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From long non-coding RNA genes to the expression of secreted effector peptides involved in *Drosophila melanogaster* defenses against toxins and virulence factors secreted by pathogenic microbes: the case of *CG44404* and *CG45045*

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Abbreviations

A

AMPs: Antimicrobial Peptides

A. fumigatus: Aspergillus fumigatus

B

BaraA: Baramicin A

BBB: Blood Brain Barrier

BLUD: Bacterial Load Upon Death

C

C. albicans: Candida albicans

CFU: Colony Forming Unit

C. glabrata: Candida glabrata

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

D

DAP-type PGN: diaminopymelic acid type Peptidoglycan

Dtx: Destruxins

Dif: Dorsal-related Immune Factor

DIM: *Drosophila*-induced Immune Molecule

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

Dredd: Death related ced-3/Nedd2-like protein

dsRNA: double-stranded RNA

DUOX: Dual Oxidase

DTT: Dithiothreitol

E

Ecc15: Erwinia carotovora carotovora 15

E. coli: Escherichia coli

E. faecalis: Enterococcus faecalis

EGFR: Epidermal Growth Factor Receptor

EntV: Enterocin O16

F

FLUD: Fungal Load Upon Death

G

GAL4: galactose4 gene

Gal80: Galactose 80

GBP: Growth-Blocking Peptide

GFP: Green Fluorescent Protein

GNBPs: Gram-Negative Binding Proteins

Grass: Gram-positive specific serine protease

H

HDR: homology directed repair

Hid: Head Involution Defective

I

IMD: immune deficiency

J

JAK: Janus Kinase

JNK: Jun N-terminal Kinase

K

KDa: Kilo Dalton

KD: Knock Down

KO: Knock Out

KI: Knock In

L

LB: Luria-Bertani

lncRNA: Long noncoding RNA

LPS: Lipopolysaccharide

Lxb: Latex beads

Lys-type PGN: Lysine-type Peptidoglycan

M

M. luteus (M. luteus, Ml): Micrococcus luteus

M. robertsii (Mr): Metarhizium robertsii

MyD88: Myeloid: differentiation primary-response gene 88

N

NF-κB: Nuclear Factor kappa-light-chainenhancer of activated B cells

NHEJ: homologous end joining DNA repair

NOX: NADPH Oxidase

O

OMVs: Outer Membrane Vesicles

P

PA: Pseudomonas aeruginosa

PDA: Potato Dextrose Agar

PGN: Peptidoglycan

PGRPs: PeptidoGlycan Recognition Proteins

PO: Phenoloxidase

PPO: Prophenoloxidase

PrtA: Protease A (Serralysin)

Psh: Persephone

PRRs: Pattern Recognition Receptors

R

Rel: Relish

RNA: Ribonucleic Acid

RNAi: Ribonucleic Acid interference

ROS: Reactive Oxygen Species

rpr: Reaper

RT-qPCR: Quantitative Reverse Transcription PCR

S

SDS: Sodium Dodecyl Sulfate

siRNA: small interfering RNA

SPE: Spätzle-processing enzyme

Spz: Spätzle

T

TCA: Trichloroacetic acid

TRiP: Transgenic RNAi Project

U

UAS: Upstream Activating Sequence

V

VitC: Vitamin C

W

wt: Wild Type

Y

Yp: Yolk protein

From long non-coding RNA genes to the expression of secreted effector peptides involved in *Drosophila melanogaster* defenses against toxins and virulence factors secreted by pathogenic microbes: the case of *CG44404* and *CG45045*

Subject: Immunology

PhD candidate: Chuping CAI

Supervisor: Dominique FERRANDON

Abstract

Background

As a valuable model organism, *Drosophila* provides ponderable advantages for the study of immunity. Several immune responses are involved in *Drosophila* host defense, including humoral and cellular ones. The humoral response is described to produce immune effectors released by the fat body into the hemolymph and plays a critical role in the host defense against many pathogens. Two relevant signaling pathways are involved, including Toll and Immune Deficiency (IMD) pathways. Some immune effectors are specifically or synergistically regulated by them. As described in the study, one cluster of immune effectors, antimicrobial peptides (AMPs), play specific, synergistic or additive functions on targeting some pathogens. Phagocytosis is another response acting in the elimination of pathogens. Melanization, which relies on the cleavage of prophenol oxidases resulting in the deposition of the melanin at the wound site, is involved in host defense against other pathogens: able to withstand other immune responses. Beyond that whether long noncoding RNAs (lncRNAs) play roles in these immune responses is still unclear. LncRNAs were previously considered to correspond to transcription noise. Yet, evidence has revealed that lncRNAs play essential roles in the immunity of mammals.

In the interactions between host and pathogens, flies succumb not only from the high burden of pathogen but also from damages inflicted by virulence factors secreted by pathogens. Pathogens produce and secrete extracellular vesicles that transport cargo, such as misfolded proteins and virulence factors that allow invading the host more efficiently. The extracellular vesicles produced by Gram-negative bacteria, such as *Serratia marcescens* and *Pseudomonas aeruginosa*, known as outer membrane vesicles (OMVs), bud out from the outer membrane of Gram-negative bacteria and filled with periplasmic content. *Drosophila* provides a potent model to study the interactions between host and pathogen.

Aims

Following an extensive RNA seq study led by previous member of the laboratory, I focused on investigating whether lncRNAs play a role in the *Drosophila* host defense against microbial infections. In chapter I, I mainly investigate the function of *Yp1* with its antisense lncRNA *CR45601*. With respect to the truth, in Chapter II, I mainly explore the function of *CG44404* and *CG45045* in *Drosophila* immune response. However, it showed that the two lncRNAs genes in Chapter II encode short peptides. A long-standing project in the laboratory is the study of *Serratia marcescens* OMVs in the host-pathogen relationships using the *Drosophila* model. Given the results reported in Chapter II, I have also contributed to the final phages of this study, which is related in a first annex.

Material and methods

The mutants of genes of interest in *Drosophila* were generated and infected with different pathogens including yeast, filamentous fungi, Gram-positive and Gram-negative bacteria. Survival rates were recorded and quantification of pathogen burdens was performed when needed. Based on the survival rate and pathogen burden, the mechanism was then further explored. For OMVs, all the canonical immune responses in *Drosophila* were tested in the OMV injection model.

Results

1. *Yp1* and *CR45601* were not found to be involved in the immunity of *Drosophila.*

2. Both *CG44404* and *CG45045* encode short secreted peptides.

3. The expression of *CG44404* is Toll and IMD pathway dependent while *CG45045* is only IMD pathway dependent.

4. *CG44404* and *CG45045* are required for the flies against *Metarhizium robertsii* and *Pseudomonas aeruginosa* but not in the action of killing pathogens.

5. The PrtA metalloprotease is the main virulence factor in *S. marcescens* OMVs mediating their pathogenicity.

6. *CG44404* and *CG45045* are involved in the host against *M. robertsii* Destruxin A and *P. aeruginosa / S. marcescens* metalloprotease.

7. *CG44404* and *CG45045* are not involved in the canonical immune responses in the action against virulence factors.

8. CG44404 associates with thioester-containing protein TEP2 and the Toll effector Baramicin A-derived Peptides.

9. *Tep2* and *Tep4* play opposite roles in the host defense against PrtA, the latter possibly by inhibiting PrtA through α2-macroglabulin-like suicide action.

10. Eiger is not involved in the activation of JNK pathway in the OMV model

11. Apoptosis is mediated by ROS upon OMVs treatment

Conclusions

1. *Yp1* and *CR45601* is not involved in the host defense different pathogens.

2. *CG44404* and *CG45045* encode short peptides in the *Drosophila*. Both peptides play roles in the host defense against Destruxin A and Protease A respectively from *Metarhizium* and *Serratia*.

3. OMVs induce apoptosis in neurons via ROS activating JNK pathway. The phagocytosis is required for the pathogenesis of OMVs.

This work shows that the resilience against several distinct classes of the virulence factors entails the combined action of several effectors of the systemic humoral immune response, including BaraA [1], Tep2/ Tep4, and CG44404/CG45045.

Key words

CG44404, *CG45045*, Destruxin A, Protease A, outer membrane vesicles, yolk protein 1, *CR45601*

General introduction

1. *Drosophila melanogaster*

1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster, also known as the fruit fly in short, has been one of the most potent model organisms to study biology for more than one hundred years. Johann Wilhelm Meigen, a well-known taxonomist, described around 4,500 names of genera and species throughout the *Diptera* in his remarkable work "*Systematische Beschreibung der europäischen zweiflügeligen Insekten*" in early 19th century. It is the first time that *Drosophila melanogaster* was unveiled. Following the efforts from scientists for decades, achievements were made in the world. Thomas H. Morgan was awarded the Nobel Prize in Physiology or Medicine in 1933 with an article about sex-limited inheritance in *Drosophila* and the chromosomal theory of heredity [2]. Hermann H. Muller obtained the Nobel Prize in 1946 with his research on the production of mutations by X-ray irradiation. In 1995, Edward B Lewis, Christiane Nüsslein-Volhard, and Eric F Wieschaus received the Nobel Prize for a study on the genetic control of embryonic development. The Berkeley *Drosophila* Genome Project Group (BDGP), led by the University of California Berkeley with the cooperation of the Celera Genomics Corporation, completed the sequence of the fly genome in March of 2000 [3]. In 2011, Jules A. Hoffmann, Bruce Beutler and Ralph Steinman shared the Nobel prize for their discoveries on Toll receptors in immunity[4]. In 2017, Jeffrey C. Hall, Michael Rosbash and Michael W. Young won the award for uncovering the molecular mechanisms that control circadian rhythms.

Drosophila melanogaster has been considered as a great organism model for a long history on account of several vital advantages. One of the main advantages of fruit flies is their short life cycle compared to other organism models that allows to generate a large number of offspring rapidly. The life cycle consists of four stages: embryo, larva, pupa, and adult. When raised at 25 degree, the fertilized eggs develope into first instar

larvae in 22 to 24 hours. The larvae then go through growth and molting every 25 hours until the third instar larval stage. At the mid-third instar larval stage, a pulse of the essential steroid hormone named ecdysone changes its behavior and results in its climbing on the wall of the vial. After 30 hours, the third instar larva transforms into a pupa. The pupa continuously develops from yellowish-white to darker in 4~5 days until the adult hatches. Taken together, it takes around 10 days to accomplish the life cycle from an embryo to an adult at 25 degree (Fig. 1). Meanwhile, the life cycle process is influenced by the temperature since the fruit fly is poikilotherm animals. For instance, it takes 19 to 20 days to develop from fertilized eggs to adults when the flies are cultured at 18 degree. Besides their rapid life cycle, fruit flies produce a great number of offspring, which allow scientists to obtain abundant material. A single female lays eggs at a rate of 50-70 eggs per day when reaching production peak, between the fourth and seventh day after the emergence.

Another relevant feature from flies is that nearly 75% of genes related to human disease are believed to have a functional homolog in *Drosophila* [5]. *Drosophila* is regarded as a valuable model to study various illnesses such as nervous system disorders [6], inflammatory disorders[7], cardiovascular disease[8], cancer[9], and diabetes[10].

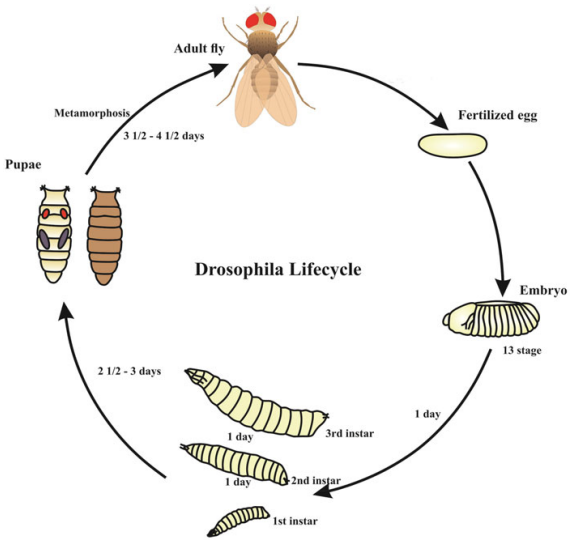


Figure 1. The life cycle of *Drosophila* at 25 degree[11].

Last but not least, *Drosophila* provides an abundant base of knowledge (www.flybase.org) and sophisticated genetic tools as a result of a century of research. Up to now, diverse collection centers of fly stocks exist in the world, such as Bloomington *Drosophila* Stock Center (BDSC), FlyORF, NIG-FLY, Tsinghua Fly Center (THFC), Vienna *Drosophila* Resource Center (VDRC), Korea *Drosophila* Resource Center (KDRC) and Kyoto Stock Center. These valuable and available stock centers provide the researchers convenience and promote the development of the investigations. The strong development of the *Drosophila* field owes a great deal to the practice of sharing fly stocks, even prior to publications. Thanks to all these advantages, *Drosophila* constitutes an invaluable model to study many biological processes, from the level of the gene to that of population and evolution.

1.2 Genetic tools in *Drosophila*

Nowadays, a vast of advanced genetic editing technique are available in *Drosophila*, allowing researchers to develop useful genetic tools. For example, ribonucleic acid interference (RNAi), Clustered regulatory interspaced palindromic repeats (CRISPR)/ Cas9, site-specific transformation via phiC31 integrase system, galactose4 (GAL4)/ upstream activating sequence (UAS) system are widely used. Here I will present the techniques used in my thesis.

1.2.1 CRISPR/Cas9 system

Clustered regulatory interspaced palindromic repeats (CRISPR) [12] were first described in *Escherichia coli* in 1987 and are constantly found in many other bacteria and archaea. Coincidentally, some genes close to CRISPR loci have been described to be involved in the CRISPR system and named CRISPR-associated genes (Cas). Several scientists independently hypothesized that the CRISPR system is part of the immune system of prokaryotes. In 2007, Philippe Horvath and his team working on yogurt cultures first noticed the mechanical action of the CRISPR system responding to viral attacks by bacteriophages [13].

Cas proteins were subsequently found to act as nuclease "genetic scissors". Cas9 is the best studied protein. These proteins function on directionally cutting foreign DNA (some others function on cutting RNA in some cases) with the dangerous genetic information-protospacer adjacent motifs (PAMs) [14]. The PAMs are essential for recognition of the foreign DNA, which is followed by the PAMs. It is impossible to assemble the CRISPR/Cas9 without an important element, the single guide RNA (sgRNA), artificially combines two RNAs, CRISPR-associated RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [14]. The crRNA sequence is 20 base pairs long and complementary to the target DNA, which is necessary and sufficient to guide the CRISPR system to the target DNA. The tracrRNA is essential for the nuclease activity. Basically, the CRISPR/Cas9 system, which consists of the "guide" sgRNA and the "scissors" Cas9, produces double-strand breaks (DSBs) with the PAMs [15].

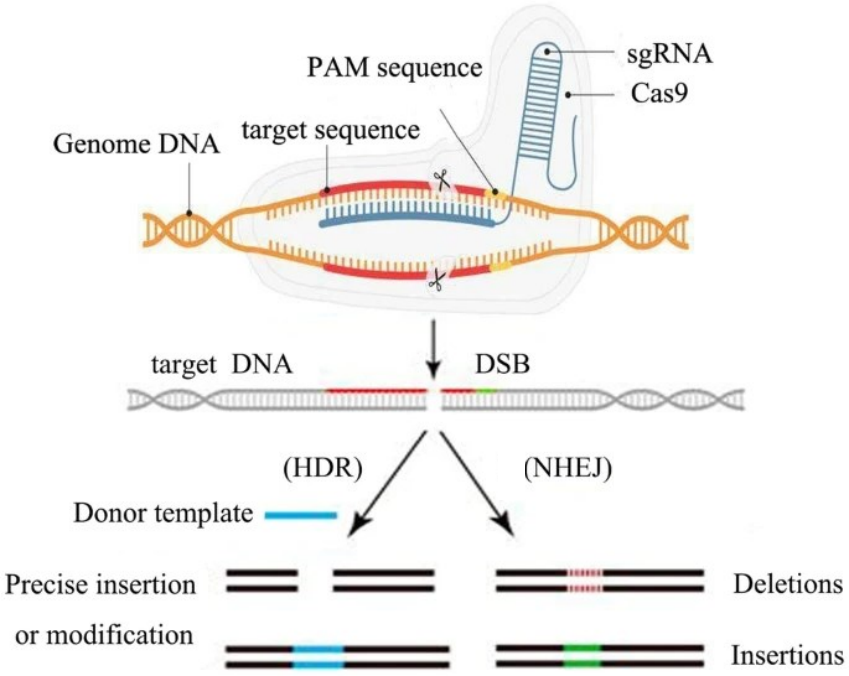


Figure 2. CRISPR/Cas9 genome-editing schematic diagram

Cas9 nuclease with sgRNA complexes can produce specific double-strand breaks (DSBs) in host DNA. The targeted DNA DSBs can stimulate endogenous cellular repair machinery. DSBs are repaired in two ways: *a* homology-directed repair (HDR) using the sister chromatid as template DNA will introduce precise genomic changes in the host's DNA; nonhomologous end joining (NHEJ), which results in the production of insertions and deletions [16].

The DNA damage DSBs can be repaired by cellular DNA repair mechanisms, non-homologous end joining DNA repair (NHEJ) and the homology directed repair (HDR) [15] (Figure 2). However, NHEJ is considered as an imprecise repair mechanism that leads to insertions or deletions in the genome and provides researchers an application for generating the knock-out mutants. On the contrary, HDR is regarded as a mechanism that can accurately repair DNA when a DNA template is provided. The repaired DNA template is considered to contain homologous sequences (homologous arms), which are homologous to the sequences upstream and downstream of the target. The DNA template can be provided from a double strand DNA plasmid called donor vector. However, HDR generally occurs at a low rate, less than 10% but substituted by NHEJ. Therefore, it is essential to improve the efficiency of HDR. It is reported that blocking the function of the NHEJ pathway in flies can effectively improve the occurrence of the HDR. It is found that coupled with a transgenic Cas9 source, HDR was improved effectively in DNA-*Ligase4* mutant germline cells [17]. In addition, they confirmed that approximately 1 kb homology arms designed in donor vector is sufficient to insert a large marker cassette at target gene loci. Here, we applied this advanced technology to the generation of knock-in mutant in *Drosophila* coupling with the mCherry reporter gene inserted in the target gene. A study from Fillip Port and Simon L. Bullock presented a flanking tRNA based vector (pCFD5) (Figure 3) for producing multiple sgRNA that markedly improved gene disruption efficiency [18].

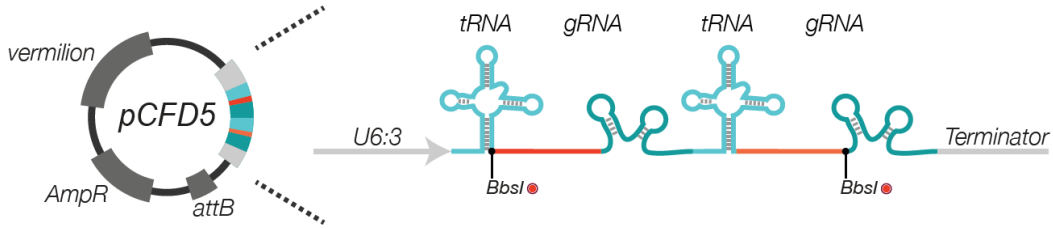


Figure 3. The diagram of pCFD5 vector.

pCFD5 is a vector for expressing one or multiple tRNA-flanked Cas9 gRNAs under the control of the strong, ubiquitous RNA pol III promoter, U6:3. [18]

1.2.2 ϕC31 integrase system

P element insertion was the first technique that allowed the production of transgenic fly line [19]. It is derived from the invasion of P element in the fly genome throughout the world through P-M hybrid dysgenesis. It has been widely used in *Drosophila* ever since. The P element encodes an enzyme called P transposase that enzymatically mediates the transposition between of the DNA present in between two inverted repeats [20]. Wild-type females are found to express an inhibitor of P transposase, which is actually produced by this very element. This inhibitor is able to reduce the disruption of genome. Without the inhibitor, the P elements are risen over all the genome, which results in the disruptions of many genes. Generally, P element insertion coupling with marker such as mini-white is extensively used as useful tool for generating mutations in *Drosophila*. But the disadvantage of this technique is that the insertion site of the P element is random. Thus, the expression of the transgene can be strongly affected by the genomic context of the insertion site, e.g., open vs. closed chromatin. With the development of the technique, an advanced system, ϕC31 integrase system, is able to make up for the shortage.

The bacteriophage ϕC31 from *Streptomyces* encodes an integrase enzyme that inserts the phage genome into the host bacterial genome [21, 22]. ϕC31 integrase recognizes a 39 base pairs long phage attachment site (attP) and a 34 base pairs long bacterial attachment site (attB) and catalyzes an efficient recombination between them [23]. The fly lines containing the attP site were created and allowed the integration of plasmid containing the attB site, which contributed to construct the site-specific insertion of flies.[24] As the integration is generated, the recombination becomes stable and cannot be excised and exchanged in the future. In this way, the ϕC31 integrase system provides an efficient and viable technique to create a reproducible genetic tool in flies, such as reporter flies used in this thesis.

1.2.3 GAL4/UAS system

The GAL4-UAS system is a method widely used to study gene expression and

function in fruit flies [25]. Galactose4 (GAL4), an 881 amino acids long transcription factor, was identified in the yeast *Saccharomyces cerevisiae*. An enhancer element, upstream activating sequence (UAS), was described to be essential for the transcriptional activation of the GAL4-regulated genes. In 1988, Fischer demonstrated that GAL4 could stimulate the transcription of a reporter gene under UAS control as a transcription factor in *Drosophila* [26]. The GAL4/UAS system was developed to express the target gene as a powerful toolkit in *Drosophila* by Andrea Brand and Norbert Perrimon in 1993 [27]. Transgenic GAL4 can be expressed in specific tissue when GAL4 is under the control of a promoter of gene which is expressed specifically in some tissues [28]. Following this, hundreds of thousands of GAL4 driver lines were generated and stocked in collections which are shared all over the world. When UAS were followed by a gene of interest, a simple cross between the GAL4 driver line and the UAS line would allow the gene of interest to be constantly expressed in the targeted tissue. On another hand, when UAS was followed by miRNA or dsRNA of the target gene, which activates the RNA interference (RNAi) and disrupts the transcriptional level of the target gene, a cross with the GAL4 driver line would lead to the decrease of the target gene expression. This system contributes largely to the studies of the function of genes. GAL80 is reported to function on the repression of GAL4 [29] and is identified to be thermo-sensitive, which provides a thermo-regulated system for GAL4 activation. At 18 degree, GAL80 binds to GAL4 and prevents GAL4 to bind with UAS, which results in the inactivation of GAL4/UAS system [27]. When the temperature is increased up to 29 degree, GAL80 will undergo a conformational disruption and release GAL4 to initiate the GAL4/UAS system. The characteristic of GAL80 allows to express gene at a specific stage (Figure 4). For instance, a gene can be specifically expressed after development when shifting to 29 degree after the adult has hatched. In this way, it is possible to exclude the potential impact on development when studying the gene function at the adult stage. The GAL4/UAS system provides a powerful toolkit for gene function study in *Drosophila* and is useful in this study.

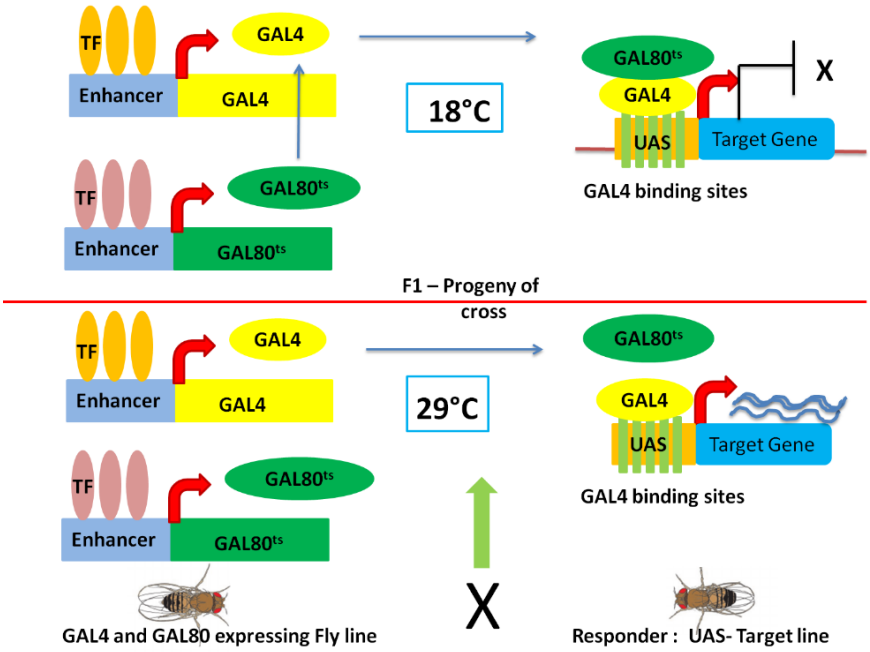


Figure 4. UAS-GAL4-GAL80^{ts} system

At 18 degree, GAL80 binds to GAL4 and prevents GAL4 to bind with UAS, which result in the inactivation of GAL4/UAS system. When the temperature is increased up to 29 degree, GAL80 will undergo a conformational disruption and release GAL4 to initiate the GAL4/UAS system. (Figure adopt from https://iflybio.com/)

1.3 Immune responses in *Drosophila* against fungi or bacteria

Evolution has selected an innate immune system in the first metazoans from which most extant immune systems are derived. Like most species, *Drosophila* defenses depends only on innate immunity which makes it a valuable model to study the interactions between host and pathogens. *Drosophila* possesses a variety of immune responses that provide at least a degree of protection against microbial infections. The first barrier to bear the response of immunity in adult fly is the epithelium such as the cuticle and the gut epithelium which prevents the microbes from invading the body cavity [4]. When the microbes overcome the first barrier of host, other immune responses are activated, including the humoral immune response and cellular response. The systemic humoral response leads to the production and releases large quantity of immune effectors, such as antimicrobial peptides (AMPs) from the fat body (composite between the mammalian liver and adipose tissue) into hemolymph. This process is

mainly under the regulation of two essential NF-κB pathways, Toll and Immune Deficiency (IMD). The cellular response involves phagocytosis/opsonization and encapsulation. In addition, an Arthropod-specific immune defense, melanization complements this response and antimicrobial activity may involve Reactive Oxygen Species (ROS). Here I will give introductions on some immune responses in details which are considered to be important in my study in following chapters.

