two families in *Drosophila* (29 serpins and about 200 SPs) and potential involvement of one gene in more than one biological pathway and possible functional redundancies makes the complete deciphering of these two families' precise roles a difficult task.

In future studies, it will be interesting to perform epistatical analysis to determine which serine protease acts downstream of Spn43Aa. This analysis could be done by crossing Spn43Aa with several SPs and check whether the Toll pathway would be shut-down. Also for the interesting candidate genes of unknown function isolated in this screen, more work will be required to understand their contribution to the Toll pathway or to flies immunity in general, but this is not a straight forward job since they do not have clear orthologues characterized in other species which could give some hints about their roles.

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