1.3.1 The production of immune effectors: the systemic humoral immune response

The systemic humoral immune response is under control of the Toll and IMD pathways. Gram-negative bacteria preferentially activate the IMD pathway, while most of Gram-positive bacteria and fungi activate the Toll pathway. The NF-κB pathways get activated upon the sensing of infections through dedicated mechanism.

The IMD pathway (Figure 5) in *Drosophila* is regarded to be similar to the Tumor Necrosis Factor Receptor (TNFR) pathway in mammals, which is involved in the inflammatory response and in cell death [30] [31]. Indeed, most of the factors from the IMD pathway have functional orthologs in the mammalian TNFR pathway. Prof. Charles Janeway [32] proposed the concept of pattern recognition receptors (PRRs) and suggested that the innate immune system relies on germline-encoded receptors. The PRRs have been selected for their ability to detect specific molecular patterns carried by microbes and their bacterial cell wall, cannot be easily altered such as cell wall components, but not from host. In the case of the *Drosophila melanogaster*, IMD pathway, peptidoglycan recognition protein (PGRP) receptors have been shown to specifically bind to a component of the Gram-negative, which is diaminopimelic-type (DAP-type) peptidoglycan (PGN).

PGNs are an essential component of the bacterial cell wall. They are sugar polymers formed by several chains of repeated motives of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide. These chains of GlcNAc

and MurNAc are linked together by short peptidic stems [33]. The type of the peptidic bridge linking the sugar chains provides the distinction between Gram (-) and Gram (+) bacteria. Indeed, PGNs from most of Gram (-) bacteria and countable Gram (+) bacteria for example *Bacillus* species, typically display a meso-diaminopimelic acid (DAP) residue at the third position of the peptidic link, while the PGN chains from many other Gram (+) bacteria possess a Lysine residue at the third position of the peptidic link. Furthermore, the subcellular localization of PGN from Gram (+) and Gram (+) bacteria call wall are different. PGNs from Gram (-) bacteria are forming to a single thin layer, which is actually hidden in the periplasmic space between the inner cytoplasmic membrane and a lipopolysaccharide (LPS) outer layer. In contrast, PGNs from Gram (+) bacteria are multilayered and exposed directly on the surface of the bacteria [33].

Recognition of DAP-type PGN in *Drosophila* is able to trigger the activation of the IMD pathway and depends on several receptors of the peptidoglycan recognition proteins (PGRPs) family. PGRP receptors are well conserved from invertebrates to humans. They are characterized by a 160 amino-acid long PGRP domain, which is similar to the domain of T7 bacteriophage amidase enzymes that degrade PGN. There are 13 different *PGRP* genes in the *Drosophila* genome, which encode at least 17 distinct PGRP isoforms after alternative splicing. They are classified into two types: small PGRP-S receptors (<200 AA) and long PGRP-L receptors (200-500 AA). PGRP-S receptors are thought to be secreted, most of them encoding a signal peptide at the N-terminus. The PGRP-L receptors are thought to be localized at the surface of the cells thanks to the transmembrane domain. However, some PGRP-L receptors (PGRP-LE, PGRP-LB) lacking a transmembrane domain are intracellular or secreted. In addition, some selected PGRP receptors (PGRP -SB, -SB2, -LB, -SCla, and -SC1b) are shown to possess amidase catalytic activity, which allows to degrade PGN and results in negative regulation of the IMD or Toll pathways [34] [35] [36].

Among all of these PGRP receptors, some are involved in the detection of DAP-type PGN that activate the IMD pathway, while others are involved in the detection of Lys-type PGN that activate the Toll pathway. PGRP-LC is the main receptor responsible

for the detection of DAP-type PGN to activate IMD pathway signaling upon Gram (-) bacterial infection or some Gram (+) bacteria with DAP-type PGN like *Bacillus* [37-39]. The PGRP-LC gene is able to be transcribed into PGRP-LCa, -LCx, and -LCy isoforms after alternative splicing. The difference among these isoforms are identical in their extracellular PGN recognition motives [40], but the PGN recognition domain in PGRP-LCy isoform is not functional but acts as a negative regulator for other isoforms. PGRP-LCx functions as a homodimer that recognizes DAP-type PGN polymers, while PGRP-LCa/ PGRP-LCx heterodimers sense monomeric PGN, known as a tracheal cytotoxin (TCT) [41]. PGRP-LC is located at the cell surface, while PGRP-LE is expressed intracellularly owing to the lack of a functional transmembrane domain. PGRP-LE is critical for bacteria recognition, specifically in the midgut [40]. PGRP-LA is described to be essential for the activation of the IMD pathway in the respiratory tract upon bacterial challenge but is dispensable for IMD pathway activation in the fat body [42]. PGRP-SD, as a secreted receptor, was found to enhance the localization of PGNs to the cell surface and promote PGN sensing by PGRP-LC [43].

Following the recognition of DAP-type PGN by PGRP-LC or PGRP-LE receptors, the PGRP receptor oligomerizes and recruits the adaptor protein immune deficiency (IMD) [4]; then, IMD recruits Fas-associated death domain (FADD) through homotypic interaction between the death domains (DD) from IMD and FADD [44]. The caspase Death-related ced-3/Nedd2- like protein (DREDD) then binds to FADD between the death effector domains (DED). Following that, the E3 ubiquitin ligase *Drosophila* inhibitor of apoptosis 2 (dIAP2), the E2 Ubiquitin-conjugation enzyme variant 1A (UevlA), together with Effete and Bendless contribute to deposit K63 ubiquitin chains on DREDD [45]. IMD is cleaved by DREDD and consequently exposes a dIAP2 binding site and recruits the same tetrameric poly ubiquitin complex to poly-ubiquitinated Dredd, which results in the K63-linked polyubiquitin chains being added on the cleaved IMD [46]. Following that, the mitogen-activated protein kinase kinase kinase (MAPKKK), transforming growth factor beta (TGF-β)-activated kinase1 (TAK1) and TAK1-associated binding protein 2(TAB2) are recruited [47] and activate the

Inhibitor of NF-κB Kinase (IKK) complex to activate the IMD pathway [48] [49].

The IKK complex consists of the catalytic IKKβ subunit and the IKKγ regulatory subunit, named kenny [50]. The IKK complex has been shown to be required for Relish cleavage and to phosphorylate Relish at two sites, on Serine 528 and Serine 529; actually, Relish phosphorylation is important to make the transcriptional activation domain functional. The actual cleavage reaction is performed by the DREED caspase [51]. However, the phosphorylation is disposable for the cleavage of Relish [46]. Relish possesses a Rel homology domain at the N terminus, and ankyrin repeats at the C terminus [52]. The N-terminus part of Relish (Rel68) is released upon cleavage and translocated into the nucleus [45]. Rel68 in dimer binds to its recognition sequence,

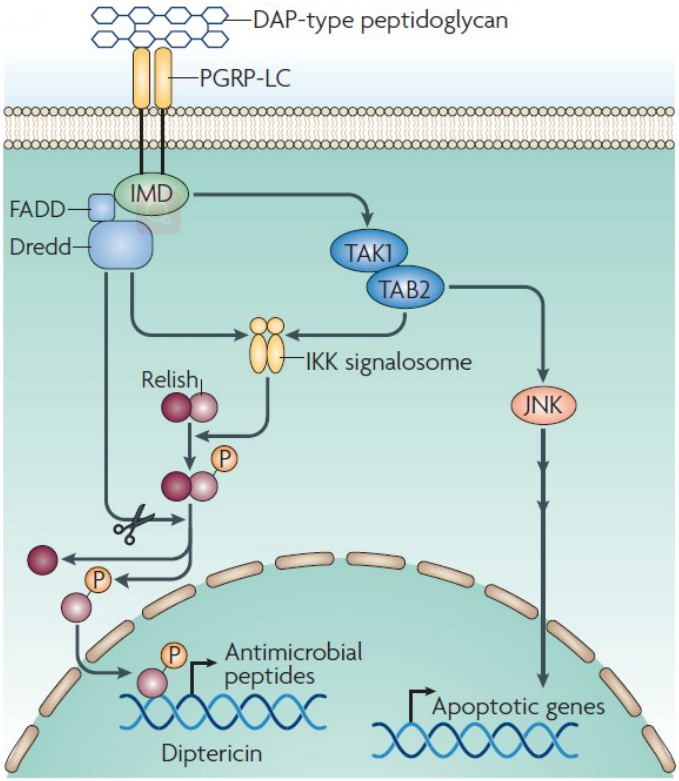


Figure 5. The activation of IMD pathway in *Drosophila*. [53]

The activation of the IMD pathway is initiated by the reorganization by PGRP-LC and results in the binding of IMD and recruitment of two other composites, dFADD and DREDD. The cleavage of IMD by the activated DREDD processes into the recruitment and the activation of TAB2/ TAK1 complex, which followed by the phosphorylation and activation of IKK complex with the consist of Kenny and IKKβ. The transcriptional factor Relish is cleaved by IKK complex or directly by DREDD and releases the N-terminus fragment (Rel-68), which translocates into the nucleus and triggers the transcription of another set of the immune effectors, including *Diptericins*, *Cecropins*.

termed κB response elements which is determined to be 5' GGGGATTYYY 3' [54]. This conserved sequence is found in the promoter of abundant genes regulated by the IMD pathway, such as the *Attacins*, *Cecropins* and *Defensins* [52]. Furthermore, Relish can form heterodimers with Dorsal or Dif, the NF-κB factors of the Toll pathway upon the simultaneous activation of both the IMD and Toll pathways, which happens upon infections by pathogens such as *Pseudomonas aeruginosa* [55]. Dif/Relish or Dorsal/Relish heterodimers is determined to bind the consensus sequence 5' GGGA (A/T) TC (C/A) C 3' in this case [56]. Following the binding with the transcriptional factors and recruitment of effectors, a set of genes are initially transcribed downstream of IMD pathway. Akirin, a transcriptional co-activator in Relish activity, is described to be required for the transcription of several IMD pathway target genes, such as *Attacin A* and *Diptericin A* but not *Attacin D* or *Cecropin A2* [57]. Akirin has been shown to physically interact with the NF-κB factor Relish and with members of the Osa-containing SWI / SNF chromatin remodeling complex [58].

The Toll pathway (Figure 6) was first described to be involved in early embryonic development [59] and was only found to play an essential role in immunity afterwards. The Toll pathway in *Drosophila melanogaster* is similar to the TLR signaling pathway in mammalian animals. In contrast to mammalian TLRs, the Toll receptor in *Drosophila* does not function as a direct microbial sensor. Instead, the activation of the Toll pathway in *Drosophila* is mediated by extracellular PRRs circulating in the hemolymph, including the PGRP family and the Gram-negative bacteria binding proteins (GNBPs) family. GNBP receptors are characterized by an N-terminus β-glucan-binding domain with β-glucanase domain at the C terminus [60].

The Toll pathway is initiated from the recognition of Lys-type PGNs from Gram (+) bacteria or β-glucans on the surface of fungi by a set of PRRs. PGRP-SA and GNBP1 are thought to recognize Lys-type PGNs from Gram (+) bacteria [61]. Whereas, it is reported that GNBP1 presents a processed form of PG for sensing by PGRP-SA and that a tripartite interaction between these proteins and PG is essential for

downstream signaling [62]. Beyond that, PGRP-SD has also been proposed to recognize Lys-PGN from Gram (+) bacteria [63]. Yet, structural studies demonstrated that PGRP-SD is able to bind DAP-type PGNs [64] and acts as a co-receptor upstream of PGRP-LC to recognize DAP-type PGN [43]. As regards fungi, the circulating receptor GNBP3 has been shown to recognize β-1,3- glucans from fungi [65] and to activate Toll signaling [66].

The direct recognition of bacterial and fungal components by these PRRs triggers a proteolytic cascade occurring in the hemolymph that will ultimately lead to the cleavage of pro-Spätzle into the active Spätzle able to activate the Toll receptor. The sensing of Lys-type PGN or β-1,3-glucans by the PRR results in the activation of the Modular Serine Protease (ModSP) [67]. In the wake of the activation of ModSP, another serine protease, Gram positive Specific Serine Protease (Grass), is activated [68] [69]. Following the action of Grass, the activation of Spätzle processing enzyme (SPE) is mediated by the redundant protease Persephone (PSH) and Hayan [70].

Beyond this, the serine protease PSH is able to directly cleave SPE. PSH, like other proteases of the immune cascade, is characterized to be expressed as an inactive zymogen that requires cleavage by microbial proteases to expose its active form. Up to now, some microbial proteases have been found to cleave PSH, such as the secreted fungal virulence factor PR1 from *Metarhizium anisopliae* and *Beauveria bassiana* [65], or proteases from Gram (+) bacteria *Bacillus subtilis* and the fungus *Aspergillus oryzea* [69]. In addition, several secrete proteases from Gram (-) bacteria species, including *Pseudomonas* and *Serratia*, are able to activate the Toll pathway by cleaving PSH [55]. The sensing of microbial protease through PSH bait domain leads to the formation of a cleaved pre-activated inactive PSH that gets further processed by the circulating 26-29-p cathepsin protease to a mature PSH. PSH can also be directly activated by subtilisin [71] (Figure 6). In general, PSH actually is an immune sensor with a specific domain that generally serves as a cleavage site for all exogenous proteases.

After its proteolytic cleavage, PSH, directly cleaves SPE, which is processed to cleave pro-Spätzle, leading to the release of a functional C-terminus end of Spätlze.

Spätzle dimmers bind to the extracellular domain of Toll and provoke the dimerization of two Toll receptors through their leucine-rich repeats (LRR) domains, which then trigger the intracellular signaling cascade [72].

There are nine Toll receptor genes (*Toll-1 to 9*) in *Drosophila*. It is indicated that over-expressing *Toll-5* and *Toll-9* is sufficient to induce the expression of several genes downstream of the Toll pathway, such as *Drosomycin* and *Metchnikowin*, which are thought to play roles in the immune response [73]. Toll-1 (also known as Toll) is the well-known receptor involved in the activation of the Toll pathway[74] [75]. Toll is a transmembrane protein containing a Leucine-rich repeat extracellular domain, a single-span transmembrane region and an intracellular Toll/Interleukine -1 Receptor signaling (TIR) domain [76].

Following activation, Toll recruits the adaptor protein Myeloid differentiation primary response gene 88(MyD88) with its intracellular TIR domain through homotypic interaction [76]. Subsequently, Tube and the kinase Pelle are recruited to MyD88 to form a complex through interactions with their death domains (DD) [77]. Tube is described to act as a scaffold protein for MyD88 and Pelle, which are not able to bind to each other with their distinct DD domains [78]. Following that, the *Drosophila* Inhibitor of κB (IκB) protein Cactus, which binds to the NF-κB factors Dif and Dorsal and kidnaps them in the cytoplasm, is then phosphorylated by Pelle at two distinct sites in its N-terminus. After its phosphorylation, Slimb, a member of the B-TrCP ubiquitin ligase family, mediates the degradation of polyubiquitinated Cactus, which result in the release of Dorsal or Dif from Cactus [79]. As a consequence, the free NF-κB factor Dorsal or Dif is translocated to the nucleus and initiates the transcription of immune-related genes.

Dif and Dorsal contain a Rel homology domain (RHD), which is responsible for their transcriptional activity. Dorsal and Dif are described to function as homodimers or heterodimers [56]. In fact, Dorsal is indicated to be involved in Toll signaling during early development and immune response in larvae and embryonic S2 cells, while Dif is only found to be involved in the immune response in adult flies [80] [81] [82]. Similar

to Relish, Dorsal and Dif bind to their kB response sequence, GGGAAA(A/T/G) YCC, to activate the transcription of hundreds of Toll downstream genes , such as the antifungal peptides genes *Drosomycin* or *Metchnikowin* [4] [54].

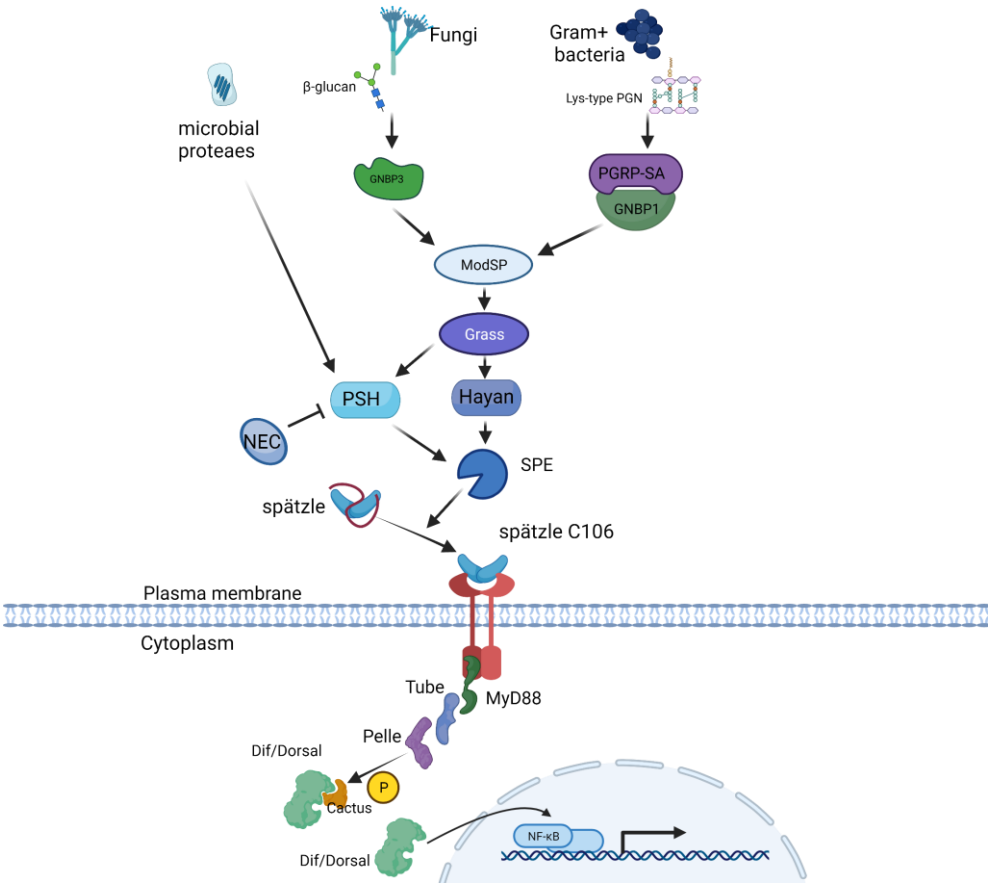


Figure 6. The activation Toll pathways in *Drosophila*

Toll pathway can be activated by the recognition from PRRs or the cleavage of PSH. Both results in the activation of the SPE and the cleavage of the Pro-Spätzle into Spätzle (Spz). The activated Spz binds to the Toll receptor and recruits MyD88, which lead to the recruitment of Tube and Pelle kinase and followed by the phosphorylation and degradation of Cactus. The transcriptional factor Drosal or Dif, which is sequestrated by Cactus, is released and translocate into the nucleus to initiate the transcription of amounts of immune effectors, such as *Drosomycin*. (Figure is adapted from the thesis of Jianqiong HUANG)

Immune effectors are induced and secreted into hemolymph as a response that defense against the microbes in the host, including antimicrobial peptides (AMPs), Bomanins, Daisho, Baramicin A as described in the following. They are described to possess a broad range of antimicrobial activities against Gram (-) bacteria, Gram (+)

bacteria and fungi. In addition, some of them have recently been found to be required for the host defense against virulence factors produced by pathogens.

1. Antimicrobial peptides

Among the immune effectors, AMPs are the better characterized ones. AMPs are those are relatively small peptides, only between 12 and 50 amino acids in length. They include a large proportion of hydrophobic amino acids and multiple arginine and lysine residues, conferring them a net positive charge that allow them to interact with negatively charged cytoplasmic membranes [83-85]. AMP genes are well conserved through evolution as the orthologs of these immune effectors can be easily found in diverse invertebrate and vertebrate species, even in plants and in prokaryote organisms [4]. “classical” AMPs are divided into seven families, Drosomycin (Drs), Defensin (Def), Drosocin (Dro), Metchnikowin (Mtk), Diptericins (Dpt-A and -B), Cecropins (Cec-A1, -A2, -B, -C and Andropin), and Attacins (Att-A, -B, -C and -D). They are described to possess a broad range of antimicrobial activities against Gram (-) bacteria, Gram (+) bacteria and fungi [86]. Some of them are reported to play a role in the disruption of membrane or targeting of bacterial cytoplasmic compounds. Owing to their amphipathic nature and positive charge, some AMPs are able to bind to bacterial membranes, which are generally negatively charged. As a consequence, pores are formed on the membrane of bacteria, leading to the disruption of membrane integrity, depolarization, and ultimately cell death [83]. It has been shown that different AMPs play distinct roles against different microbes. Attacins, Cecropins, Diptericins and Drosocin have been shown to target Gram (-) bacteria [83, 87], while Defensin is the AMPs with in vitro activity against Gram (+) bacteria. Besides, Drosomycin and Metchnikowin are efficient to kill the invading fungi [88, 89]. Interestingly, some AMPs pocess broad antimicrobial activities, while others show a remarkable specificity in the response against a pathogen. A study from the team of Prof. Bruno Lemaitre, using individual and multiple knockouts, including lacking some or all these ten AMP genes, has revealed that some AMPs act specifically against certain pathogens, for example, DptA is the only AMPs responsible against the Gram (-) bacteria *Providencia rettgeri*,

which is consistent to the previous study [90] and *Drosocin* is specifically required for the defense against the Gram (-) bacteria *Enterobacter cloacae*. In addition, some AMPs act additively and synergistically against pathogens, for example, Dpts and Atts have a synergistic effect against the Gram (-) bacteria *Providencia burhodogranariea,* while Mtk and Drs contribute additively to the defense against the yeast *Candida albicans* [91]. IMD-dependent AMPs, do defend against Gram (-) bacteria whereas they do not appear to play such important role in the host defense against Gram (+) bacterial and fungal infection. Once the AMPs are produced and secreted in hemolymph, the AMPs can persist in the hemolymph for several weeks. They can reach concentrations from 0.5μ M (Diptericins) to 100 μM (Drosomycin), while their combined concentration can reach 300 μM [92].

2. **Bomanins**

Except for these well-characterized AMPs, other diverse immune effectors are secreted upon an immune challenge. A mass-spectrometry analysis was performed on the hemolymph sample from a single fly upon the immune challenge. The results showed that more than 30 peaks, regarded to be the *Drosophila* immune-induced molecules (DIMs), were identified [93]. Some of them correspond to some known AMPs described above, whereas some of them belong to a family that contains a Bomanin domain [94]. There are ten Bomanins (Boms) related genes located on the second chromosome on band 55C. Most of the Bomanins at the 55C locus are short (BomS), the secreted form of which essentially contains a single domain characteristic of this family of peptides. Other members include a tail after the Bomanin domain (BomT), whereas bicipital members are characterized by the inclusion of two domains separated by a linker domain (BomBc). It has been reported that *Bombardier* is required to stabilize the expression of short-form *Boms* but not required for the stability of three bicipital *Boms*. The deficiency of *Bombardier* leads to the reduction of survival as *Boms* mutants [95].

Surprisingly, the deletion of a locus encoding ten Bomanins at the 55C locus largely phenocopies the *Toll* mutant phenotype, even though the Toll pathway regulates

the expression of more than 150 genes [94]. This suggests that these peptides are somehow involved in mediating the defenses resulting from Toll pathway activation. A recent study suggests that some BomS are likely active against *Candida glabrata* and can function somewhat redundantly [96]. Another study from R. Xu et al. indicates that *BomS6* is required for the host against some mycotoxins from *Aspergillus fumigatus*, including Verruculogen and Restrictocin [97]. Nevertheless, the exact function of most Bomanins in host defense remains to be defined.

3. Daisho

Daisho1 (also known as IM4) is 15-amino acid (aa)-long peptide, and IM4 and Daisho2 (also known as IM14) is 24 aa long. Based on the finding from the team of Steven A. Wasserman [98], they have named them Daisho1 and Daisho2, for 大小, the Japanese term for a matched pair of samurai swords, one short and one long. Both peptides are found in hemolymph following Toll pathway activation. Through generating a CRISPR/Cas9 knockout mutants of both genes, *daisho1 and daisho2*, they found that the Daisho peptides are required for defense against a subset of filamentous fungi, including *Fusarium oxysporum*, but not other pathogens, such as *Enterococcus faecalis* and *Candida glabrata*. The *daisho* genes are each required for defense. They also found the interaction between epitope-tagged Daisho2 peptide in hemolymph and the hyphae of *F. oxysporum* in vitro. Altogether, these results identify the Daisho peptides as other innate immune effectors with humoral activity against a select set of filamentous fungi.

4. Baramicin A

Several DIMs (5, 6, 8, 10, 12, 13, 22, 24) are actually derived from a polyprotein precursor known as IMPPP via furin cleavage sites, and until recently, their function has not been understood [99]. In a recent study, they renamed this protein as Baramicin A (BaraA) a for the Japanese idiom Bara Bara (バラバラ), meaning “to break apart” a reference to the fragmenting structure of the Baramicin precursor protein and its many peptidic products, as well as the locus is actually duplicated in several wild-type strain including the one that was originally separated. It has been found *BaraA* is strongly

immune-induced in the fat body downstream of the Toll pathway but also exhibits expression in other tissues. They show that flies lacking *BaraA* are susceptible to the entomopathogenic fungus *Beauveria bassiana*. Consistent with *BaraA* being directly antimicrobial, overexpression of *BaraA* promotes resistance to fungi, and the IM10-like peptides produced by *BaraA* synergistically inhibit the growth of fungi in vitro when combined with a membrane-disrupting antifungal [100].

Another study from J. Huang et al. has revealed that *BaraA* mutant exhibits sensitivity to entomopathogenic fungi *Metarhizium robertsii* and Gram-positive bacteria *E. faecalis*, but not to other pathogens. Interestingly, the pathogen burden does not appear to be altered in the mutants upon *M. robertsii* but upon *E. faecalis*, which indicates that BaraA is not involved in targeting *M. robertsii* themselves but that it is required in the resistance to *E. faecalis*. Besides, we found a major function of *BaraA* is in the resilience against distinct toxins, Destruxin A (DtxA), a pore-forming toxin, and Enterocin V (EntV), a bacteriocin, respectively, secreted by *Metarhizium robertsii* and *E. faecalis*. BaraA helps the host recover from DtxA-induced paralysis and appears to be required in glial cells but not in neurons [1].

In the study of the interaction between *Drosophila* and pathogens, there are two concepts were provided, **resistance** and **resilience** (also known as tolerance). Resistance describes the host's ability to acts on the pathogen itself and reduce the microbial load, while resilience describes the ability to acts on the detrimental effects exerted by pathogens on the host own immune response, pathogens but not directly decreasing the microbial load.

Toll and IMD pathway mutants exhibit a stable and strong sensitivity to certain pathogens. It suggests that the immune effectors under the control of both pathways provide critical functions in *Drosophila* defense against pathogens. There are numerous genes under the regulations of both pathways. For example, the Toll pathway regulates the expression of at least 150 genes. Based on what was found for some immune effectors described in this review, it is revealed that these immune effectors are involved

in the resistance and resilience. Some immune effectors, such as AMPs and Daisho, function on pathogens directly and are considered to function in resistance, while some others such as Bomanins act in resilience and are involved in the host defense against the virulence factors produced by pathogens. Interestingly, BaraA play a role both in resistance and resilience upon the infections of *E. faecalis* possibly through distinct BaraA derived peptides. We consider that the pathogens beat down the flies with a high burden of pathogens and a high toxicity in flies. Plenty of immune effectors are considered the critical part of the immune response in *Drosophila* in killing the pathogens or fighting against the virulence factors from pathogens. When knocking out one or few genes encoding the immune effector, for example BaraA, always exhibits a subtle susceptibility comparing to the mutant of signaling pathway, which indicated that there are some immune effectors still remaining to be charactered and discovered.

1.3.2 Phagocytosis: a cellular response

Phagocytosis is described to engulf small particles, apoptotic bodies or invading pathogens by plasmatocytes which are rich in adults among hemocytes. 90% of the plasmatocytes are sessile, while 10% are circulating [4]. Phagocytosis is normally initiated by the receptors recognizing the molecules exposed on the surface of pathogens or apoptotic cells, for example, the PGNs, lipopolysaccharides (LPS) or l β-1,3 glucans from the pathogens and the phosphatidylserine (PS) from apoptotic cells. Studies have revealed plenty of receptors involved in phagocytosis [101] [102]. They can be classified into four types, Scavenger receptors (SRs), PGRPs, Nimrods and Integrins. The class C scavenger receptor I (dSr-CI), similar to mammalian class A SR, is expressed on the surface of the plasmatocytes and involved in the recognition of both Gram (+) and Gram (-) bacteria, but not yeast. Croquemort (Crq), a class B SRs with homology to the mammalian CD36, was shown to function as a receptor for apoptotic cells and the recognition of the Gram (+) bacteria *Staphylococcus aureus (S. aureus)*[103]. Peste, another class B SRs, is required to engulf Gram (+) bacteria *Mycobacterium fortuitum* and *Listeria monocytogenes*, but not *Escherichia coli (E. coli)* or *S. aureus*. PGRP-LC, is involved in the uptake and phagocytosis of Gram (-) bacteria

but not Gram (+) bacteria. Draper, a member of the Nimrod family, has been identified to function in the clearance of apoptotic cell conservatively and the phagocytosis of both *S. aureus* and *E. coli* bacteria. NimC4, also called Six-Microns-Under (SIMU), acts upstream of Draper, triggering the engulfment of apoptotic cells [104, 105]. Eater and NimC1, another type of Nimrods family, are essential for the phagocytosis of bacteria in flies. Knocking out the two genes resulted in the deficiency of phagocytosis to all types of bacteria [106]. Eater plays a dual role as a phagocytosis receptor and is adhered to tissue [107]. Whereas Eater has been proposed to bind to bacteria in general [108]. The study of an eater KO mutant suggested a role only in the phagocytosis of Gram (+) bacteria. Yet, eater is required for the phagocytosis of Gram (-) bacteria such as *S. marcescens* and *P. aeruginosa* that has escaped from the digestion tract [108, 109]. They likely display altered surface properties. αPS3 and βv integrin subunits function as a heterodimer in the phagocytosis of both apoptotic cells and *S. aureus* bacteria [110].

It is worth mentioning that, beyond these receptors, opsonins are molecules that bind to microbes and contribute to the engulfment by macrophages in mammals. Opsonins act as adaptors between microbial surfaces and immune phagocytic cells. According to the structure, six thioester-containing proteins (TEPs, TEP1-6) in *Drosophila* are related to the mammalian complement factor C3 family that act as opsonin [111]. Phylogenetically, TEPs share sequence similarities with both the vertebrate complement factors C3/C4/C5 and the α_2-macroglobulin family of protease inhibitors. Most TEPs share the common 4-amino-acid sequence (CGEQ) defining the thioester site, which allows the formation of covalent bond to microbial surface [112]. Compared to other insects, the TEPs in *Drosophila* are less described. In mosquito *Anopheles gambiae*, TEP1 has been described as an opsonin involved in the phagocytosis of bacteria, the lysis of the malaria parasite *Plasmodium ookinetes* as well as entomopathogenic fungi, and the melanization of entomopathogenic fungi. Besides, TEP1 also plays a role in removing damaged cells during spermatogenesis [113]. TEP1 is described to bind to the surface of bacteria or *Plasmodium ookinetes* [41]. In *Drosophila*, TEP1-TEP4 are thought to function as opsonins, while *TEP5* is thought to

be a pseudogene and TEP6 is involved in the formation of septate junctions in the gut [114]. Most of the *TEPs* are expressed in plasmatocytes, fat body, and some barrier epithelia and secreted in hemolymph. However, two isoforms of the *TEP4* lack of a signal peptide suggesting that they encode intracellular proteins, and TEP3 is considered to be anchored to the plasma membrane owing to a predicted glycosyl-phosphatidylinositol (GPI)-anchoring site at the C-terminus. It was suggested that *Tep2*, *Tep3* and *Tep6* are required for phagocytosis of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* respectively when using RNA interference (RNAi) silencing in S2 cells [115]. However, the deficiency of *Tep2*, *Tep3* and *Tep4* were found with no impact on survival upon infection with several species of bacteria and a fungus [112]. A study by using the entomopathogenic nematode *Heterorhabditis bacteriophora* in *Drosophila* larvae indicates that TEP3 but not TEP2 and TEP4 are required for host defense [116]. However, it is found in *Drosophila* adults, TEP1, 2, 3 and 4 proteins are required in the defense against fungi, Gram (+) bacteria and parasitoid wasps. These TEPs have been shown to regulate the Toll pathway and phagocytosis of some Gram-positive bacterial strains [117]. The team of Ioannis Eleftherianos have demonstrated that the mutants of Tep2, Tep4 or Tep6 exhibited the resistance to *Photorhabbus* species which could be due to the increased AMPs expression, decreased phagocytosis or increased melanization leading to the higher metabolism [118, 119].

The recognition of the receptors leads to F-actin branching at the engulfment site and generating a phagosome. The phagosomes become mature through a series of fission and fusion events with cellular organelles (endosomes and lysosomes) and leads to the formation of a highly acidic phagolysosome. Finally, the phagolysosome fuses the final particle with the important degradative enzymes such as DNases and proteases to finish the destruction.

1.3.3 Melanization

Melanization has been characterized to be an important immune response of arthropods. It is the immune response that is described to deposit a black pigment at the

wound site that is catalyzed by phenol oxidases (POs) that are activated from prophenol oxidases (PPOs) by the proteolytic processing of the PRO-domain [120]. The sensing of infections by PRRs initiates melanization through the activation of the proteolytic cascades, which include the Hayan protease, that ultimately process PPOs into active POs. For instance, PGRP-LE is involved in melanization from the report that overexpression of PGRP-LE led to constitutive melanization, while the mutation in PGRP-LE blocked PO activation after *E. coli* infection [121]. Overexpression of full-length or PGRP domain-deleted PGRP-LC caused a mass of melanization in larvae and adults [122] (Figure 6). How a membrane-bound or an intracellular receptor manage to activate an extracellular proteolytic cascade remains to be defined. As regards Gram-positive bacteria and fungi, it has been shown that GNBP3, an extracellular sensor, activates melanization through the cleavage of PPO in a Toll-independent manner upon the challenge with *Candida albicans* and *Beauveria bassiana* [123]. The genetic overexpression of both *PGRP-SA* and *GNBP1* is sufficient to trigger melanization. It has further been shown by several biochemical methods that GNBP3 assembles a complex that comprises PO and the Necrotic serpin, which suggests that GNBP3 may muster an attack complex directed at the invading pathogen [123] (Figure 6).Besides, studies have revealed that clip proteases, melanization protease 2 (MP2, also known as sp7 or PAE1) [124] but not melanization protease 1 (MP1) [70] is involved in the activation of melanization.

PPOs are encoded by three genes in *Drosophila*, *PPO1*, *PPO2* and *PPO3*. A study of *Drosophila* hemocytes reported that larval PPO1 and PPO2 are mainly expressed in crystal cells, while PPO3 is expressed in lamellocytes [125]. It has been shown that PPO1 and PPO2 are responsible for all the PO activity in the hemolymph [126]. A study on null mutants of PPO genes has revealed that melanization is critical for the resistance of flies to Gram-positive bacterial and fungal infections; however, the pathogen burden was not altered in all the infection [126]. Another study has shown that PPO1 is essential to the flies upon the infection of low doses of *Staphylococcus aureus*. Unexpectedly, loss function of serine protease *Hayan* flies, which almost completely lack the

blackening reaction, were still as resistant to *S. aureus* as wild type [70] (Figure 6). Thus, the blackening reaction can be separated from a microbial killing activity. Instead, the production of reactive oxygen species (ROS), which is toxic to the pathogens, may be involved in mediating this killing activity.

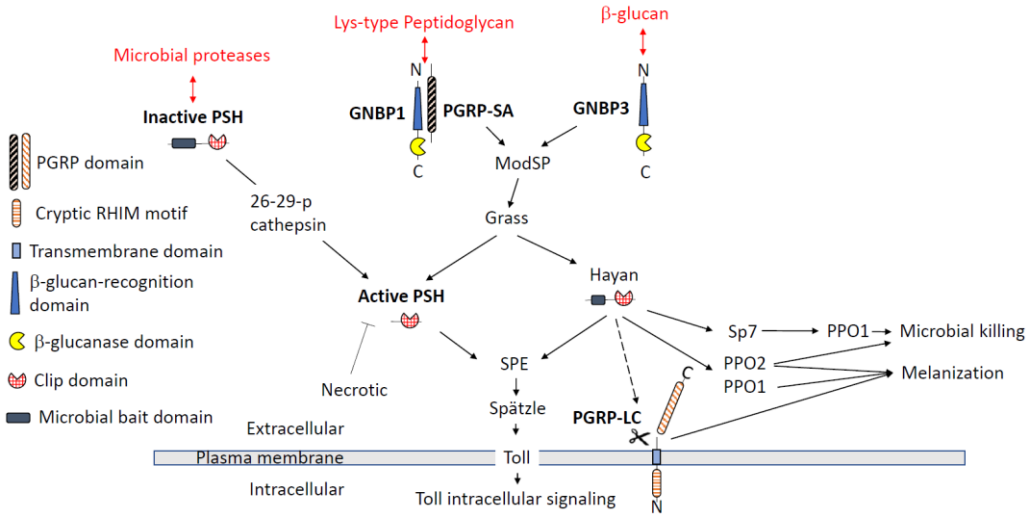


Figure 6. The activation of Toll extracellular signaling and the cascade of melanization. [127]

At the beginning of melanin biosynthesis, phenylalanine hydroxylase (PAH) contributes to the conversion of hydroxylate phenylalanine into tyrosine in the presence of tetrahydrobiopterin (BH4). Tyrosine is then converted to 3,4-dihydroxyphenylalanine (DOPA) through PO or tyrosine hydroxylase (TH). Following this, DOPA is converted to dopaquinone by PO or to dopamine by dopa decarboxylase (DDC). Dopaquinone can convert non-enzymatically to dopachrome, which is subsequently decarboxylated to 5,6-dihyroxyindole (DHI) by dopachrome conversion enzyme (DCE). Finally, after a few reaction steps, both DHI and dopamine can be converted into melanin, a PO-dependent process (Figure 7) [128].

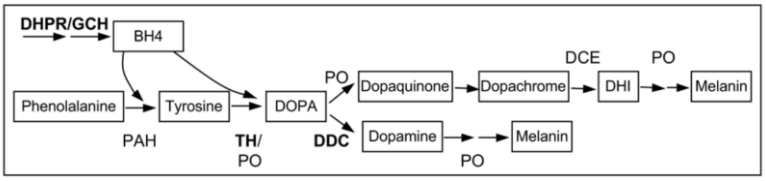


Figure 7. The biosynthesis of melanin [128]

1.3.4 ROS

The production of reactive oxygen species (ROS) is well conserved during evolution. The ROS consist of hydrogen peroxides (H_2O_2), hydroxyl radical (OH), superoxide anion (O_2^-), hydroxyl chloride (HOCl), nitric oxide (NO) and singlet oxygen (O_2) [129]. They play roles in acting against a variety of cells, including microorganisms, in an unspecific manner through their high reactivity. At low doses, they can be used as Redox signaling molecules. In *Drosophila*, it is described that the production of ROS is mainly contributed from the enzymes Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) [130] and dual oxidase (DUOX) [131]. Other important sources may generate ROS, for instance the electron transport chain in mitochondria.

DUOX is a transmembrane protein located in different cells and able to catalyze the synthesis of extracellular ROS. The expression of DUOX is regulated by the uracil-dependent activation of the mitogen-activated protein (MAP) kinase pathway. During the invasion, Gaq-PLCB triggers P38a phosphorylation through activating MEKK1/MKK3 kinases, which results in the transcription Factor 2(ATF2) translocating into nucleus and launching the transcription of DUOX. Besides, Gaq-PLCB is able to hydrolyze phosphatidylinositol 4,5- biphosphate (PIP2) and generate inositol 1,4,5- triphosphate (IP3), which will lead to the release of Ca2+ and then activates DUOX activity regard to the calcium-sensing EF-hand domain [132] [133]. NOX, which is less-studied, is shown to produce intracellular ROS at the level of phagosomes. It has been shown that the ingestion of *Lactobacillus plantarum* in *Drosophila* larvae generates the NOX-dependent ROS and leads to the proliferation in intestinal stem cells [134]. Besides, in adults, *Lactobacillus plantarum* triggers NOX activation and leads to intestinal damages [135]. The ROS contribute to limiting the invading microorganisms but could be deleterious to host cells simultaneously since the ROS act indiscriminately on microorganisms as well as on host cells.

1.3.5 JNK pathway

The JNK pathway is an conserved eukaryotic signaling response that plays a crucial role in the development of flies [136] [137]. This pathway is one of the three *Drosophila* mitogen activated protein kinase (MAPK) pathways, along with extracellular regulated kinase (ERK) and p38 MAPK pathways [136] [138]. The activation of the JNK pathway is triggered by several stressors such as UV irradiation, reactive oxygen species (ROS), DNA damage, heat, infections and inflammation [139] [140]. These stressors stimulate the activation of a JNK Kinase Kinase (JNKKK), such as Mixed Lineage Kinase 2 (MLK2) [140], Apoptotic Signal-regulating Kinase 1 (ASK1) [141], MEK Kinase 1 (MEKK1) [142] and TAK1 (after IMD pathway activation) [48] present in the cytoplasm (Figure 8). As a consequence, JNKKK phosphorylates and activates two JNK Kinases (JNKK) that act in parallel, Hemipterous (Hep) [143] and dMKK4 [144]. Then, these two JNKK phosphorylate the MAP1 Kinase Basket (Bsk), which targets several transcription factors in the cytoplasm, such as Jun and Fos [138] that belong to the AP-1 transcription factors family and the Forkhead box O (FOXO) [145]. The phosphorylated Jun, Fos and FOXO are translocated to the nucleus, where they activate numerous target genes such as Reaper and Head Involution Defective (HID) (for apoptosis) [139] or unpaired (for proliferation) [146]. The function of JNK varies depending on the stimuli and is context-specific. In fact, the JNK pathway can promote apoptosis, autophagy, resistance to ROS, metabolism, cell proliferation and tissue repair [139]. Of note, Puckered, one of the Activating Protein -1 (AP-1) transcriptional targets, is a central negative regulator of the JNK pathway. Once expressed, Puckered is a Basket-specific phosphatase that restricts the activity of the JNK pathway in a negative feedback loop [147].

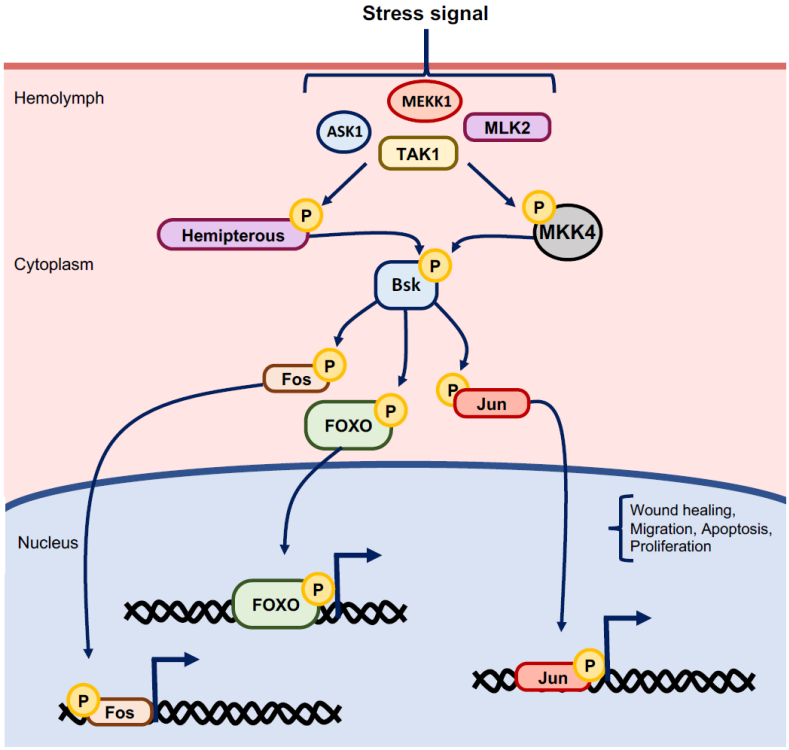


Figure 8. The JNK pathway.

(adapted from the thesis of Dr. Bechara SINA RAHME)

2. Pathogenic microorganisms

2.1 *Metarhizium robertsii*

Metarhizium species, which are part of the estimated 1.5 million entomopathogenic fungal species [148], is used worldwide as a chemical insecticide in agriculture [149]. It belongs to the order Hypocreales of the Ascomycota phylum. It was first described in 1897 by Metchnikoff and used as a microorganism to infect the wheat grain beetle *Anisoplia austriaca*. *Mertarhizium robertsii* was previously called *Metarhizium anisopliae*, which is used in this study. *Metarhizium* is thought to infect insects with conidia by contacting the surface of body in nature. Once they have crossed the cuticle and invaded the hemocoel, the invading fungus will produce blastospores (also known as hyphal bodies), which can proliferate efficiently in hemolymph without forming hyphae; they ultimately kill the host fly [150] (Figure 9). We can perform the

infection on flies using different models: “natural infection” and septic injury. It is worth to mention the technique of the former infection model that consists in mixing the anesthetized flies into a spore solution for 30 seconds, which contrasts with the direct injection of spores into the hemocoel.

An analysis of seven *Metarhizium* species with different host ranges indicated that specialists evolved first, followed by transitional species with intermediate host ranges and then the most recently diverged generalists. This speciation/evolution trajectory was paralleled by insect host speciation [151]. The analysis of the genomes of insect pathogenic fungi has revealed on one hand the existence of hundreds of putative secreted proteins, most predicted to be proteases, and on the other hand the presence of dozens of secondary metabolic gene clusters, mainly nonribosome peptide synthetase (NRPS), polyketide synthase (PKS), NRPS-PKS hybrids, and terpene synthases. The deletion of NRPS or PKS genes for cyclopeptide destruxin biosynthesis in *M. robertsii* revealed that these genes are required for fungal virulence [152]. It has been identified that a NRPS gene cluster is responsible to the biogenesis of Destruxins (Dtxs) [153]. Dtxs were first characterized by Yoshinori Kodaira in 1961 from *Oopsra destructor*. As cyclic hexadepsipeptides, Dtxs have been shown to dispaly a variety of detrimental effects, such as V-ATPase inhibition, and insecticidal, cytotoxic, antiviral, immunosuppressant properties. The Dtxs may act as insecticides through their ionophoric properties: they are able to form complexes with specific cations and cause the selective permeability for the complexed ions across the membrane [154] [155]. As recorded, in the interaction with host insects, the Dtxs can cause host membrane depolarization due to the opening of calcium channels in muscle resulting in tetanic paralysis and possibly thereby inhibit cellular immune responses [156]. It has been reported that Dtxs have the capacity to inhibit phagocytosis in a dose-dependent manner [157]. In addition, Dtxs are able to target muscles, Malpighian and mesenteral epithelial cells [158] [155]. A study from the team of Chengshu WANG has revealed that Dtxs are important for *M. robertsii* ability to counteract the encapsulation and melanization immune responses in silkworm through the use of Δ*dtxS1* (unable to produce Dtxs) and

Δ*dtxS2* (can only produce DtxB, B_2, and desmethyl-B) mutants [159]. Nevertheless, the mechanism of host defense against Dtxs is still unclear.

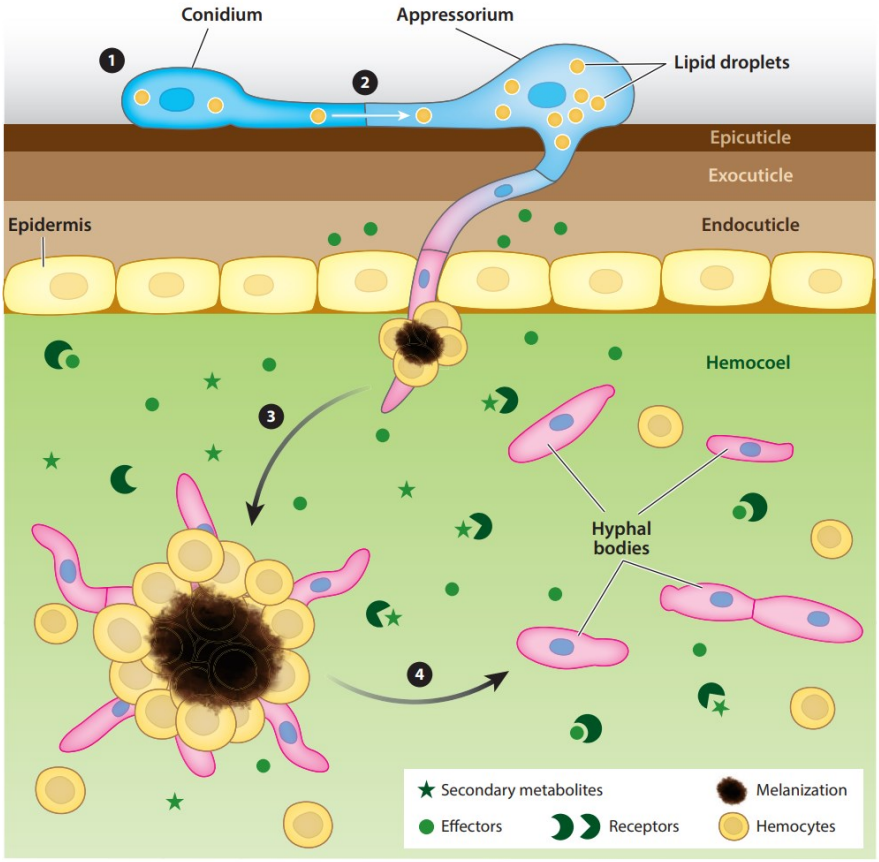


Figure 9. Schematic of infection process of *Metarhizium robertsii*. [160]

(1) A spore adheres to the insect cuticle, germinates, and forms the infection structure, the appressorium. (2) In addition to the secretion of proteases and chitinases, lipid droplets are translocated from the mother conidium to the appressorium for hydrolysis, which generates a high concentration of glycerol to build up turgor pressure and breach the cuticle. (3) During this process and after reaching the host hemocoel, the fungal cells modify their cell wall structures (represented in pink) in response to hemocyte recognition, encapsulation, and melanization. In addition, fungal cells secrete effector proteins and secondary metabolites to evade host immunity by counteracting host receptors (resistant proteins). (4) To successfully colonize the hemocoel and kill the insect, escaped filamentous cells switch to a yeast-type propagation strategy to form hyphal bodies (also called blastospores) for quick growth and host nutrient deprivation.

2.2 *Serratia marcescens*

Serratia marcescens, as Enterobacteriaceae species, is usually found in soil, water, air or on plant and animal surfaces [161]. As a facultative anaerobic Gram (-) bacteria, *Serratia marcescens* is described to generate energy via respiration, fermentation and extract nutrients from organic matter as a saprophytic microorganism. Peritrichous flagella is required for its motility. *Serratia* is easy to contaminate and grows on starch-rich foods. Many strains of *Serratia* produce prodigiosin, a red pigment, and thus was regarded as a sign of "miraculous blood" in middle age when it contaminated in red the bread shown in red. *S. marcescens* is able to infect a wide range of species, including plants, insects and humans [161]. As a human opportunistic pathogen, *S. marcescens* usually causes nosocomial infections [162] [163] and easily infects several human tissues such as the urinary [164], respiratory epithelia, and digestive tract [165]. These infections generally cause inflammation and fever and can be critical for immune-deficient patients. *S. marcescens* is widely used in the study of infection in *Caenorhabditis elegans* [166] and *Drosophila* [167]. It has been described that *S. marcescens* is insensitive to the systemic humoral immune response in the flies and efficiently kills the flies in less than one day [168]. In addition, *Serratia* infections are often associated with multiple-antibiotic resistance mediated by the bacterial production of β-lactamases and active multi-drug efflux pumps [169] [170]. The pathogenicity of *S. marcescens* is mainly mediated by the quorum sensing, the secretion of several virulence factors and the formation of outer membrane vesicles (OMVs). Almost all kinds of microbes are able to produce extracellular vesicles, which play roles in delivering virulence factors or communication between cells [171]. In the case of Gram-negative bacteria, the extracellular vesicles are budded out from the outer membrane and constitute so-called outer membrane vesicles (described in detail in 2.4). OMVs can serve as a cargo to deliver misfolded proteins under stress and virulence factors to enhance the pathogenesis of bacteria. In this study, we mainly focus on the virulent factors targeting the host. It has been shown that *S. marcescens* RM66262

(isolated from Human urinary tract infection) [165] produced more OMVs at lower temperature 30 degree than 37 degree, but without affecting virulence upon infection of *G. mellonella* larvae [172]. From mass spectrometry analysis, a number of proteins, including some extracellular proteins like lipase A, phospholipase, and serralysin (PrtA), were detected in OMVs samples.

2.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium, which was previously considered as an obligate aerobe previously but is now categorized as a facultative aerobe. It can adapt to an oxygen-free environment by using nitrogen or other alternative electron acceptors [173]. As *Serratia marcescens,* it can be easily found in the environment, like in soil and in water. It is an opportunistic pathogen which is able to cause infections in the lungs, urinary tract and blood in patients, especially burn-wounds patients. Its infections lead to high morbidity and mortality in patients with cystic fibrosis (CF), traumas, burns, cancer, chronic obstructive pulmonary disease (COPD), and ventilator-associated pneumonia (VAP), particularly in the immunocompromised situation and it is considered as one of the "critical" categories of the World Health Organization's (WHO) priority list of bacterial pathogens [173]. *P. aeruginosa* strains PAK (clinical strain), PAO1 (reference strain) and PA14 (clinical strain) are commonly used in two types of infections in *Drosophila*: the septic injury and the oral infection models [174]. Here, we only used the septic injury infection model based on PAO1 in this thesis study. Once infected with even a few *P. aeruginosa*, the *Drosophila* succumbed in less than 27 hours under a large quantity of *P. aeruginosa* proliferated in the body cavity. It is reported that the humoral immune response from *Drosophila* is important for fighting *P. aeruginosa* infections by controlling the bacterial burden in the host.

P. aeruginosa displays a vast virulence systems as weapons to its pathogenesis in the host, such as the production of outer membrane vesicles (OMVs) [175], the formation of biofilm, flagellum, type III secretion system. It is reported that *P.*

aeruginosa secretes OMVs, around 10 to 300 nm in diameter, which diffuse through the mucus layer and fuse with lipid rafts in the apical plasma membrane of airway epithelial cells, which results in delivering virulence factors into the cytoplasm of lung epithelial cells. It has been reported that the content of OMVs displays variability in the studies from different laboratories. In the study from Katja Koeppen, after comparing the content with other studies from different teams, 66 proteins, including AprA, CbpD, FliD, LasA, MexA, was found commonly [176, 177]. They found that Tobramycin, used to treat *P. aeruginosa* lung infection in patients, decreased the abundance of several proteins, including AprA in OMVs, thereby reducing the virulence of OMVs. It has been reported in another study that the purified AprA from *Pseudomonas entomophila* exhibited high virulence when injected into the Bean bugs (*Riptortus pedestris*) [176]. Taken together, AprA could play a role in the virulence of OMVs.

2.4 Outer membrane vesicles

As described previously, OMVs are those extracellular vesicles produced by Gram (-) bacteria. The OMVs are nanoparticles 20 - 250 nm in diameter, and their envelope consists of three different compartments: the outer membrane (OM) made of lipopolysaccharide (LPS), the periplasm containing the peptidoglycan layer and the inner membrane. The peptidoglycan is connected to the OM through Outer membrane proteins (Omp) and lipoproteins (Lpp). Generally, these nanoparticles package enzymes, DNA and RNA fragments, peptidoglycans and other factors [178] [179]. The formation of OMVs is initiated with protein condensation in the periplasm, continued with a part of the upper OM bulging away from the bacteria and ending with pinching off from the bacteria (Figure 10). The production of OMVs can be impacted by several environmental conditions such as temperature. For instance, increased growth temperature can activate stress response pathways or increase the fluidity of the OM, that may lead to enhanced OMV production through hypervesiculation. The disruption of the peptidoglycan layer by antibiotics also triggers OM bulging and vesicle formation [180]. In addition, it is reported that the deficiency of Omp increases OMVs formation

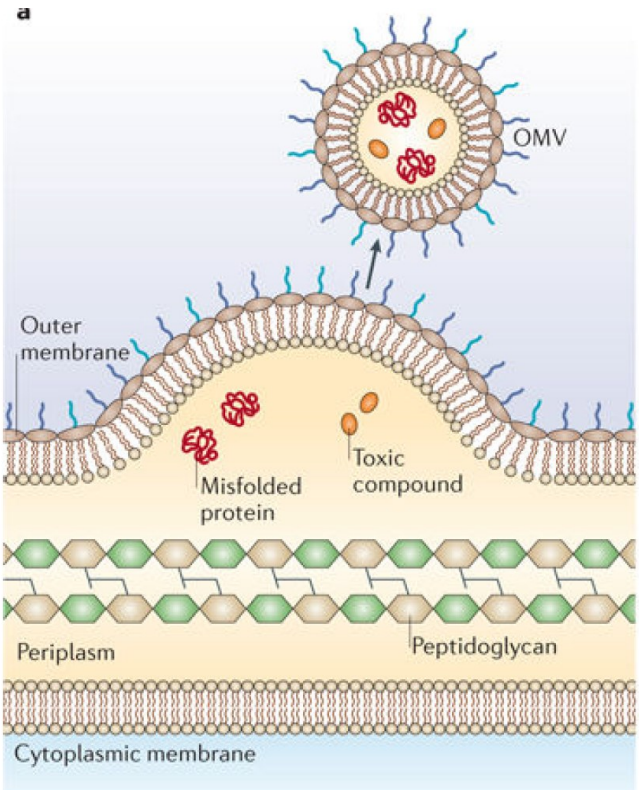


Figure 10. Outer-membrane vesicles (OMVs)

OMVs can serve as a mechanism to remove toxic compounds, such as misfolded proteins, from bacterial cells under stress conditions. [181]

in several bacteria [182] [183]. Following a transposon mutant screen of *Escherichia coli*, several genes, which are crucial for the integrity of the OM, and some genes related to sigmaE pathway, are identified to be involved in the vesiculation [184]. The mutation in the candidate genes belonging to this pathway induced hypervesiculation [185]. The sigmaE pathway is critical for the bacteria to eliminate misfolded proteins in the periplasm. The loss of function of sigmaE candidate genes led to the accumulation of misfolded proteins in the periplasm, which may provoke bulging of the OM resulting in hypervesiculation. In fact, there has been no report of Gram-negative bacteria or mutants that lack OMVs formation until now, which allows us to assume that OMVs formation from bacteria is either essential for the bacteria or is manipulated by multiple pathways.

The comparison between the proteome of OM and the one of OMVs has revealed the existence of a protein selection mechanism [172], which indicates that OMVs play

different biological functions, such as secretion system, nutrient delivery, interspecies interaction, biofilm formation and virulence factor delivery. Considered as a new secretory system, OMVs provide protection to soluble proteins from the action of extracellular proteases [186] or contribute to the secretion of non-soluble proteins through OMVs [187]. For instance, the PQS (Pseudomonas Quinolone Signal) molecule from quinolone sensing system, which is hydrophobic, is found in the OM and is released together with OMVs [188] [189]. OMVs act on nutrient acquisition, such as amino acids and iron. Since OMVs can release enzymes and proteases in the environment, they may contribute to the degradation of proteins into amino acids. The amino acids can be then utilized by the bacteria [190] [191]. On another hand, PQS molecule of *Pseudomonas* in the OM contributes to binding and delivering iron to bacteria [188]. Beyond that, two different species of bacteria can interact with each other through OMVs. When two different species compete for nutrient and tend to eliminate the other, bacteria can envelop bacteriolytic enzymes in OMVs and deliver them to the other competing species via fusing with the membrane, which results in the degradation of peptidoglycans and lysis of cells compacting [192]. On the other hand, OMVs can also contribute to the survival of other neighboring species. For example, OMVs transfer virulence genes and DNAse-resistance sequences between *E. coli* and *Salmonella enterica* [182]. It is reported that the OMVs contribute to the biofilm formation of bacteria. For instance, the supplementation of OMVs to a *Helicobacter* culture is found to promote biofilm formation [193]. In fact, OMVs from *Pseudomonas* actually form a part of the biofilm matrix [180]. Last but not least, OMVs play an important role in virulence factor delivery, especially when invading a host. Enterotoxin of pathogenic *E. coli* that is bound to LPS is delivered to epithelial cells through OMVs [194]. OMVs are able to enter the host cell by membrane fusion [195] or through an endocytosis-mediated receptor [186] to deliver virulence factors to the host cell.

It has been shown in mammals that OMVs activate both the innate and adaptive immune response. In innate immunity, OMVs possess peptidoglycan, LPS, LPP, RNA and DNA that can act as a ligand for PRR [178] [196]. For instance, vesicles from

Salmonella and *Yersinia* stimulate inflammatory response through Toll-like receptor (TLR) 4 [197]. The activation of PRRs by OMVs is then followed by the activation of intracellular pathways which are required to produce pro-inflammatory molecules. In fact, OMVs produced by *Helicobacter, Neisseria* and *Pseudomonas* have the capacity to penetrate into the host cell and deliver PGN which is then detected by the intracellular receptor NOD1, resulting in the secretion of IL-8 [198]. In the adaptive immune response, the antigens from OMVs can be presented to CD4+ T cells, which mediate a B cell antigen-specific response [199]. Interestingly, immunization with OMVs generates a strong IgG and IgA antigen-specific response that enhances the protective immunity against infection [200]. Furthermore, the ability of OMVs to activate the adaptive immune system by mediating an antigen-specific B cell response contributes to their usage in vaccination [201] [178]. Beyond that, OMVs also can act to silence the immune response. For instance, the OMVs produced by *H. pylori* trigger apoptosis in T cells [202], while OMVs secreted from *P. aeruginosa* possess a short RNA which reduces the secretion of IL-8 and neutrophil recruitment in the lungs of mice [203]. On the contrary, compared to these pieces of information in mammals the immune response against OMVs in *Drosophila* is still mysterious and needs to be discovered.

The Strasbourg laboratory in collaboration with a team of microbiologists led by Prof. Eleonora Garcia-Vescovi has actually studied whether *S. marcescens* plays a role for its unusual strong pathogenicity. It was found that the injection of purified OMV preparations led to the demise of injected flies within hours, which were actually first paralyzed. Whereas the IMD pathway appeared to be involved in the host defense against injected OMVs, the cellular immune response and the key melanization protease Hayan promoted the pathogenicity of OMVs, independently of POs. Mitochondria-generated ROS in neurons also promoted the virulence of injected OMVs, an effect that may be mediated by the JNK pathway in this cell type. Taken together, the studies suggest that the injection of OMVs lead to the generation or ROS by mitochondria in the nervous system and induces the apoptosis of some neurons, which likely mediate the initial paralysis phenotype. PrtA was found to be the major virulence

factor carried by OMVs, which unexpectedly requires the activity of Hayan for full virulence. Finally, it was suggested that OMVs contribute to the virulence of *S. marcescens* in the septic injury model but are not major mediators of the pathogenicity of this entomopathogenic bacterium. A first version of the manuscript relating these discoveries is included as an Annex to this doctoral thesis as I have participated in the work and as it provides the background that led to some of the experiments described in Chapter II.

3.Long non-coding RNAs

With the incredible advances of sequencing technologies, the sequencing of the genome of *Saccharomyces cerevisiae* was the first genome of a eukaryotic model organism to be completed in 1996 [204]. The sequencing of the genome of *Drosophila melanogaster* was completed in 2000 [3] and the Human genome project was completed in 2004 [205]. The fast-developing sequencing technologies allowed the generation of an increasing amount of data with a reduced cost. It was reported that only a few percents of the human genome are actually coding into about 20, 000 proteins [205]. Numerous studies have reported that transcription is actually widespread across the genome, even in some non-coding regions, which was regarded as transcriptional noise at that time. After the discovery of some non-coding RNAs (ncRNAs), such as ribosomal RNAs (rRNAs), transfer RNA (tRNAs), small nuclear RNAs (snRNA) and micro RNAs (miRNAs), a new class of ncRNA, long non-coding RNAs (lncRNAs), was identified.

LncRNAs are defined not to be translated into protein and more than 200 nucleotides in length [206] [207, 208] [209]. In some aspects, lncRNAs are similar to mRNAs. For instance, lncRNAs are transcribed by RNA polymerase II, are almost always capped and poly-adenylated, and can contain introns as well [210] [209] [211]. The lncRNAs can be located between protein-coding genes, called long intergenic non-coding RNAs (lincRNAs), and located at the region which overlap with coding genes, within introns or exons sequences, either in sense or antisense of the coding gene [209,

212]. LncRNA transcripts can be present in the cytoplasm, nuclear, or even inside mitochondria. Besides, lncRNAs are considered to evolve fast with poor conservation among species.

Numerous studies showed that lncRNAs contribute to the regulation of transcription. Generally, it is thought that lncRNAs could regulate transcription as described here: 1) Recruitment: lncRNA can recruit some regulatory protein and process to a gene or an entire chromosome in cis or in trans. 2) Inhibition: lncRNA can inhibit the binding of the transcriptional regulatory factor. 3) Indirectly: the transcription of a lncRNA may regulate the transcription of neighbor genes through maintaining active chromatin structure or competing for polymerases.

It is indicated that lncRNAs play roles in the immunity of mammals, such as regulating the development and differentiation of several different immune cell lineages and mediating the circuit of the inflammatory response. Compared to the abundant studies about the lncRNA in mammalian immunity, our understanding of the roles of lncRNAs in *Drosophila* immunity remains limited. The study of lncRNA VINR in the immunity of flies sets an excellent example. It has been reported that *VINR* is induced upon viral infection and required for the host defense against *Drosophila* C virus. VINR was shown to prevent the degradation by the proteasome of Cactin and resulted in the secretion of AMPs mediated by a non-canonical IMD pathway.

Although by definition lncRNA should have no capacity to encode into proteins, but with the improvements in bioinformatics and high-throughput technologies, some lncRNAs from plants and invertebrates have been shown to be translated into peptides or micropeptides with open reading frames (ORFs) and found to play roles in different biological processes. For example, a 679nt lncRNA from soybean can encode two small peptides and interact with sucrose synthase. lncRNA HOXB-AS3 can be translated into a 53 amino acid (aa) peptide and functions in inhibiting colon cancer [213]. The lncRNA Toddler in zebrafish is able to code for a 58aa peptide and promotes gastrulation movements. It is still not easy to evaluate the coding capacity of lncRNA even with abundant computational technology, such as PhyloCSF [214], Coding potential

calculator (CPC) and so on. It could be due to the high similarity to mRNA in structure. Up to now, only few lncRNAs have been functionally annotated, and a large number of lncRNA-encoded peptides still need to be discovered.

PhD objective

Drosophila melanogaster is a powerful model organism with a long research history. Nearly 75% of genes related to human disease are thought to have functional homologs in *Drosophila*. With a lack of adaptive immunity and a plethora of advanced genetic tools, *Drosophila* has been regarded as an excellent model to study immune responses. Up to now, several critical immune responses have been revealed thanks to the efforts of scientists. Initially considered as transcriptional noise, lncRNAs actually play important roles in biological processes. Many studies have revealed the functions of lncRNAs in mammals. Nevertheless, there are only few studies documenting their roles in *Drosophila* immunity. Thus, whether and how the lncRNAs are involved in *Drosophila* innate immunity is a theme worth investigating. Dr. Wenhui WANG in our team performed RNA sequencing on total RNAs extracted from whole flies upon the challenged of entomopathogen *Metarhizium robertsii* in both injection and natural infection model, including the transcription profile of ncRNAs. In the beginning, I aimed to investigate the role of lncRNAs in *Drosophila* immunity. With the contribution from Prof. Samuel LIEGEOIS, upon the statistical analysis using Audic-S method, we found that 23 lncRNAs were significantly elevated or suppressed upon infection (shown in table). The most significantly and consistently induced and suppressed genes among these lncRNA genes in both injection and natural infection are *CR44404* and *CR45601* respectively. To understand the function of these lncRNAs of interest, we generated full-length deletion mutants that were subsequently tested in survival assays upon challenges with different types of pathogens to determine whether these lncRNAs are involved in the host defense against pathogens.

In Chapter I, I study a noncoding gene *CR45601* that completely overlaps in the opposite direction a coding gene, *yolk protein 1* (*Yp1*). It was however difficult to study *CR45601* without disrupting *Yp1*. Based on the transcriptional profile that *CR45601* behaved the same as *Yp1* upon the treatment of pathogens, we hypothesized that *CR45601* might regulate the expression of *Yp1*. Thus, we worked with both genes

together to find a positive immune readout.

In Chapter II, during my study of *CR44404*, an article was published revealing that *CR44404* might function as a lncRNA in *Drosophila* immunity, based solely on genetic overexpression studies (as this gene is already upregulated by infections, this approach might not have been the most relevant one to understand the function of the gene). In fact, we found that *CR44404* has been mis-annotated as a noncoding gene as it does encode a short-secreted peptide, which is similar to a peptide encoded by *CR45045* another mis-annotated noncoding gene. *CR45045* was actually being worked on by Adrian ACKER, a student from the team of Prof. Nicolas MATT at University of Strasbourg. Thus, we initiated a collaboration to study both genes. Through genetic analysis, we found that the two peptides actually play roles in the host defense against some virulence factors produced by pathogens rather than by acting directly against the pathogens. Nevertheless, the mechanism of *Drosophila* fighting against the virulence factors is still unclear. We made many efforts in Chapter II to uncover their mechanism of action.

In the annex, I was also involved in finishing a project that was initially started in Strasbourg as a collaboration between the host immunity group animated by Dr. D. Ferrandon and a team of microbiologists working on *Serratia marcescens* led by Prof. Eleonora Garcia-Vescovi. It was discovered that OMVs likely constitute a major virulence factor of *S. marcescens* in the septic injury model. I have put in annex a preliminary version of this work that provides an overview of our current understanding of the biology of *S. marcescens* OMVs in host-pathogen interactions in *Drosophila*. This ground work provided one of the major foundations of my doctoral work (Chapter II). Another foundation is provided by the BaraA study that I co-authored, which is therefore placed as an additional annex.

Table.

lncRNAs	Injection	Natural infection	Overlapping gene
Gene name	**Fold change (compared to PBS)**	**Fold change (compared to untreated)**	
CR44404	induced 17x (18h) 5x (33h), 4x (48h)	induced 15x (18h), 30x (33h)	No
CR44366	induced 7x (33h)		No
CR45161	induced 2.5x (6h), repressed 3x (48h)	induced 2x (18h)	fln
CR44367	induced 3x (6h, 18h, 33h), 2.5x (48h)	induced 3x (18h), 4.5x (33h)	CG6553
CR44138	induced 3x (18h, 33h, 48h)	induced 3.5x (33h, 48h)	CG9813
CR45701	induced 3x (33h), 2x (48h)	induced 3x (33h)	CG10680
CR45601	repressed 3x (18h, 33h), 15x (48h)	repressed 7.5x (33h), 12x (48h)	Yp1
CR43887	repressed 12x (6h)		No
CR31084	repressed 4x (6h, 33h)	repressed 4.5x (33h), 15x (48h)	CG34291
CR45024	repressed 3.5x (48h)	repressed 2x (33h), 5.5x (48h)	CG4000
CR45955	repressed 2.5x (18h, 33h)	repressed 2x (18h), 5x (33h), 6x (48h)	CG32444
CR45485	repressed 2.5x (33h)	repressed 4x (33h)	CG9914
CR44850	repressed 2x (33h), 3x (48h)	repressed 3x (33h)	lethal (2) 34Fc
CR43152		induced 16x (33h), 20x (48h)	CG15721
CR40469		induced 18x (33h)	No
CR43636		induced 13x (33h), 16x (48h)	CG15721
CR46018		induced 12x (48h)	CG18530
CR46204		induced 6.5x (33h), 9x (48h)	No
CR44478		induced 4.5x (33h), 4x (48h)	Socs36E
CR44953		induced 3.5x (33h), 4.5x (48h)	No
CR43264		induced 2.5x (33h), 5x (48h)	CG15765
CR43684		repressed 11x (48h)	Sytbeta (CG42333)
CR46269		repressed 3.5x (33h), 5x (48h)	CG7882

Chapter I

Preliminary investigations of the function of *CR45601* and *Yp1* in *Drosophila*

Abstract

Many publications have revealed that lncRNAs play diverse roles in several biological processes. Antisense lncRNA is a common lncRNA in transcriptomes and was found to tune gene transcription. From the transcriptomic profile of RNAseq, we found that the expression level of an antisense noncoding gene *CR45601* behaved almost the same as *Yp1*, its overlapping protein-coding gene on the opposite strand. The expression of both genes was significantly reduced upon infection. We also found that the reduced expression of *Yp1* was *MyD88*-dependent. Following the infections with different pathogens, including yeast, filamentous fungi, Gram-negative, and Gram-positive bacteria in three independent mutants of *Yp1*, we failed to find any interesting phenotype. Until now, we still have not uncovered the function of *Yp1* in *Drosophila* immunity and the role of lncRNA CR45601.

Introduction

Previously regarded as transcriptional noise, lncRNAs were actually reported to contribute to diverse biological processes. According to their genomic location with respect to nearby protein-coding genes, lncRNAs are classified into several different groups, including intergenic lncRNA (lincRNA), which are located between two protein-coding genes; intronic lncRNA, which are transcribed from the introns of protein-coding genes; antisense lncRNA, which contain one or more regions of sequences complementary to the overlapping protein-coding gene. Antisense lncRNA is very common in human and eukaryotic transcriptomes [215]. It is reported that antisense lncRNA mediates the regulation of neighboring gene expression, through

DNA-RNA, RNA-RNA or protein-RNA interactions [216]. For instance, in rat sarcoma, *RASSF1A* (RAS-association domain family member 1A) encodes a protein similar to the RAS effector proteins and is involved in multiple functions at apoptotic and cell cycle checkpoint pathways. *ANRASSF1* (antisense intronic non-coding *RASSF1*), a non-coding gene, is transcribed in the antisense direction relative to the protein-coding gene *RASSF1* locus. ANRASSF1 is localized in the nucleus and interacts with genomic DNA, forming an RNA/DNA hybrid. It recruits the polycomb repressive complex 2 (PRC2) to the promoter of RASSF1A, then PRC2 induces the accumulation of the repressive mark H3K27me3, leading to a specific reduction in the *RASSF1A* transcriptional activity [217]. Several publications indicate that antisense lncRNA plays an indispensable role in the regulation of the neighboring genes.

We performed a transcriptome analysis on flies after *Metarhizium robertsii* infection and we found a lncRNA gene, *CR45601*, which is significantly reduced upon infection among lncRNAs. It completely overlaps with the sense protein-coding gene, yolk protein 1 (*Yp1*), when we refer to Flybase.

Yolk protein is essential for female fertility and is involved in vitellogenesis [218] . In *Drosophila melanogaster*, there are three yolk proteins characterized (*Yp1, 2,* and *3*). Yolk proteins are synthesized by two tissues, the fat body and the ovarian follicle cells, at specific stages of oogenesis [219]. A study published in 1991 showed that through genetic crosses, each yolk protein gene makes an equivalent contribution to the fecundity and fertility of the female. It is shown that the egg laying rate of a female depends upon the number of genes encoding yolk proteins presented in the genome. In addition, the rate of eggs hatching into an adult also generally depends on the number of yolk protein genes presented in their mother [220].

It has been reported that the oviposition of *Drosophila* females is reduced after *E. coli* infection. The peptidoglycan from Gram (-) bacteria activates the NF-κB signaling pathway in octopaminergic neurons in *Drosophila*, and thereby reduces egg laying rate. It may thus constitute a case of behavioral immunity [221]. If *M. robertsii* infection were to have a similar impact on egg laying as the activation of the IMD pathway in

octopaminergic neurons, a decreased expression of *Yp1* may underlie such a phenomenon. Indeed, we found that the expression of the three yolk protein-encoding genes, *Yp1*, *Yp2*, and *Yp3*, were reduced upon challenge with this entomopathogenic fungus. Because the antisense *CR45601* lncRNA may mediate this downregulation, we decided to study this locus and asked whether *Yp1* might be indirectly involved in host defense against infections. The work presented below starts answering this question.

Results

The transcriptional expression of *CR45601* behaves the same as the overlapped coding gene *Yolk protein 1* upon *Metarhizium robertsii* infection

Except for the well-known mechanisms involved in the *Drosophila* against pathogens described in the general introduction, there are still other immune responses that need to be discovered. Dr. Wenhui WANG from our team performed dual RNAseq on whole files upon the entomopathogenic fungus *M. robertsii* in both natural infection and injection model. She optimized the concentration of *M. robertsii* to 10^7 spores/ml for infections in the injection and natural infection models so that the survival rate kinetics would be similar, thereby allowing a direct comparison between the two models (Fig 1A). Flies upon natural infection might have a minor response at an early stage, because the fungi take time to penetrate the cuticle and invade flies. Therefore, we collected flies at 6h, 18h, 33h, 48h post septic injury infection and only 18h, 33h, 48h post natural infection. 50 flies were harvested for each condition. Total RNA was isolated and was fragmented into around 200nt oligonucleotides and rRNAs-depleted. rRNA-free samples were then reverse-transcribed into cDNA libraries with ligated adaptors and sequenced. The resulting sequencing reads of mRNAs and ncRNAs were mapped to the reference genome of *Drosophila*.

We have analyzed predicted lncRNAs that display altered expression upon *M. robertsii* challenge. The transcription level of *CR45601* was significantly reduced upon *M. robertsii* in both infection models (Fig 1B). CR45601 is an 875nt-long antisense lncRNA fully overlapped with *Yolk protein 1 (Yp1)* at the genomic locus (Fig 1C).

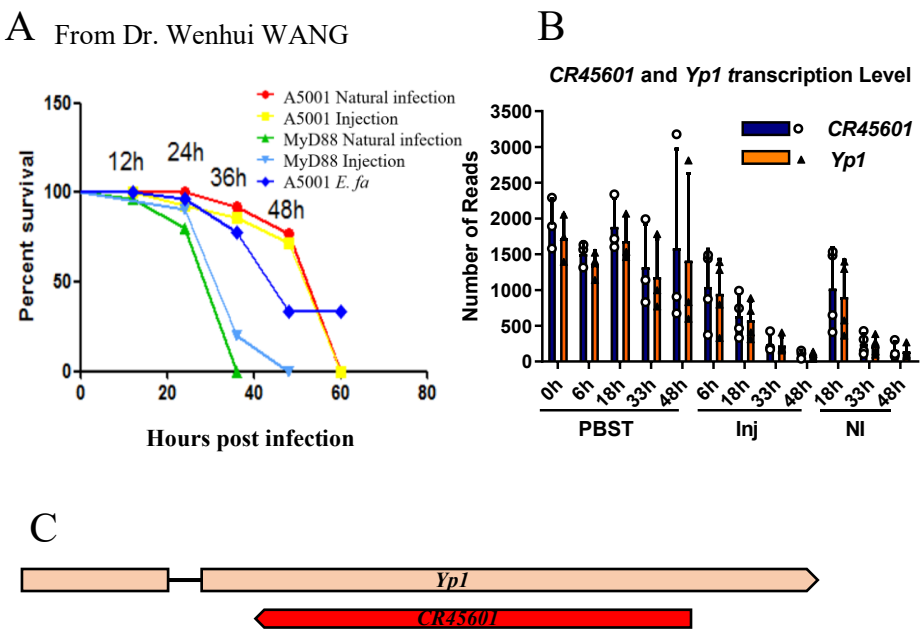


Figure 1 : CR45601 et Yp1. *CR45601* et *Yp1*. (A) Le test de survie a été réalisé par le Dr Wenhui WANG. La concentration de *Metarhizium robertsii (Mr)* utilisée dans cette expérience est de 10^7 spores/ml. Sur la base de ce résultat de survie, une RNAseq a été réalisée. **(B)** Les niveaux de transcription des gènes *CR45601* et *Yp1* après injection de *Mr* (inj) et infection naturelle (NI) à partir des données RNAseq. Les valeurs RPKM ont été indiquées. **(C)** Schéma des locus des gènes *Yp1* et *CR45601*.

Interestingly, *Yp1* displays a similar transcriptional profile as *CR45601* (Fig 1B). Since the dual RNAseq approach is based on the ligated adaptors at 3-end only while both genes are transcribed from opposite directions and shown in base-complementary transcripts, the possibility of mixing up two genes could be excluded. Based on one of the functions of antisense lncRNAs that regulates the transcription of the opposite gene, lnc-CR45601 might play a role in the regulation of *Yp1*.

The expression of *Yp1* is reduced upon infections and dependent on *MyD88*

Since the expression of both *Yp1* and *CR45601* were reduced upon *M. robertsii*, it was interesting to monitor their expression level upon challenge with different microorganisms. Here we used non-pathogenic Gram-negative bacteria *Escherichia coli (E. coli)* and Gram-positive bacteria *Micrococcus luteus (M. luteus)* as representatives. Regarding to the importance of the IMD pathway and the Toll pathway

in host defense, it is worth studying whether both pathways are involved in the expression of both genes. It has been described that the IMD pathway is activated rapidly and transiently from 3 to 24 hours by *E. coli*, while the peak of activation of Toll pathway occurs at 24 hours upon *M. luteus* challenge. We thus measured the expression in *kenny* (a scaffold protein of the IMD pathway) mutant flies at 6 hours, 18 hours and 24 hours post *E. coli* infection and likewise in *MyD88* (a protein adaptor functioning in the Toll pathway) mutants at 6 hours, 24 hours and 48 hours post *M. luteus* infection compared to wild type flies. We designed a pair of primers at the first exon of *Yp1* (Fig 2A) for quantitative PCR (qPCR) without interference from *CR45601*. We found that *Yp1* was downregulated under both bacterial infections. Knocking out *kenny* has no impact on the expression of *Yp1* (Fig 2B). However, the reduction of *Yp1* upon *M. luteus* infection vanished in *MyD88* mutants (Fig 2C). These data indicate that the reduction of *Yp1* upon infections is Toll but not IMD pathway-dependent.

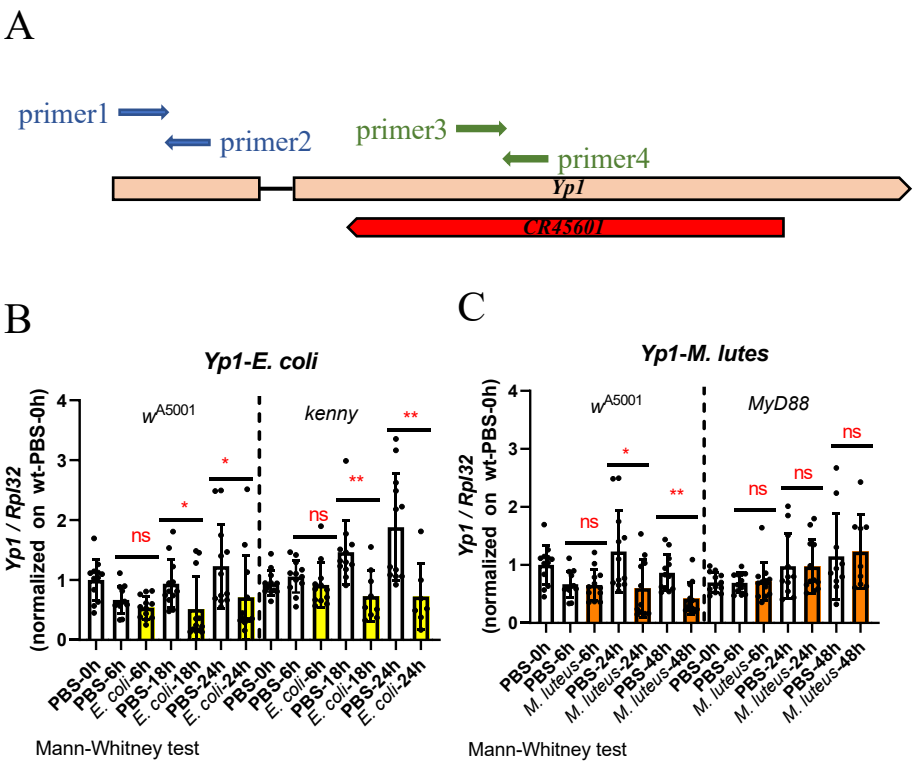


Figure 2. Niveau de transcription de *Yp1* lors d'infections. (A) Le locus de *Yp1* est utilisé pour la qPCR. **(B)** L'expression transcriptionnelle de *Yp1* à différents moments de l'infection par *E. coli* (OD_{600} =50) chez les mouches mutantes w^{A5001} et *kenny*. **(C)** L'expression transcriptionnelle de *Yp1* à différents moments après l'infection par *M.luteus* (OD_{600} =50) chez les mouches w^{A5001} et *MyD88* mutantes.

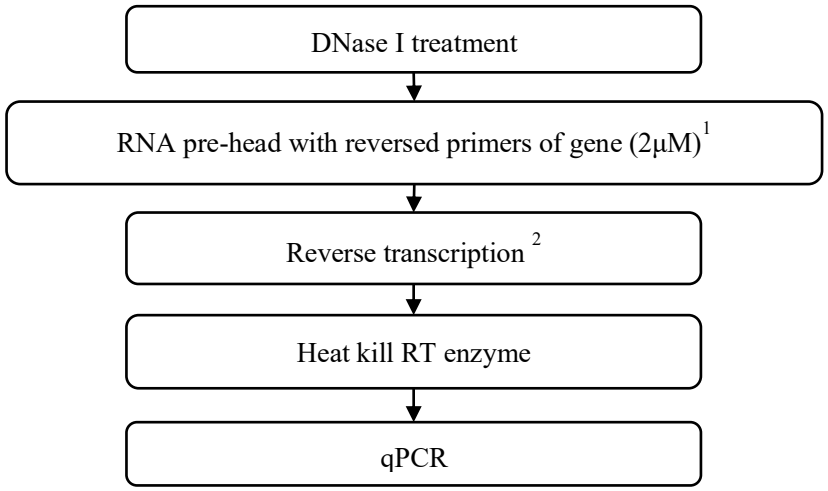
Measuring the transcriptional level of *CR45601* by strand-specific reverse transcription quantitative PCR

It is challenging to monitor the transcriptional expression level of *CR45601* since it overlaps with *Yp1* (Fig. 1C). It is not possible to measure the expression of *CR45601* by using oligo dT or random primers for reverse transcription (RT). Here we employed another method that used a *CR45601* strand-specific primer (primer 3 in Fig. 2A) but not oligo dT or random primers for the reverse transcription step. The cDNAs were then amplified by qPCR using a pair of *CR45601* primers (primer 3 and 4 in Fig. 2A) (Fig 3A). We designed several control samples as checkpoints for this method. During the the RNA and primers preheating step[1], primers were not supplied for checking if the RT enzyme was completely killed by heat and no longer function during qPCR step. During the RT step[2], RT enzyme was not supplied to check if the reaction was disrupted by contaminating genomic DNA. We only detected the expression of *CR45601* or *Yp1* respectively following this method but not in the other control samples, which indicates that we successfully monitored the expression of *CR45601* without interference from *Yp1* transcripts (Fig 3B). Of note, a full validation of the strategy would be provided by monitoring the expression of both genes after a *M. robertsii* challenge. The results obtained with this strategy as regards *Yp1* should be similar to those obtained by RNAseq (Fig. 1B) or by classical RTqPCR (Fig. 2B-C).

***Yp1* is not involved in the host defense against different microorganisms**

In order to better understand the roles of *Yp1* and *CR45601* in *Drosophila* immunity, we obtained two independent frame shift mutants, which are 7 or 13 base pairs depleted at the first exon of *Yp1* by CRISPR cas9 kindly from the platform of Sino-French Hoffmann Institute (Fig 4A). Meantime, we generated a knock-in mutant by replacing the whole CDS region of *Yp1* with mCherry sequences by CRISPR Cas9 technique which is deficient of both *Yp1* and *CR45601*. $Yp1^{KI}$ flies are able to be used for reporting the expressed tissue of *Yp1* (Fig 4B). The interesting point about the frameshift mutants is that they occur in the first exon, which is not covered by the *CR45601* antisense

A



B

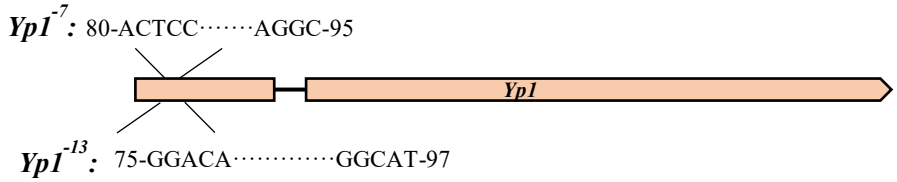
Target gene	SRT-qPCR	without primers at 1	without RT at 2
CR45601	20.56	30.10	30.44
Yp1	22.59	33.80	30.52

shown with average Ct values

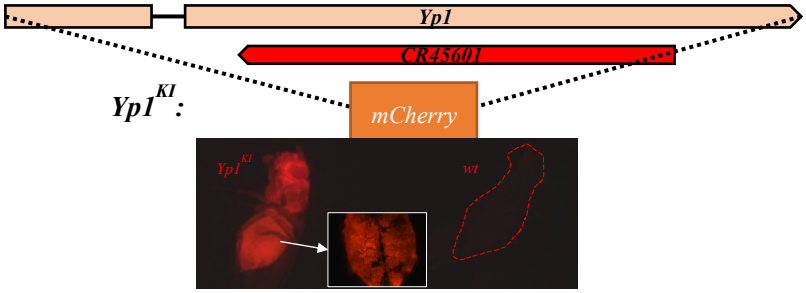
Figure 3. Transcription inverse spécifique d'un brin qPCR. (A) Déroulement de la RT-qPCR à brin spécifique (SRT-qPCR). Le traitement à la DNase I est important pour éliminer la contamination de l'ADN génomique. Le préchauffage de l'ARN avec des amorces inversées peut augmenter l'efficacité de la RT. L'enzyme RT doit être inactivée avant l'étape qPCR en cas de perturbation de la transcription du gène antisens pendant l'étape qPCR. **(B) La** SRT-qPCR a été réalisée sur les échantillons de mouches entières de w^{A5001} . Les résultats des valeurs Ct ont été indiqués. Les réactions de *CR45601* et *Yp1* ont été réalisées séparément. 1 : à cette étape, les amorces n'ont pas été fournies pour vérifier si l'ADN génomique était contaminé. 2 : à cette étape, l'enzyme RT n'a pas été fournie pour vérifier si l'enzyme RT est inactivée.

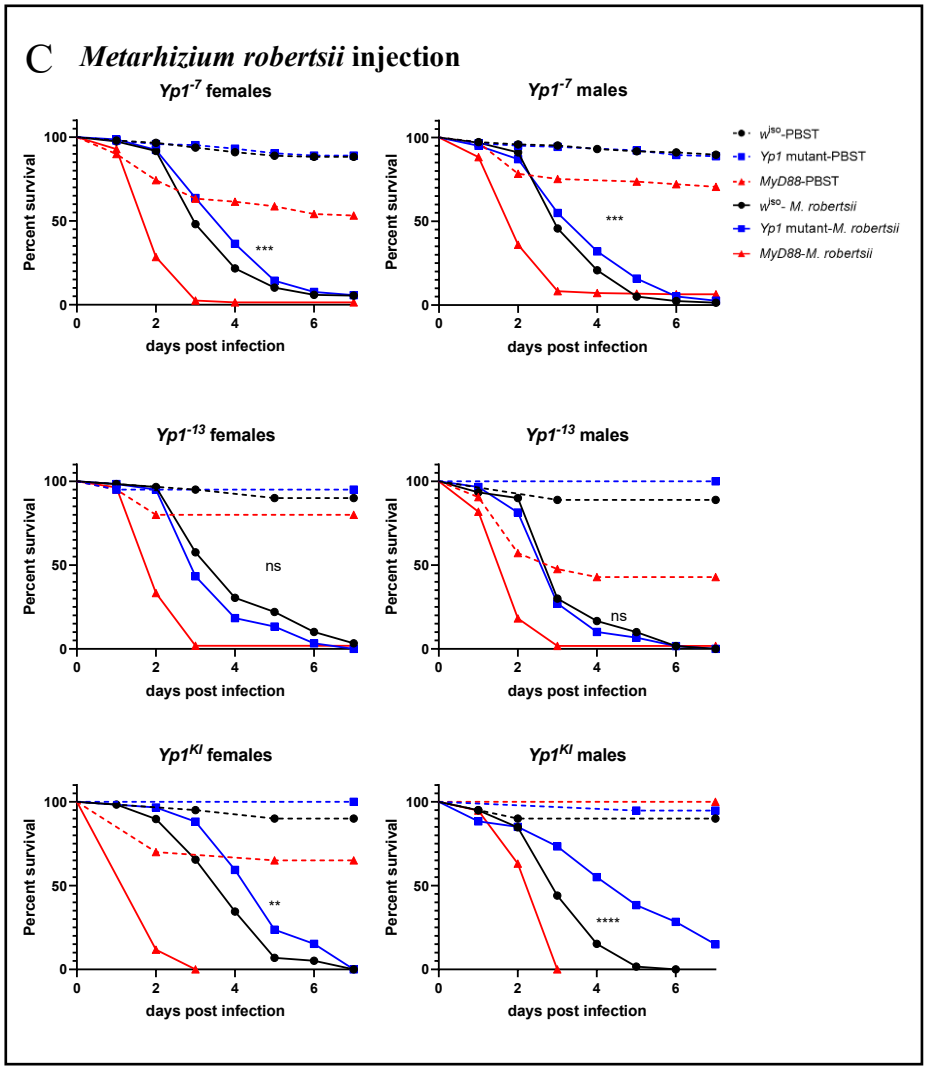
transcript. Thus, these mutants should alter only the Yp1 protein, or at worst its transcripts, without altering the expression of *CR45601*. They affect solely *Yp1*. Previous studies have demonstrated that *Yp1* is strongly expressed in the fat body and delivered to the ovary. We successfully detected the strong fluorescence in the fat body of $Yp1^{KI}$ (Fig 4B). Since it is shown that *Yp1* was reduced upon infections of different microorganisms, including fungi, Gram (-) bacteria and Gram (+) bacteria, we therefore infected all the mutants mentioned above with different pathogens, including *M. robertsii* (filamentous fungi), *Candida albicans* (*C. albicans*, yeast), *Pectobacterium*

A

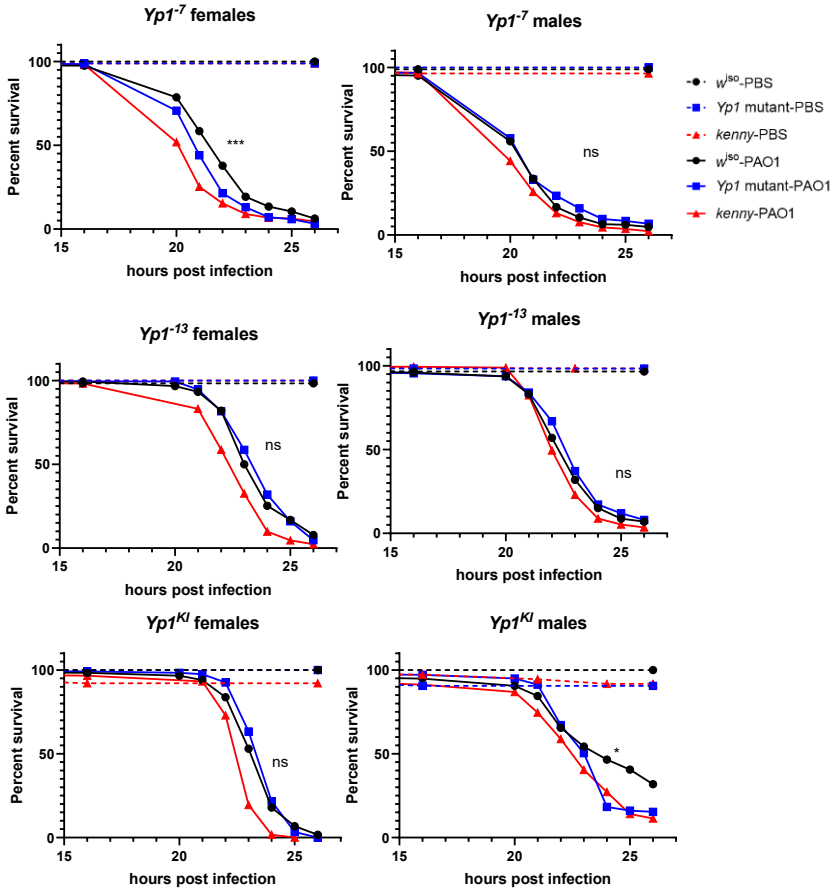

Yp1-7: 80-ACTCC·····AGGC-95
Yp1
Yp1-13: 75-GGACA·····GGCAT-97

B

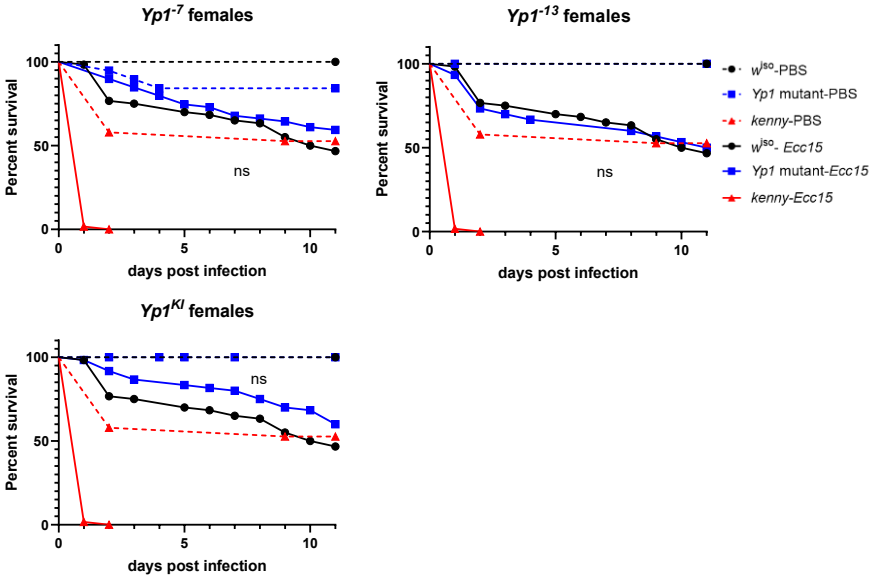

Yp1
CR45601
Yp1KI:
mCherry
Yp1ki
wt


C Metarhizium robertsii injection
Yp1-7 females
Yp1-7 males
Yp1-13 females
Yp1-13 males
Yp1KI females
Yp1KI males
Percent survival
days post infection
w1so-PBST
Yp1 mutant-PBST
MyD88-PBST
w1so- M. robertsii
Yp1 mutant-M. robertsii
MyD88-M. robertsii
***
ns
**
****

D *Pseudomonas aeruginosa* injection



E *Pectobacterium carotovorum (Erwinia carotovora carotovora)* injection



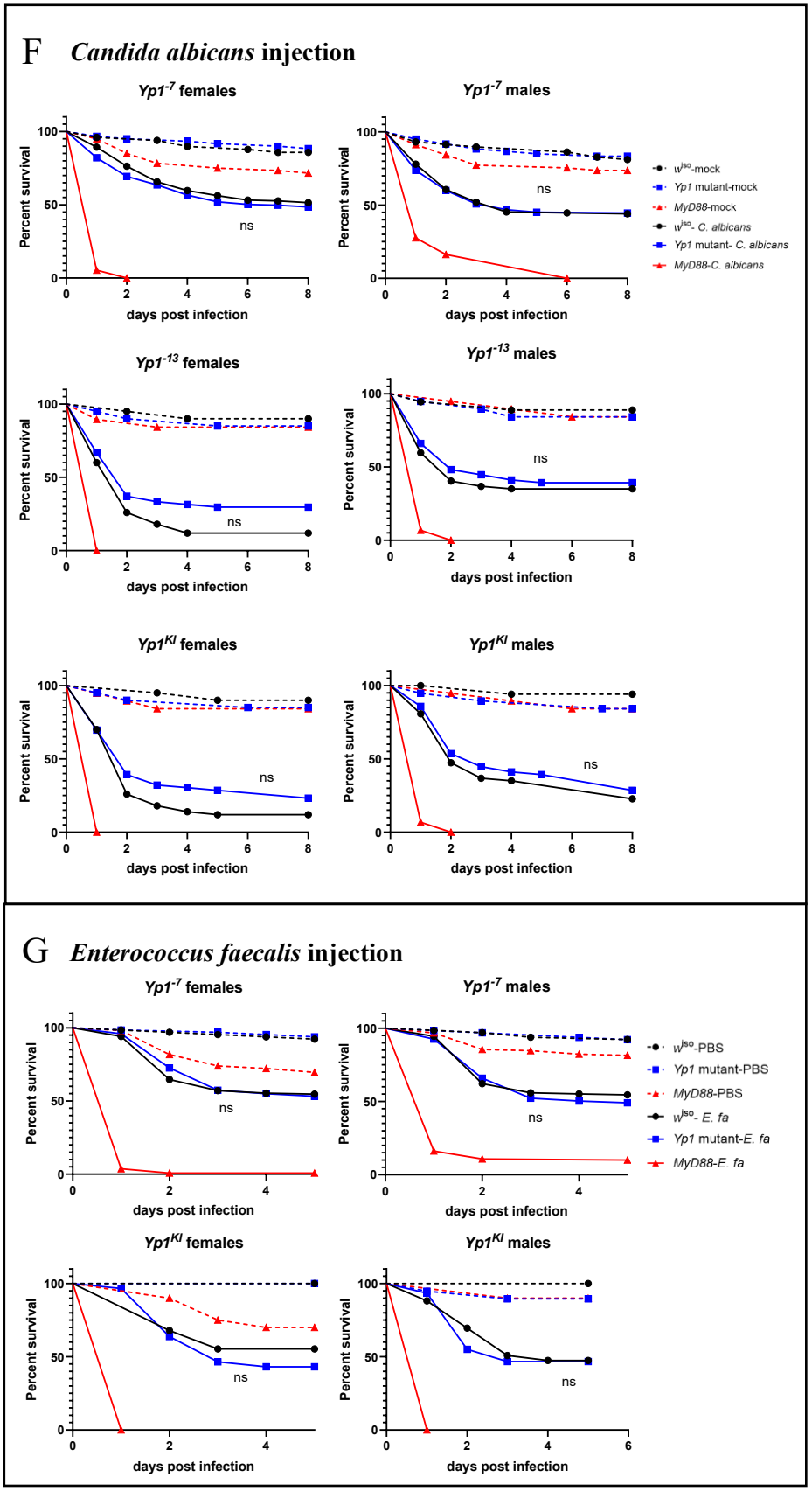

F Candida albicans injection
Yp1-7 females
Yp1-7 males
Yp1-13 females
Yp1-13 males
Yp1KI females
Yp1KI males
Percent survival
days post infection
ns
wiso-mock
Yp1 mutant-mock
MyD88-mock
wiso- C. albicans
Yp1 mutant- C. albicans
MyD88-C. albicans
G Enterococcus faecalis injection
Yp1-7 females
Yp1-7 males
Yp1KI females
Yp1KI males
Percent survival
days post infection
ns
wiso-PBS
Yp1 mutant-PBS
MyD88-PBS
wiso- E. fa
Yp1 mutant-E. fa
MyD88-E. fa

Figure 4. Taux de survie des mutants *Yp1* lors de différentes infections. (A) Schéma de la génération de mutants de décalage de cadre de *Yp1*. $Yp1^{-7}$ présente une délétion de 7 paires de bases entre les sites 85 et 91. $Yp1^{-13}$ présente une délétion de 13 paires de bases entre les sites 80 et 92. **(B)** Schéma du mutant $Yp1^{KI}$. La région du gène *Yp1* allant de l'ATG au codon d'arrêt a été remplacée par le gène *mCherry*. L'image de $Yp1^{KI}$ et des mouches de type sauvage est montrée en bas. **(C)** Les taux de survie de trois mutants isogéniques de *Yp1*, femelles et mâles, après injection septique de 10^7 spores/ml de *Metarhizium robertsii* (*M. roobertsii*). w^{iso} est considéré comme le contrôle de type wile, tandis que le mutant *MyD88* est considéré comme le contrôle positif. **(D)** Taux de survie de trois mutants femelles isogéniques de *Yp1* après injection septique de 13,8nl OD_{600} =0,001 *Pseudomonas aeruginosa* (*PAO1)*. w^{iso} est considéré comme le contrôle de type wile tandis que le mutant *kenny* est considéré comme le contrôle positif. **(E)** Taux de survie de trois mutants femelles isogéniques de *Yp1* après injection septique de 4,6nl OD_{600} =*50 Pectobacterium carotovorum* (anciennement *Erwinia carotovora carotovora,* souche *Ecc15*). w^{iso} est considéré comme le contrôle de type wile tandis que le mutant *kenny* est considéré comme le contrôle positif. **(F)** Taux de survie de trois mutants isogéniques de *Yp1*, femelles et mâles, après piqûre avec un clone de *Candida albicans (C. albicans)*. w^{iso} est considéré comme le contrôle de type wile, tandis que le mutant *MyD88* est considéré comme un contrôle positif. **(G)** Taux de survie de deux mutants femelles isogéniques de *Yp1* après injection septique de 4,6nl OD_{600} =0,1 *Enterococcus faecalis* (*E. faecalis*). w^{iso} est considéré comme le contrôle de type wile tandis que le mutant *MyD88* est considéré comme le contrôle positif. Le test Log-rank a été utilisé pour les statistiques.

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carotovorum (formerly *Erwinia carotovora carotovora, Ecc15* strain, Gram (-) bacteria), *Pseudomonas aeruginosa* (*PAO1,* Gram (-) bacteria) and *Enterococcus faecalis* (*E. faecalis,* Gram (+) bacteria). It is reported that *Yp1* is only expressed in female flies but rarely in male flies, which provide a negative control. We found both $Yp1^{-7}$ and $Yp1^{KI}$ mutants but not $Yp1^{-13}$ mutants were more resistant to *M. robertsii* than wild type adult flies. However, the resistant survival rates were observed in both female flies and male flies (Fig 4C). We also found that the $Yp1^{-7}$ female mutants were slightly more susceptible to *PAO1* but not $Yp1^{-13}$ nor $Yp1^{KI}$ mutants (Fig 4D). We did not find any difference between *Yp1* mutants and wild type upon infection of *Ecc15* (Fig 4E), *C. albicans* (Fig 4F) and *E. faecalis* (Fig 4G). Here we conclude the difference between those independent mutants could be due to the genetic background even though all of the fly strains were isogenized, and actually knocking out *Yp1* did not display susceptibility to infection upon tested pathogens.

Yp1 is not critical for the egg laying

It is reported that deficiency of multiple yolk proteins but not a single yolk protein

resulted in reduced eggs production. Here we want to confirm whether deficiency of *Yp1* alone impacts egg laying. We collected the eggs from different *Yp1* mutants compared to wild type using method described in the publication from C Leopold Kurz. The result showed that there is no difference between several mutants of *Yp1* and the wild type raised at both 25 degree (Fig 5A) and 29 degree (Fig 5B), which indicates that *Yp1* is not critical for the egg laying of flies. These results could be due to the compensation of other yolk proteins.

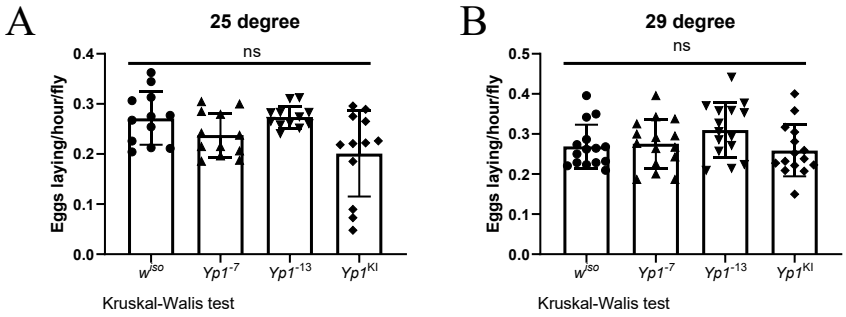

Figure 5. L'oviposition des mutants *Yp1*. (A) 20 femelles et 20 mâles sont collectés dans un flacon en trois exemplaires et conservés à 25 degrés et **(B)** 29 degrés sur un milieu bleu pour la ponte. Des plaques ont été prélevées pour compter le nombre d'œufs. w^{iso} est considéré comme le contrôle de type sauvage. Les données de trois expériences indépendantes ont été regroupées. Le test de Kruskal-Wallis a été utilisé pour les statistiques.

Discussion

The study of the role of lnc-CR45601 in *Drosophila* is not easy since this non-coding gene is located in the antisense orientation of the longest exon of *Yp1*, making it difficult to tamper with the expression of one without affecting that of the other, especially as regards loss-of-function genetic analysis. Here, we have optimized the RT step and succeeded to specifically reverse transcribe the *CR45601* and *Yp1* mRNA respectively, opening the possibility to monitor their respective expressions in different infectious conditions. At the same time, we also kept exploring the phenotype in loss-of-function mutants affecting either *Yp1* or both genes together by treating them with different pathogens since *Yp1* was reduced upon at least *M. robertsii* infections. Indeed,

a similar reduction of *Yp1* expression is observed in the RNAseq data from Troha and Buchon (Flysickseq) after challenges with several Gram-positive bacterial species; however, the data were collected on males...

There was a difference in terms of survival to injected *M. robertsii* spores between the two KO mutants, while the KI mutant that removes both loci behaved as $Yp1^{-7}$and survived this infection somewhat better, in both males and females. This suggests that the difference is not directly linked to *Yp1* expression since this gene is essentially transcribed in females, as checked on the Flybase site (FlyAtlas2 and ModEncode data).

It is not clear why the phenotypes of the two *Yp1* KO mutants differ somewhat, as both lines were isogenized against the same w^{A5001} "wild-type" background. One possibility would be that the two mutations may have a differential effect on *CR45601* expression, which ought to be checked. Given that the KI mutant yields a phenotype similar to that of $Yp1^{-7}$, an observation that requires further validation in the case of the KI line that has been tested only once, one would expect that *CR45601* expression is strongly reduced in the latter mutant and not in the $Yp1^{-13}$ mutant.

Of note, the *Yp1* mutants were viable and fertile, with no measurable effect on the egg laying rate of three to six-day-old flies, suggesting that the two other yolk protein loci may be able to compensate for the loss of *Yp1*.

We had actually made a transgenic line that allows the expression of either *CR45601* or *Yp1* under the control of UAS sequences. These constructs would allow us to determine whether *CR45601* functions by an antisense effect simply by overexpressing it prior or during infections and monitoring an impact on the endogenous expression at the endogenous *Yp1* locus. However, because we did not observe a reproducible susceptibility phenotype to the tested infection for the three *Yp1* loss-of-function mutants, we did not push further the analysis. However, we should point out that we have tested only a limited array of bacterial and fungal pathogens and did not test the impact of endosymbionts or parasites such as *Tubulinosema ratisbonensis*. Thus, we cannot exclude that the decreased expression of *Yp1* might be physiologically relevant to limit the spread of a parasite. For instance, *Spiroplasma*

species are endosymbionts found in 5% of insect species that are related to Gram-positive bacteria. They are transmitted through the female germ line: the bacteria use the yolk particles that transit from the hemolymph to the egg via the follicle cell epithelium as Trojan horses [222]. However, this endosymbiont lacks peptidoglycan and at least one species does not apparently elicit the Toll pathway thus would not be expected to induce a decreased expression of *Yp1*. However, we cannot exclude that other vertically-transmitted endosymbionts/parasites may use a similar strategy but would induce a systemic immune response.

Material and Methods

Fly strains and maintenance

Fly lines were raised on the media prepared for flies at 25°C with 65% humidity. The receipt of 100 liter of fly medium is that 4.8 kg cornmeal (Priméal), 4.8 kg glucose (Tereos Syral), 6 kg yeast (Bio Springer), 360 g nipagin (VWR Chemicals) were diluted into 1400 mL ethanol (Sigma-Aldrich), 480 g agar (Sobigel) and distilled water were used to cook.

The positive controls for infection assays for Gram-positive/fungal infections and Gram-negative infections were respectively *MyD88* and key^{1} on the w^{A5001} background. All mutant flies including $Yp1^{KI}$, $Yp1^{-7}$, $Yp1^{-13}$, were isogenized on the w^{A5001} background. w^{iso} flies were served as wild type controls.

Generation of CRISPR/Cas9-mediated mutants

The *Yp1* frame shift mutants were generated by the platform in Sino-French Hoffmann Institute using CRISPR/Cas9 technology based on the expression of gRNA transgenes that were then crossed to a transgenic line expressing a *pnos-Cas9* transgene. The 20bp-long gRNAs for the target genes were devised using web-based CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/). The plasmids carrying DNA sequences for the production of single strand gRNAs were constructed using standard

methods. Briefly, the oligonucleotides were synthesized, denatured, and annealed to get double strand DNA before ligation into the expression vector, in which the gRNA coding sequences were transcribed under the control of the U6:3 promoter.

The generation of *Yp1* knock-in mutants was mediated by CRISPR/Cas9 through HDR pathway. The pCFD5 (U6:3-(t :: RNA^{Cas9})) plasmid vector was used to clone two gRNA as described in paper. Two gRNAs (TCCACGCCTGGTGGACACCGTGG and CCACGGTGTCCACCAGGCGTGGA) were designed on CRISPR Optimal Target Finder. We used a pSK vector as donor plasmid with the homology arms flanking the mCherry: a fragment 1000bp upstream and downstream of the *Yp1* locus had been amplified as a left and right arm. Several fragments including Left arm, mCherry, right arm have been ligated and assembled (Gilson Assembly) in pSK vector based on to the double-digestion at Pst1-Spe1 site and checked by sequencing. The plasmid mixture containing the two plasmids at a ration pCFD5:pSK=3:1, was then injected into recipient y^1 M{Act5C-Cas9.P.RFP- ZH-2A} w^{1118} DNAlig4 embryos.

Pathogen infections

The filamentous fungus used in this study is *Metarhizium robertsii (*10^7 spores/ml*).* The yeast species we used in this study is *Candida albicans* (Caf 2.1 strain). The bacterial strains used in this study include the Gram-negative bacterium *Pectobacterium carotovora* (strain *Ecc15,* OD_{600}=50), *Pseudomonas aeruginosa* (*PAO1*, OD=0.001) and the Gram-positive strains *Enterococcus faecalis (ATCC 19433)* (OD=0.1). The following media were used to grow the strains: Potato Dextrose Agar (PDA for *Metarhizium robertsii*); Yeast extract- Peptone-Glucose Broth Agar (YPDA for *C. albicans*); Luria Broth (LB for both Gram-positive and Gram-negative bacteria). Most of the infections were performed with injection of 4.6nl per fly except 13.8nl per fly in the case of *PAO1*. Flies were infected with *C. albicans* through pricking with a needle dipped in a clone cultured on a plate.

Gene Expression Quantitation.

Four to five whole flies were smashed into 100µl of Trizol. Samples were filled with

900µl of Trizol and mixed with 200µl of chloroform. Samples were centrifuged at 10, 000g for 10 min at 4ºC. The 400µl liquid at the upper phase of the samples was collected carefully without disruption of other phase into a 1.5ml Eppendorf tube containing 400µl of isopropanol. The samples were vortexed well and incubated at room temperature for 5 mins and then centrifuged at 12,000 g for 15 min at 4ºC. The pellet was washed in 1ml of 75% ethanol and dried. RNAs were then re-suspended in DEPC water. A volume of 10 µl was used to generate cDNA by reverse transcription, using the Transcript II all in one first strand synthesis supermix for qPCR (one step gDNA removal) synthesis kit (transgen biotech #AT341-02). The quantitative Polymerase Chain Reaction (qPCR) was performed with the same kit on cDNA diluted 20 times. The program-used was the following: 30 sec at 98ºC; 34 cycles of 5 sec at 95ºC, 30 sec at 98ºC and finally 30 sec at 65ºC. The data were analyzed using the CFX384 software (Bio-Rad). The Ct (Cycle threshold) values of the genes were normalized with the Ct values of Rpl32 (housekeeping gene that codes for a ribosomal protein). Furthermore, the normalized values of treated conditions were normalized with the normalized values of untreated conditions (delta delta Ct). All primers used in this report are listed in table S2. Quantification of mRNA levels was calculated relative to levels of the ribosomal protein gene *Rpl32* (forward primer: 5'- GACGCTTCAAGGGACAGTA-TCTG-3' ; reverse primer 5'-AAACGCGGTTCTGCATGAG-3'). Primers for *Yp1* sequences are as follow: forward primer 5'- CAACTCCGTCAACCAGGCATT-3'; reverse primer 5'-CAACTCCGTCAACCAGGCATT-3'. Primers for *CR45601* sequences are as follow: forward primer 5'-CGTTGAGCCCAACTATGTGC-3'; reverse primer 5'-ATCATCTCGTTCAGGGGAGCA-3'.

Data are expressed as means ± SEM. Data were analyzed by Mann-Whitney test, with a significance threshold of $p = 0.05$. Details are included in the legend of each figure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p<0.0001$.

Survival tests

Survival tests were performed using around 20 flies per vial in biological triplicates.

Adult flies used for survival tests were 3-6 days old. Infected flies were raised in 29°C. Statistical analysis was performed with Log-rank test.

Egg laying quantification assays

In order to ease the quantification of the laid eggs, a blue food dye (1%) was supplied into the media used for the oviposition assays. The blue media was applied on a plate. Males and females were mixed in one tube with no more than 40 individuals per tube. Tubes were kept at 25°C and flies shifted to fresh tubes every 12 hours. Flies were kept 24 hours at 25°C in these tubes then individually shifted to another new tube with blue medium. Eggs were counted on the blue medium. The egg laying index corresponds to the number of the average number of eggs laid by one fly in an hour.

Chapter II

Drosophila melanogaster CG44404 and *CG45045* encode short peptides that act in concert against biochemically-distinct virulence factors produced by prokaryotic and eukaryotic pathogens

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Abstract

The *Drosophila* systemic humoral immune response is mediated by two NF-κB pathways, Toll and Immune deficiency (IMD) that regulate the expression of effector genes that may either target microbes present in the hemocoel such as antimicrobial peptides (AMPs) or may counteract the action of secreted virulence factors. We report here that two immune inducible genes that were initially annotated as lncRNAs, *CR44404* and *CR45045* actually encode two related short secreted peptides. Genetic analysis of null mutants suggest that they are separately or jointly required for host defense against respectively injected spores of entomopathogenic fungus *Metarhizium robertsii* or systemic infections by the Gram-negative pathogen *Pseudomonas aeruginosa*. We have not detected an altered microbial titer of these pathogens in the

double mutant. We have however found that they participate to the protection of flies from the action of DestruxinA (DtxA), a mycotoxin secreted by *M. robertsii*, or Outer Membrane Vesicles (OMVs) secreted either by *P. aeruginosa* or the entomopathogenic *Serratia marcescens* Gram-negative bacterium. Interestingly, CG44404 and CG45045 together counteract the detrimental effects of the *S. marcescens* metalloprotease PrtA, the major virulence determinant carried by *S. marcescens* OMVs, without specifically inhibiting its catalytic activity. The *CG44404-CG45045* phenotype is reminiscent of that recently described for *BaramicinA*, a Toll pathway effector that protects the fly from the noxious activities of DtxA and the Enterocin V (EntV) bacteriocin secreted by *E. faecalis*. We find that the *CG44404-CG45045* displays an enhanced sensitivity to EntV. Conversely, *BaraA* mutants are sensitive to OMVs and PrtA. Immunoprecipitation experiments suggest the association of CG44404 with specific BaraA-derived peptides and also with a complement protein, TEP2. We finally report that *Tep2* and *Tep4* mutants display opposite phenotypes of decreased or increased sensitivity to PrtA, but not to DtxA. Altogether, our data suggest the existence of a host response able to counteract the virulence of secreted microbial factors of distinct origins with different biochemical activities and that involve several immune-related proteins.

Introduction

Drosophila melanogaster is a powerful genetic model organism that is well-adapted for the study of innate immune mechanisms, which are largely evolutionarily conserved from a primeval common ancestor of flies and of humans. The adult fly relies on multiple defense responses that allow them to combat infections [4], which are 1) a systemic humoral immune response: the fat body (a functional composite of the liver and adipose tissue of mammals) secretes in the hemolymph varied effectors such as antimicrobial peptides (AMPs) that attack the microbes. The expression of AMP genes is mainly regulated by two pathways [127], the Toll and Immune Deficiency (IMD) pathways. Whereas many Gram-positive bacteria and fungi activate the Toll pathway, the IMD pathway is preferentially triggered by Gram-negative bacteria, which however

can also activate the Toll pathway to some extent upon the secretion of pathogenic proteases [65, 71]; 2) a cellular immune response: mature plasmatocytes (a functional equivalent of the mammalian macrophage) phagocytose the microbes directly and have addtional roles such as secretion of cytokines; 3) melanization: relying on the cleavage of pro-phenoloxidase (PPO) into phenoloxidase (PO) followed by the oxidation of phenols, melanin is deposited at the wound and infection site [128]; it may contribute to killing microbes through the generation of Reactive Oxygen Species (ROS) [70]. Most immune responses are known to target pathogens by killing them or preventing their proliferation. Besides, systemic immune respones, local immune respones occur at barrier epithelia.

The sequencing of genomes confirmed that most of chromosomal DNA is noncoding and revealed an array of RNAs that include long noncoding RNAs (lncRNAs). Advanced computational biology using programs such as PhyloCSF [214] and the analysis of RNAs associated with polysomes, ribosome profiling [223], revealed that many lncRNAs genes actually encode micropeptides [224]. The well-characterized secreted short peptides in *Drosophila* are AMPs that act in resistance against microbes. For instance, AMPs are generally described to interact with the negatively charged bacterial surface through their cationic feature, some of them leading to the lysis of the bacterial membrane[83-85].

However, recent studies in *Drosophila* have revealed that some Toll pathway effectors do not solely function in resistance against infections [91]. The analysis of *Aspergillus fumigatus* infections in *Drosophila* has shown that this pathogen kills Toll pathway mutant but not wild-type flies through secreted mycotoxins, most of which are secondary metabolites. The wild-type host is largely protected by a family of Toll-regulated related genes, *Bomanins*, that encode secreted peptides, some of which are able to protect the host from the action of mycotoxins. Since these peptides do not directly target the pathogen for protection, they function in resilience, also known as disease tolerance [97]. In addition, Baramicin A (BaraA, also known as IMPPP), a polyprotein, can be cleaved post translation into several short peptides, including DIM

5, 6, 8, 10, 12, 13, 22, and 24 [93, 99]. A recent study has revealed that BaraA plays a role against distinct microbial toxins, Destruxin A (DtxA), a circular hexadepsipeptide that may function as an ionophore, and Enterocin V (EntV), a bacteriocin, secreted respectively by the entomopathogenic fungus *Metarhizium robertsii* and the Gram-positive bacterium *Enterococcus faecalis*. BaraA helps the host recover from DtxA-induced paralysis and appears to be required in glial cells but not in neurons [1]. Interestingly, it has been proposed that long peptides evolutionarily related to DIM24 may function in the nervous system [100].

In order to better understand the interactions between the host and pathogens, we performed a transcriptomic analysis on flies after *M. robertsii* infection. We dug out *CR44404* (now known as *CG44404* or *Induced by infection (IBIN)* [225]), a putative long noncoding RNA (lncRNAs), which was one of the most highly induced genes among lncRNAs after *M. robertsii* infection. Here, we report that these two putative lncRNAs, *CG44404* (known as *CR44404*) and *CG45045* (known as *CR45045*), encode similar peptides and are required in host defense against injected *M. robertsii* spores or injected *Pseudomonas aeruginosa*. Finally, we found that both short peptides actually counteract the actions of secreted virulence factors, DtxA secreted by *Metarhizium robertsii* and protease A (PrtA) produced by *Serratia marcescens* and packaged in Outer Membrane Vesicles (OMVs) released by the bacteria.

Results

The annotated lncRNAs CR44404 *and* CR45045 *encode related secreted short peptides*

We first confirmed using RTqPCR that *CR44404* is indeed strongly induced by *M. robertsii* in both injection and natural infection models (Fig.1 A-B). To determine whether this lncRNA was evolutionarily conserved, we blasted two kb of genomic sequence against *Drosphila* genome sequences deposited in Flybase that includes *CR44404* and found that this region was relatively well conserved in members of the *Drosophila melanogaster* subgroup (*D. melanogaster*, *D. biarmipes*, *D. eugracilis*, *D. ficusphila*, *D. elegans*, *D. rhopaloa, D. simulans*, *D. sechellia*, *D. yakuba,* and *D. erecta*). There were some gaps however in *D. melanogaster* the sibling species *D. sechellia* and *D. simulans*. By blasting the sequence of *CR44404*, we found hits in *D. biarmipes*, *D. eugracilis*, *D. ficusphila*, *D. elegans*, and *D. rhopaloa*, besides *D. yakuba* and *D. erecta*. Unexpectedly, the *CR44404* locus was interrupted at the same position for *D. simulans* and *D. sechellia*. The degree of conservation was poorer for more distant *Drosophila* species with e-values higher than 6. 10^{-20} and a Blast score lower than 100. As long ncRNAs may encode micropeptides [224], we looked for open reading frames (ORFs) in the sequence and found one, which was evolutionarily conserved in the above species, *D. simulans* and *D. sechellia* excepted as the coding sequence has conserved only in the proximal part of the ORF (Fig. 1C). A consensus Kozak sequence was found for all of them. Interestingly, all of them were predicted to encode a signal peptide, suggesting that the micropeptides may be secreted.

Next, we used the tBlastN program in NCBI on *D. melanogaster* sequences and found one hit in the *Drosophila* genome, which actually corresponds to the lncRNA locus of *CR45045*. This locus is also evolutionarily conserved in *D. sechellia*, *D. simulans*. *D. erecta*, *D. biarmipes* and *D. takahashii* and absent in the other species cited above. The ORFs encode peptides related to CR44404, including the signal peptide (Fig. 1C). Thus, the two genes may have been selected for unrelated functions

in some species such as *D. simulans/D. sechellia* or *D. yakuba*. Alternatively, one peptide might be sufficient to fulfill the functions of the other missing peptide. We found that *CR45045* was not specifically induced by *M. robertsii* (Fig. 1D-E).

The putative secreted CR44404 peptide is expected to be 23 amino-acid long with a 2931.14 Da molecular weight with a 6.7 pI and a Grand average of hydropathicity (GRAVY) of -2.05, in the absence of any post-translational modifications. As regards the hypothetical secreted CR45045 peptide, the 24 amino-acids are computed to have a 3027.27 Da MW and a more basic 8.44 pI with a GRAVY of -2.47. To experimentally determine whether these peptides are secreted, we first expressed both genes in S2 cells as HA-TAG peptides. The CR45045-HA peptide was expressed more efficiently than CR44404-HA as observed in cell lysates and both peptides were detected in the supernatant (Fig. S1). We also constructed the TR44404 and TR45045 transgenes containing the genomic regions of interest in which the *CR44404* and *CR45045* coding sequences were replaced after the initial ATG respectively by *eGFP* and *mCherry* coding sequences. Whereas the fluorescent reporters were hardly detected in uninfected control flies, both reporters were induced in the fat body after an *Escherichia coli* immune challenge thus establishing the potential expression of these two peptides (Fig. 1F-G). We also analyzed by MALDI-TOF mass spectrometry the hemolymph of flies (Fig. S2). We did find an *E. coli*-infection inducible peak at the expected MW for CR45045 but not for CR44404. This peak was absent in a *CR45045* deletion mutant (see below). We conclude that *CR45045* is a *bona fide* immune-inducible peptide. We next addressed the issue of the potential secretion of CR44404 in the absence of direct biochemical evidence for its existence. We generated two further transgenes in which we either fused the *mCherry* coding sequence to those encoding the signal peptide at its predicted cleavage site or tagged the *CR44404* full coding sequences with those of *mCherry*, which would be expected to yield a secreted CR44404-mCherry C-terminal fusion protein. We next collected the hemolymph of transgenic flies and measured its fluorescence intensity using a fluorometer. Control flies carrying a TR45045 transgene that encodes a nonsecreted version of mCherry did not yield any signal as expected

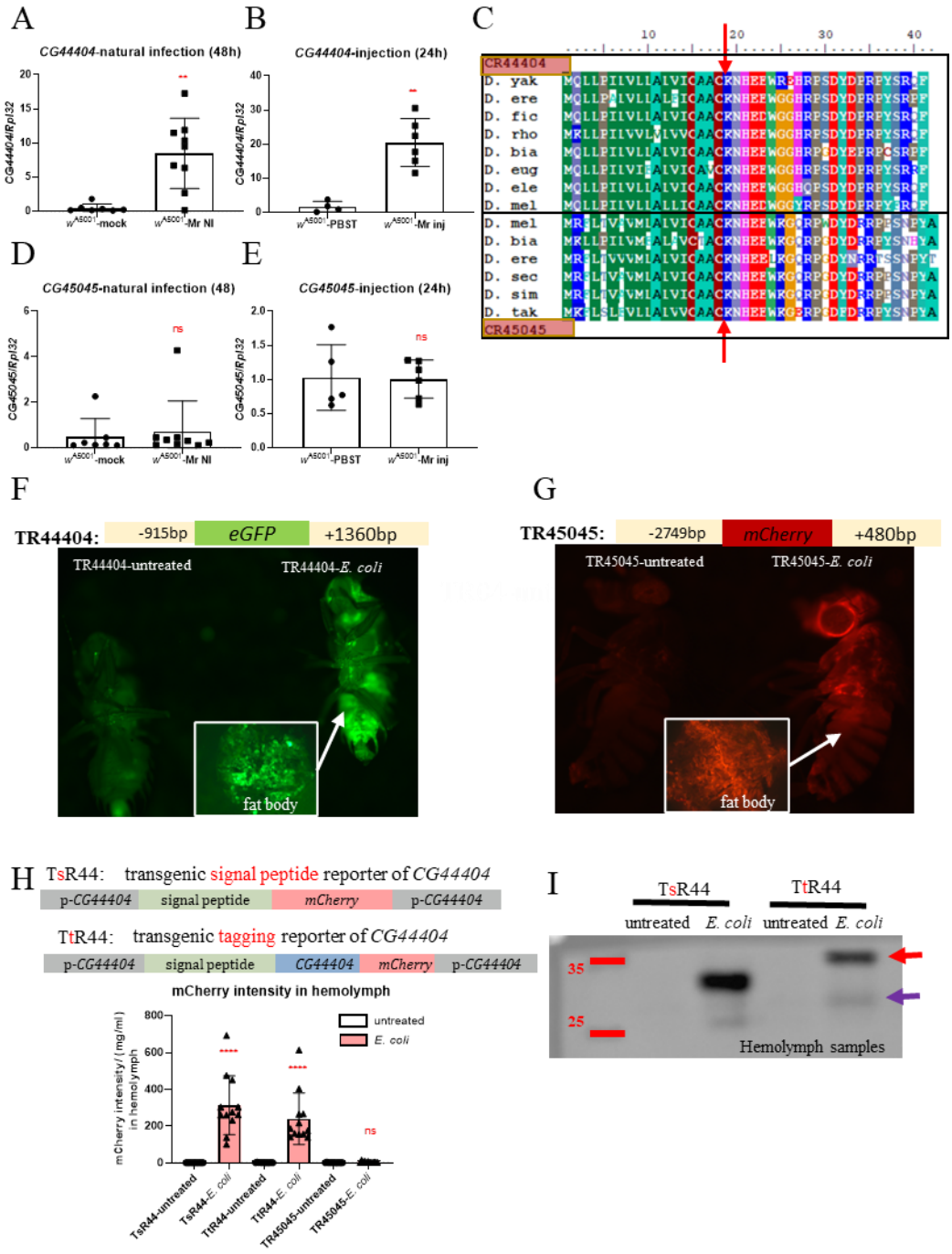

Figure 1. *CG44404* et *CG45045* codent pour des peptides sécrétés courts. (A) L'expression du gène *CR44404* a été contrôlée par RTqPCR 48 heures après l'infection naturelle (NI) et **(B)** 24 heures après l'injection (inj) de *Metarhizium robertsii (Mr)*. Les moyennes ± SEM sont indiquées. Les données regroupées de deux ou trois expériences indépendantes ont été montrées. Le test de Mann-Whiney a été utilisé pour l'analyse statistique. **(C)** Alignement des peptides prédits codés par *CR44404* et *CR45045* dans différentes espèces de *drosophile*. Les flèches indiquent le site de clivage prédit des peptides de signal à partir de SignalP-5.0. **(D)** L'expression du gène *CR45045* a été contrôlée par RTqPCR 48 heures après le NI et **(E)** 24 heures après l'injection de Mr. Les données regroupées de deux ou trois expériences indépendantes ont été montrées. Le test de Mann-Whiney a été utilisé pour l'analyse statistique. **(F. G) Le** schéma de la construction de TR44404 et TR45045 (TR=transgenic reporter) a été

montré en haut. Image du TR44404 et du TR45045 à 24h sans (à gauche) et avec (à droite) traitement par *E. coli. Les* images du corps gras dans l'abdomen sont montrées au milieu avec une boîte blanche. **(H) Le** schéma des constructions TsR44 et TtR44 est présenté en haut. L'intensité du mCherry dans l'hémolymphe collectée à 24 heures avec ou sans traitement par *E. coli* a été détectée par un fluoromètre. TR45045 a servi de contrôle négatif. Le test de Kruskal-Wallis a été utilisé pour l'analyse statistique. Les moyennes ± SEM sont indiquées. Les données regroupées de trois expériences indépendantes ont été montrées. **(I)** Un Western blot a été réalisé sur les échantillons d'hémolymphe prélevés sur les mouches. Trois expériences indépendantes ont montré la même tendance que la figure présentée. Les flèches indiquent les bandes décalées.

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whether transgenic flies were untreated or challenged with *E. coli.* In contrast, both the secreted mCherry and the secreted CG44404-mCherry fusion protein produced a similarly strong fluorescence upon immune challenge (Fig. 1H). These data were further confirmed by Western blot analysis of the hemolymph in which the CG44404-mCherry fusion protein migrated with a lower velocity as the mCherry protein as expected (Fig. 1I).

All together, these data establish that the lncRNAs *CR44404* and *CR45045* actually encode immune-inducible secreted peptides, thus justifying the change of annotation of Flybase of these two genes into coding genes, *CG44404* and *CG45045,* a correction that was introduced which was made while this work was underway.

The expressions of CG44404 *and* CG45045 *are induced by immune challenges but are not strictly coincident*

We challenged flies with either *E. coli* or *Micrococcus luteus*, which respectively stimulate preferentially the IMD and the Toll pathways. Whereas the expression of *CG44404* was induced by both stimuli, that of *CG45045* was essentially increased upon *E. coli* challenge, whereas the injection of PBS alone as an injury control did also trigger its expression, albeit at lower levels (Fig. 2A-D). Of note, both genes were better induced by the Gram-negative than the Gram-positive bacterial challenge. The expression of *CG44404* induced by *E. coli* was largely mediated by the IMD pathway, as witnessed using the *kenny* (*key*) or *Relish* (*Rel*) mutants (Fig. 2A, E). This pathway also contributed to the increased expression of *CG44404* upon *M. luteus* injection at

early time point at 6h (Fig. 2B), which mirrored its induction by a mock injection with PBS, in keeping with the distinct kinetics exhibited by the IMD and Toll pathways upon Gram-negative or Gram-positive bacterial injury [81]. Indeed, the Toll pathway mediated to a large extent the induction of *CG44404* expression by *M. luteus* (Fig. 2B, F). As expected, the induction of *CG45045* was dependent on *key* and *Rel* (Fig. 2C, G) and not on the Toll pathway genes *Myeloid Differentiation Factor 88* (*MyD88*) and *Dorsal-related immunity factor* (*Dif*) (Fig. 2D, H).

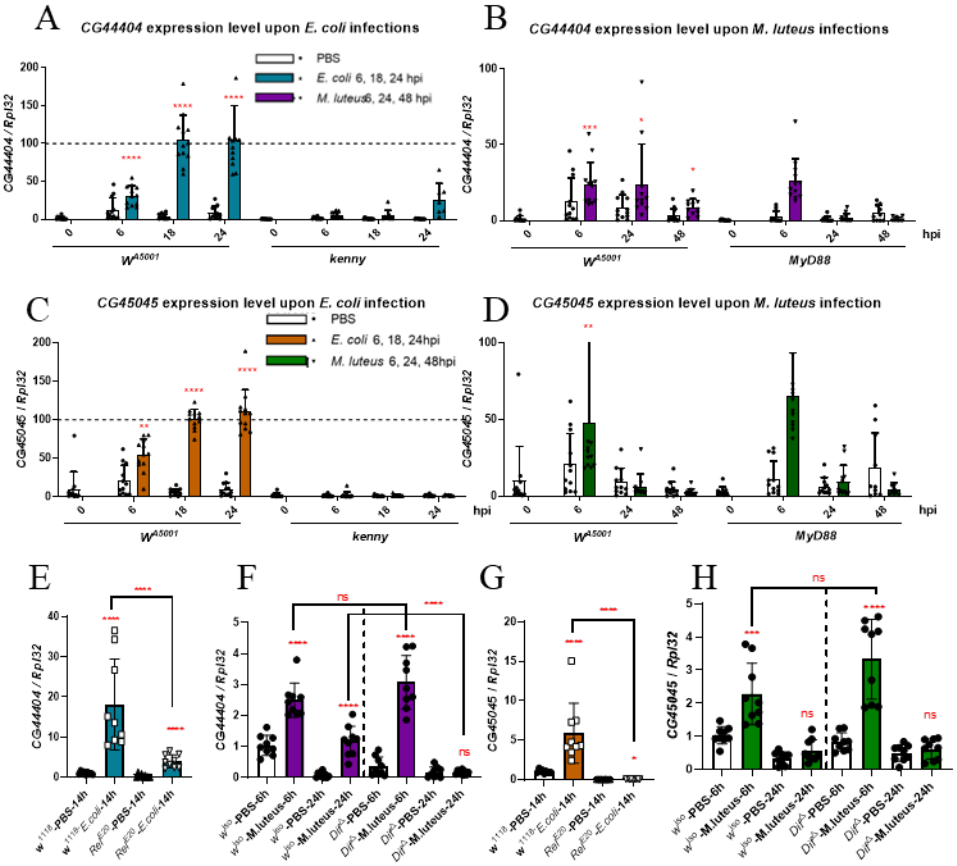

Figure 2. L'expression transcriptionnelle des gènes *CG44404* et *CG45045* après l'injection septique de différentes bactéries chez les mouches de type sauvage et les mutants. (A) L'expression du gène *CG44404* chez le type sauvage et les mutants a été contrôlée par RTqPCR à différents moments après l'injection septique d'*E. coli* (OD_{600} =50, 4.6nl) et **(B)** *M. luteus* (OD_{600} =50, 4.6nl). **(C) L'**expression du gène *CG45045* chez le type sauvage et les mutants a été contrôlée par RTqPCR à différents moments après l'injection septique d'*E. coli* et **(D) de** *M. luteus*. **(E) L'**expression du gène *CG44404* chez le type sauvage, le mutant *Relish* et **(F) le** mutant *Dif* a été contrôlée après différentes infections bactériennes. **(G) L'**expression du gène *CG45045* chez le type sauvage, le mutant *Relish* et **(H) le** mutant *Dif* a été contrôlée après différentes infections bactériennes. Les moyennes ± SEM sont indiquées dans toutes les figures. Les données regroupées de trois ou quatre expériences indépendantes ont été présentées. Le test de Mann-Whiney a été utilisé pour l'analyse statistique.

The CG44404 *and/or* CG45045 *null mutants are susceptible only to* M. robertsii *and* Pseudomonas aeruginosa

We have generated two genetically null mutants for *CG44404* and *CG45045* by deleting their respective coding sequences (Fig. S3A) and then isogenized the mutant lines in the w^{1118} (A5001) line. We have challenged these mutants, or the double-mutant, with a variety of pathogenic microorganisms. These mutants did not display any enhanced susceptibility to *Enterococcus faecalis*, *Pectobacterium carotovorum* (formerly *Erwinia carotovora carotovora*, *Ecc15* strain), *Aspergillus fumigatus*, and *Candida glabrata* (Fig. S4A-D). However, we noted a mild but nevertheless reproducible sensitivity of double mutants to injected *M. robertsii* spores (Fig. 3A). Interestingly, the single mutants were equally susceptible to the injected *M. robertsii* spores. Unexpectedly, the *CG44404* mutant but not the *CG45045* mutant or the double-mutant exhibited a mild sensitivity to *M. robertsii* spores applied in a "natural" infection paradigm (Fig. S4E). As we have not been able to directly show that the *CG44404* null mutant is really not expressing the CG44404 peptide, we performed a genetic rescue experiment in which we overexpressed the *CG44404* coding sequences using the Gal4/UAS system in a *CG44404- CG45045* double mutant background. As shown in Fig. S3B, there was a significant rescue of the fly line overexpressing *CG44404* coding sequences as compared to several *CG44404- CG45045* negative control lines, including one that expresses the ubiquitous driver *pubi-Gal4* in the absence of the *UAS-CG44404* transgene. The rescue was partial when compared to wild-type control flies. Because *CG45045* mutants are susceptible to injected *M. robertsii* spores, it was somewhat unexpected that this rescue experiment would work. We therefore checked the degree of expression of the *CG44404* transcripts by RTqPCR. The transgene was strongly overexpressed as compared to wild-type control flies (Fig. S3C), which opens the possibility that the CG44404 peptide may functionally fill in for the missing CG45045 peptide when strongly overexpressed, at least to some degree.

As regards Gram-negative bacteria, we noted a mild sensitivity of the double mutant to *Enterobacter cloacae* and to the *P. aeruginosa* (PAO1 strain) pathogen (Fig.

S4F and Fig.3B). In contrast to the situation with injected *M. robertsii* spores, we did not observe any enhanced susceptibility of the single mutants to the *P. aeruginosa* challenge (Fig. S4G-H).

To determine whether the *CG44404* and the *CG45045* genes play a role in resistance or resilience to infections, we monitored the microbial burden in the *CG44404-CG45045* double mutant upon a challenge with the injection of *M. robertsii*

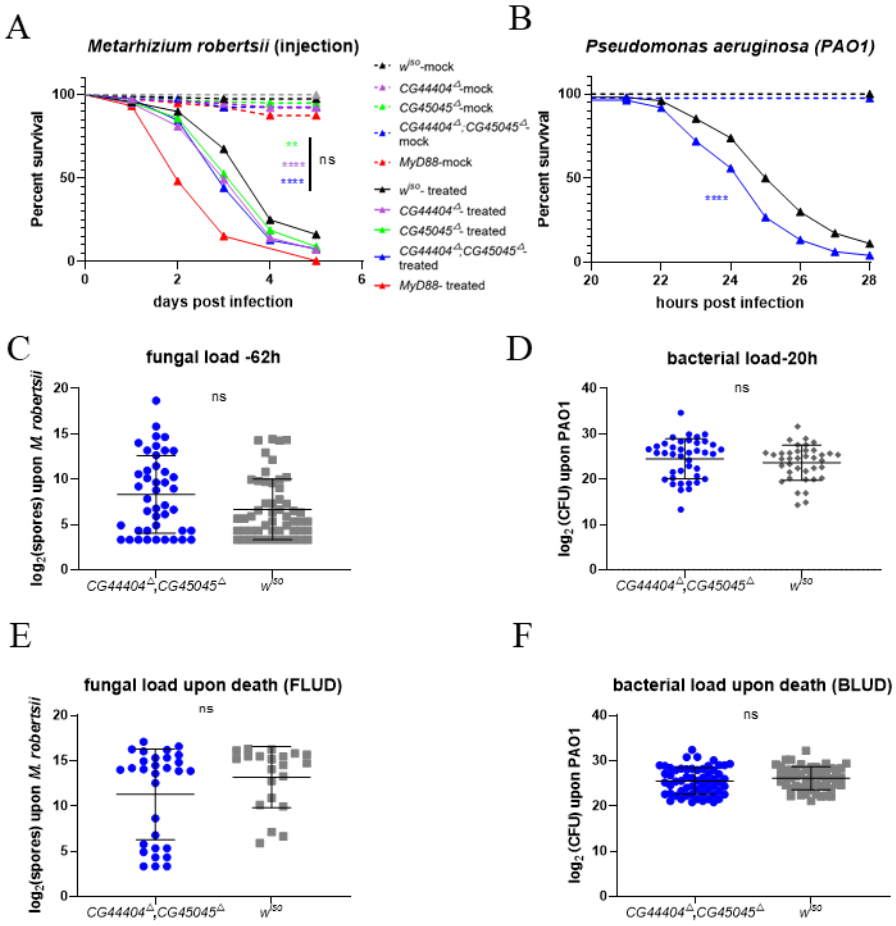

Figure 3. Taux de survie et charge pathogène *sur Metarhizium robertsii* et *Pseudomonas aeruginosa.* **(A)** Courbes de survie des mutants simples isogéniques de *CG44404* et *CG45045* et du double mutant infecté par 4,6 nl 10^7 spores/ml *M. robertsii* et **(B)** 13,8 nl OD_{600} =0,001 *PAO1*. *Les* données regroupées de quatre et huit expériences indépendantes ont été montrées. Le w^{iso} a servi de contrôle de type sauvage tandis que le mutant *MyD88* a servi de contrôle positif. Le log-rank a été utilisé pour l'analyse statistique. Les couleurs de * correspondent aux couleurs des mutants par rapport à w^{iso} . L'analyse statistique en noir a été réalisée entre trois mutants. **(C, E)** Charge pathogène de *M. robertsii* et **(D, F)** Charge *PAO1* chez les doubles mutants et le type sauvage. Les données regroupées de trois expériences indépendantes ont été montrées. Les moyennes ± SEM ont été indiquées dans toutes les figures. Le test de Mann-Whitney a été réalisé pour l'analyse statistique.

spores or to PAO1 bacteria. As shown in Fig. 3C-D, we did not detect any significant difference in the microbial burden between the double mutant and isogenic control. We next measured the microbial load upon death and found that the double mutant succumbed to the same microbial load as control flies (Fig. 3E-F). Thus, these tests did not reveal a clear-cut implication of CG44404/CG45045 in either resistance or resilience.

CG44404 ***and/or*** **CG45045** ***mutants are susceptible to secreted fungal and bacterial virulence factors***

In a previous study, we have reported that mutants affecting the Toll pathway effector BaramicinA (BaraA) are sensitive to injected *M. robertsii* spores yet do not display altered fungal load or fungal load upon death. Interestingly, we had discovered that the *BaraA* flies were sensitive to one secreted toxin known as DestruxinA (DtxA) [1]. We therefore tested whether *CG44404* and *CG45045* single and double mutants are also susceptible to this toxin and found that the single and double mutants displayed a significant susceptibility to injected DtxA (Fig. 4A). Similar to the *BaraA* phenotype, the enhanced susceptibility of single mutants to injected *M. robertsii* spores was lost when mutant flies were injected with a strain that is unable to synthesize destruxins (Fig. 4B). The double-mutant was however still slightly sensitive to the challenge with the mutant *M. robertsii* strain. Thus, we conclude that both CG44404 and CG45045 peptides play a role in the host defense against DtxA.

BaraA mutants were also mildly susceptible to *E. faecalis* and we reported that actually *BaraA* was involved in the host defense against an *E. faecalis* secreted virulence factor, the bacteriocin EntV [1]. We therefore tested whether a killing activity is present in the supernatant of *P. aeruginosa* PAO1 bacteria grown in Brain-Heart-Infusion (BHB) liquid culture medium. Because only the *CG44404-CG45045* double mutant was susceptible to PAO1, we only tested the double mutant to the injection of unconcentrated supernatant of stationary phase bacteria: it was more susceptible to this challenge than control flies (Fig. 4C). We next tested whether the killing activity was

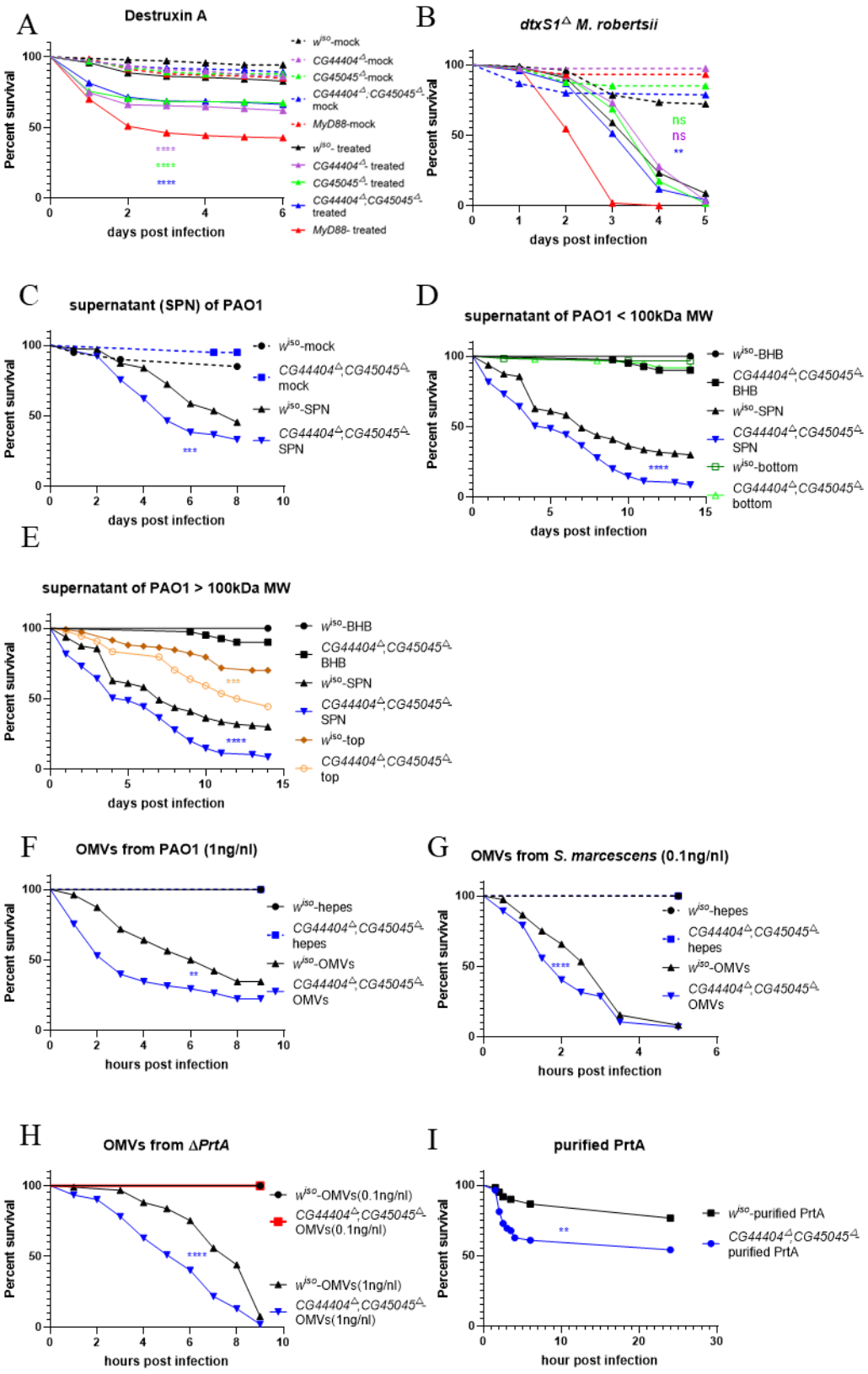

Figure 4. Taux de survie à différentes substances virulentes. (A) Courbes de survie des mutants simples isogéniques de *CG44404* et *CG45045* et des mutants doubles infectés par la toxine Destruxin 4,6nl, 8mM. 80% de DMSO a été injecté comme contrôle du véhicule. Données regroupées provenant de plus de cinq expériences indépendantes. **(B) Les** mutants ont été injectés avec 50 spores de la souche mutante *DestruxinS1⁻ M. robertsii* dans laquelle la biosynthèse des Destruxines est bloquée. Données regroupées provenant de plus de trois

expériences indépendantes. Le log-rank a été utilisé pour l'analyse statistique. Les couleurs de * correspondent aux couleurs des mutants. **(C)** Les doubles mutants ont été infectés par le surnageant des cultures *PAO1*. Données regroupées de trois expériences indépendantes. (**D)** Les doubles mutants ont été infectés avec la fraction du surnageant passant à travers le filtre amicon 100k Da MW (fraction du bas) et **(E)** la fraction retenue sur le filtre (fraction du haut). Données regroupées de deux expériences indépendantes. **(F) Les** doubles mutants ont été injectés avec les OMVs produites à partir de *PAO1* et **(G)** *S. marcescens*. Données regroupées de deux ou cinq expériences indépendantes. **(H) Les** doubles mutants ont été injectés avec la protéase A purifiée (PrtA) de *S. marcescens.* Une seule expérience a été montrée. **(I) Les** OMVs produites à partir de *S. marcescens* mutant *DPrtA* n'ont montré aucune virulence pour les mouches à la même concentration (0,1ng/nl) que *S. marcescens* wt. Les OMVs 10 fois provenant de DPrtA ont retrouvé leur virulence et tué les doubles mutants plus rapidement. Données regroupées de deux expériences indépendantes. Le log-rank a été utilisé pour l'analyse statistique.

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of a molecular weight higher or lower than 100kDa by centrifuging the supernatant on Amicon Centricon™ filters. Whereas no activity was found in the fraction containing molecules of MW lower than 100 kDa (Fig. 4D, green curves), it was present in the unfiltered fraction that contains molecules or complexes above 100 kDa (Fig. 4E, orange-brown curves), albeit with a lower level than the native supernatant positive control (Fig. 4E, blue-black curves). In our studies on the virulence of the highly pathogenic bacterium *Serratia marcescens* that kills flies in less than 24 hours, we have discovered that Outer Membrane Vesicles (OMVs) secreted by the pathogen are especially potent effectors that paralyze flies within six hours of their injection. We therefore prepared OMVs from both *P. aeruginosa* PAO1 and *S. marcescens* RM66262 and injected them. For both preparations, we detected an enhanced sensitivity of the *CG44404-CG45045* double mutant as compared to control flies (Fig. 4F-G). In the case of RM66262 OMVs, we had documented by mass-spectrometry that it contains the metalloprotease PrtA, also known as Serralysin, a major virulence factor of *S. marcescens*. In keeping with those results, we found that OMVs prepared from a *S. marcescens* RM66262 *PrtA* mutant were no longer virulent when injected at a 0.1 ng/nl concentration we normally use (Fig. 4H, red curve). However, we were able to demonstrate that the 10x concentrated *PrtA* OMVs do still contain another activity to which the double mutant is sensitive (Fig. 4H, blue curve). Finally, we directly injected purified PrtA and found that again the double mutant was more sensitive to this

challenge than the control flies (Fig. 4I).

All together, these data establish that *CG44404* and/or *CG45045* play a role in the host defense against secreted microbial toxins, the circular hexadepsipeptide DtxA and the 56kD protease PrtA.

The deletion of* CG44404 *and* CG45045 *leads to an impaired induction of some AMP genes and does not impact other arms of the immune defense

We checked whether the *CG44404* and *CG45045* might play a role in the innate immune system. We first checked whether the systemic humoral immune response might be impacted by the deletion of the two genes. Unexpectedly, we observed a lessened induction of the *Attacin D*, *Defensin*, and *Diptericin A* genes, and an almost abolished induction of the *Attacin A&B* genes upon an *E. coli* challenge, but not of the *Drosocin* and *Cecropin A2* genes (Fig. 5A-D; Fig S5A-B). Because all of the above-cited genes are under the control of the IMD pathway upon an *E. coli* challenge, it appears that the two peptides may not be directly regulating the IMD pathway. Since the functions we have identified so far for *CG44404* and *CG45045* is in the host defense against *M. robertsii* DtxA and *S. marcescens* PrtA, we tested whether a line that lacks *Attacin*, *Drosocin*, and *Diptericin* genes (group B genes) was sensitive to these two virulence factors. The Group B mutants were not significantly susceptible as compared to isogenic controls (Fig. 5E-F), suggesting that the decreased induction of some IMD pathway regulated AMP genes observed in the *CG44404-CG45045* double mutants does not contribute to the virulence factor sensitivity phenotype, although we cannot formally exclude a contribution of *Defensin*. PAO1 is resistant to the action of AMPs and Group B, but not the *pilJ* mutant, which would be worth testing in the *CG44404-CG45045* double mutant. We do not want to go into this in this manuscript.

We also tested whether the induction of Toll pathway-regulated AMP genes were similarly reduced in the double mutant flies and found that the expressions of the *Drosomycin, IM1,* and *Metchnikowin* were normally up-regulated upon a *M. luteus* injection (Fig. S5C-E). Of note, *Defensin*, which is hardly induced by a *M. luteus*

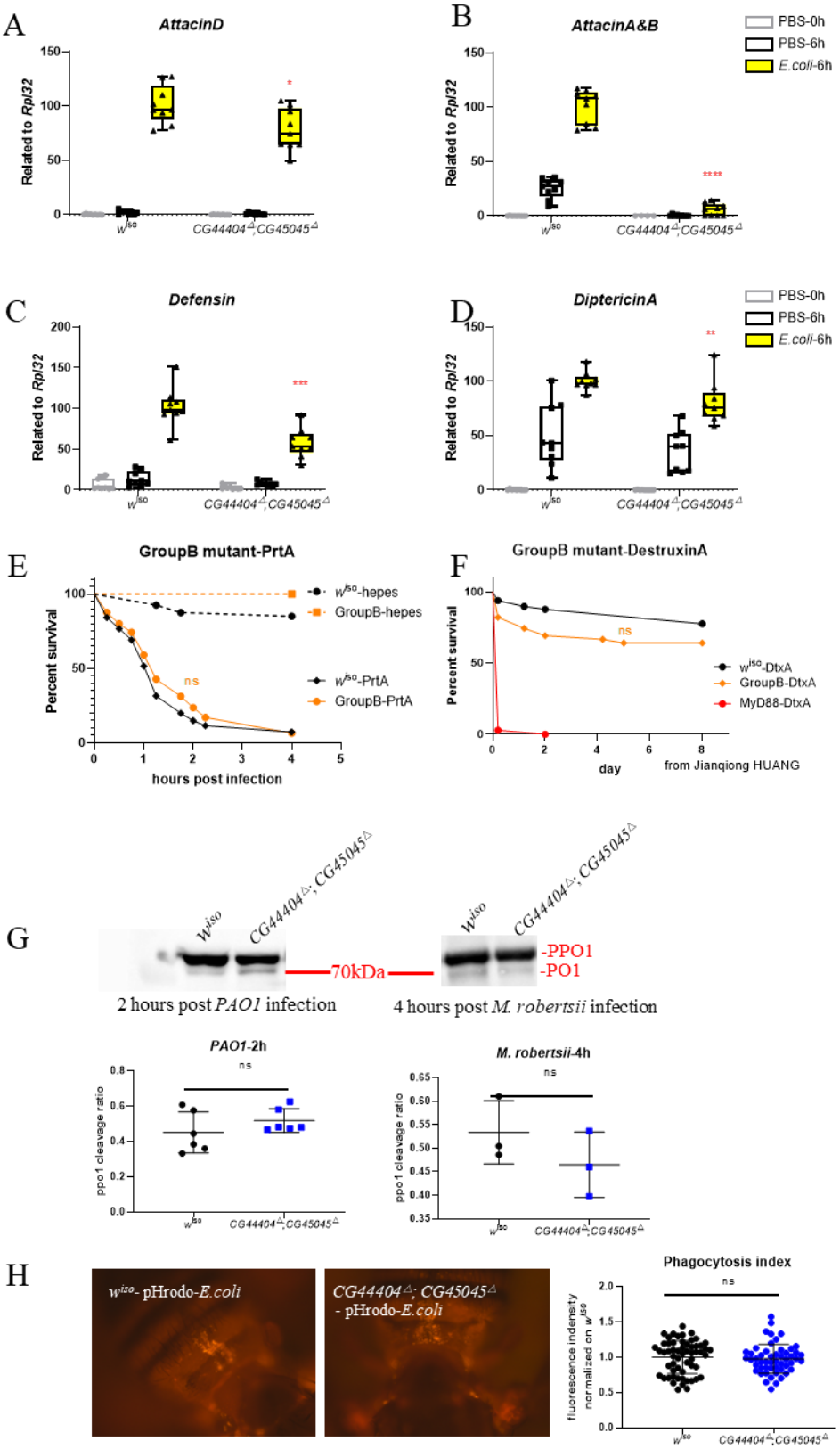

Figure 5. Réponses immunitaires canoniques chez le double mutant. (A) L'expression de l'*attacine D*, **(B) de l'***attacine A&B,* **(C) de la** *défensine* et **(D)** *de la diptéricine A* a été contrôlée par RTqPCR chez w^{iso} et les doubles mutants 6 heures après l'infection par *E. coli.* Toutes les données ont été normalisées sur le w^{iso} infecté à 100. Les données regroupées de trois expériences indépendantes ont été présentées. Le test de Mann-Whitney a été effectué entre le w^{iso} infecté et le double mutant. **(E) Les** mutants du groupe B portent les mutations de toutes les *attacines, des* deux *diptéricines* et de la *drosocine. Les* mutants isogéniques du groupe B ont

été injectés avec de la PrtA purifiée et de la DtxA commerciale**.** Les données regroupées de deux expériences indépendantes ont été montrées. Le log-rank a été utilisé pour l'analyse statistique. **(G) Un** Western blot a été réalisé sur des échantillons d'hémolymphe de mouches infectées par *PAO1* (à gauche) et *M. robertsii* (à droite). Des bandes de PPO1 et de PO1 clivé ont été détectées. La quantification du ratio de clivage (PPO1/(PP1+PO1)) a été analysée par Fiji et est présentée en bas respectivement. Les données regroupées de trois ou quatre expériences indépendantes ont été présentées. Le test de Mann-Whitney a été utilisé pour l'analyse statistique. **(H)** *E. coli* marqué au pHrodo a été injecté dans des mouches. Les mouches ont été photographiées 30 minutes après l'injection. L'intensité de la fluorescence a été analysée par Fiji. Les données regroupées de trois expériences indépendantes ont été montrées. Les moyennes ± SEM ont été indiquées. Le test de Mann-Whitney a été utilisé pour l'analyse statistique.

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challenge was expressed at lower levels in the *CG44404- CG45045* flies (Fig. S5F).

We did not observe an altered induction of the melanization cascade as monitored by Western blot analysis of prophenol oxidase 1 cleavage upon a *P. aeruginosa* or *M. robertsii* challenge in *CG44404- CG45045* double-mutant flies (Fig. 5 G). The phagocytic uptake of pH-RODO-labeled bacteria was not impacted in the double-mutants (Fig. 5H).

The function of CG44404 and CG45045 in the host defense against OMVs does not involve a direct action on the PrtA protease nor on redox-signaling

Since PrtA recapitulates to a large extent the pathogenicity of *S. marcescens* OMVs, we tested whether the CG44404 and CG45045 peptides might simply act as protease inhibitors. We therefore established a simple *in vitro* assay in which purified PrtA is incubated with an azocasein substrate; the cleavage activity of the protease can then be monitored using a colorimetric assay. Since PrtA is a zinc metalloprotease that can be inhibited by EDTA, we first determined which EDTA concentration was needed to inhibit its activity *in vitro* so as to provide a positive control. PrtA activity was strongly decreased in a 50 mM final EDTA concentration (Fig. 6A). Next, we added synthetic CG44404 or CG45045 peptides to the azocasein PrtA mix. We could not use concentrations of peptides equal or above 6 mM as the azocasein was otherwise precipitated by the peptides (Fig. 6B). Indeed, a similar behavior was observed upon adding a mix of the two scrambled peptides (sc). Thus, we repeated the experiment

using a 3 mM final concentration of each peptide or of the combined CG44404 and CG45045 peptides and noted a mild inhibition of the proteolytic activity of PrtA. This inhibition was however not specific since it was also observed with the scrambled peptide mix (Fig. 6C). We conclude that CG44404 and CG45045 are unlikely to function by directly inhibiting the enzymatic activity of PrtA since we used them at concentrations likely one or two orders of magnitude higher than those found *in vivo*.

In another study, we have documented that reactive oxygen species (ROS) signaling is important to mediate the pathogenicity of injected OMVs which can be alleviated by the co-injection of anti-oxidants such as vitamin C. As shown in Fig. 6D-E, the double-mutant flies were more susceptible to the injection of *S. marcescens* or *P. aeruginosa* OMVs whether vitamin C was co-injected or not. We conclude that *CG44404* and *CG45045* are unlikely to be required in the host defense against OMVs through an action on ROS signaling.

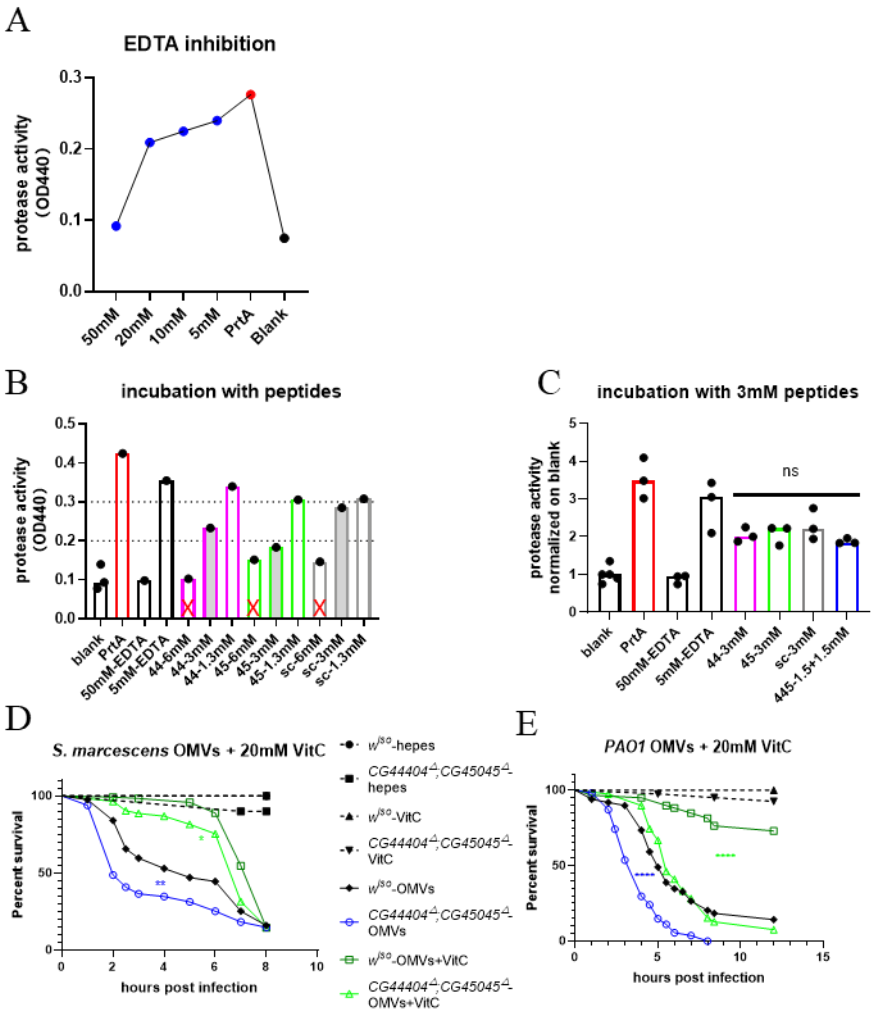


Figure 6. Activité protéasique de la PrtA incubée avec des peptides. (A) La PrtA a été incubée avec différentes concentrations d'EDTA *in vitro*. L'activité protéasique a été contrôlée avec le substrat azocaséine à OD440. La PrtA seule sans EDTA est représentée en rouge. **(B) La** PrtA a été incubée avec différentes concentrations de peptide CG44404 (44), de peptide CG45045 (45) et de peptides brouillés (sc). Le résultat de l'incubation des peptides 6mM n'était pas disponible en raison de la précipitation de l'azocaséine. La concentration de 3mM dans la colonne grise était optimiste. **(C)** 3mM de peptides ont été incubés avec PrtA. Les données regroupées de trois expériences indépendantes ont été montrées. Le test de Kruskal-Wallis a été utilisé pour l'analyse statistique. **(D)** Des mutants doubles ont été co-injectés avec 20 Mm de vitamine C (VitC) et des OMVs de *S. marcescens* et **(E)** PAO1. Les données regroupées de deux ou d'une expérience indépendante ont été montrées. Une analyse log-rank a été réalisée entre w^{iso} et le double mutant avec ou sans VitC respectivement.

DtxA does not appear to alter the permeability of the blood brain barrier (BBB) in wild-type of CG44404-CG45045 *mutants*

Almost immediately upon its injection, DtxA induces a partially reversible paralysis of injected flies. As had been observed for *BaraA* mutants, a lower percentage of *CG44404-CG45045* mutants recovered from the DtxA-induced paralysis (Fig. 7A). As paralysis is likely mediated by an action of DtxA on the neuro-muscular system, we asked whether DtxA might differentially affect the permeability of the BBB in the double mutants as compared to control flies. We first monitored the permeability of the BBB in larvae using a well-established protocol that relies on the penetration of a dye within the nervous system. Whereas the BBB permeability was increased upon an experimental direct brain injury, we did not observe any enhanced permeability upon DtxA injection in either double-mutant or control larvae (Fig. 7B). We next tested the permeability of the BBB of adult flies and obtained similar results (Fig. 7C). Thus, we did not obtain any evidence that the CG44404 or CG45045 peptides protect the host from the action of DtxA by modulating the permeability of the BBB, although we cannot exclude that they may specifically affect the passage of DtxA through this barrier.

BaraA *and the* CG44404-CG45045 *double mutant share a similar phenotype with respect to host defense against secreted virulence factors*

We have previously reported that *BaraA* mutants are sensitive to *E. faecalis* and to injected *M. robertsii* spores. We have discovered that BaraA-derived peptides play a

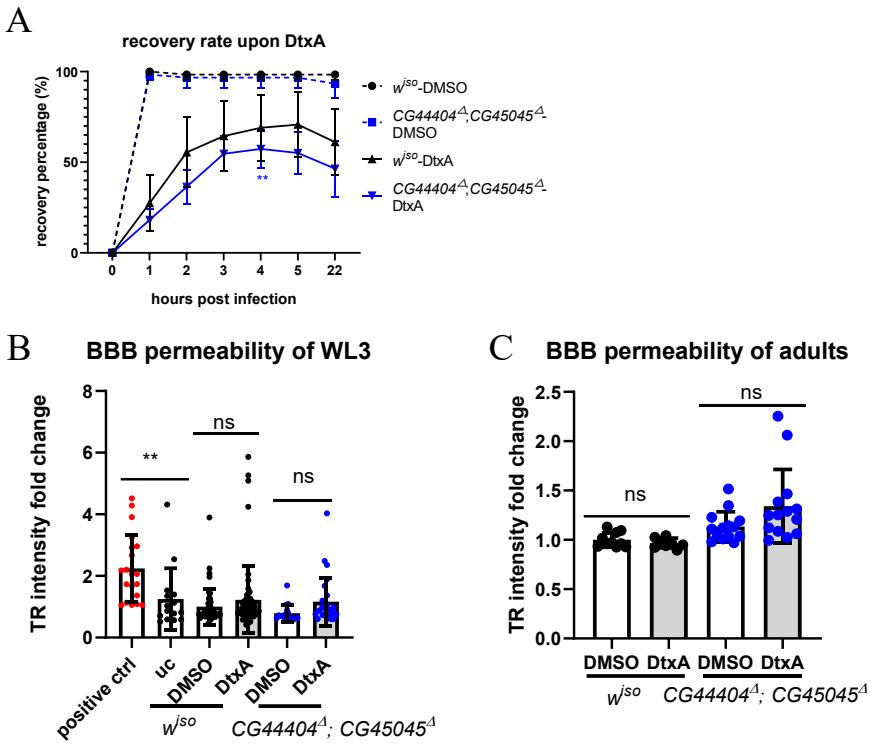
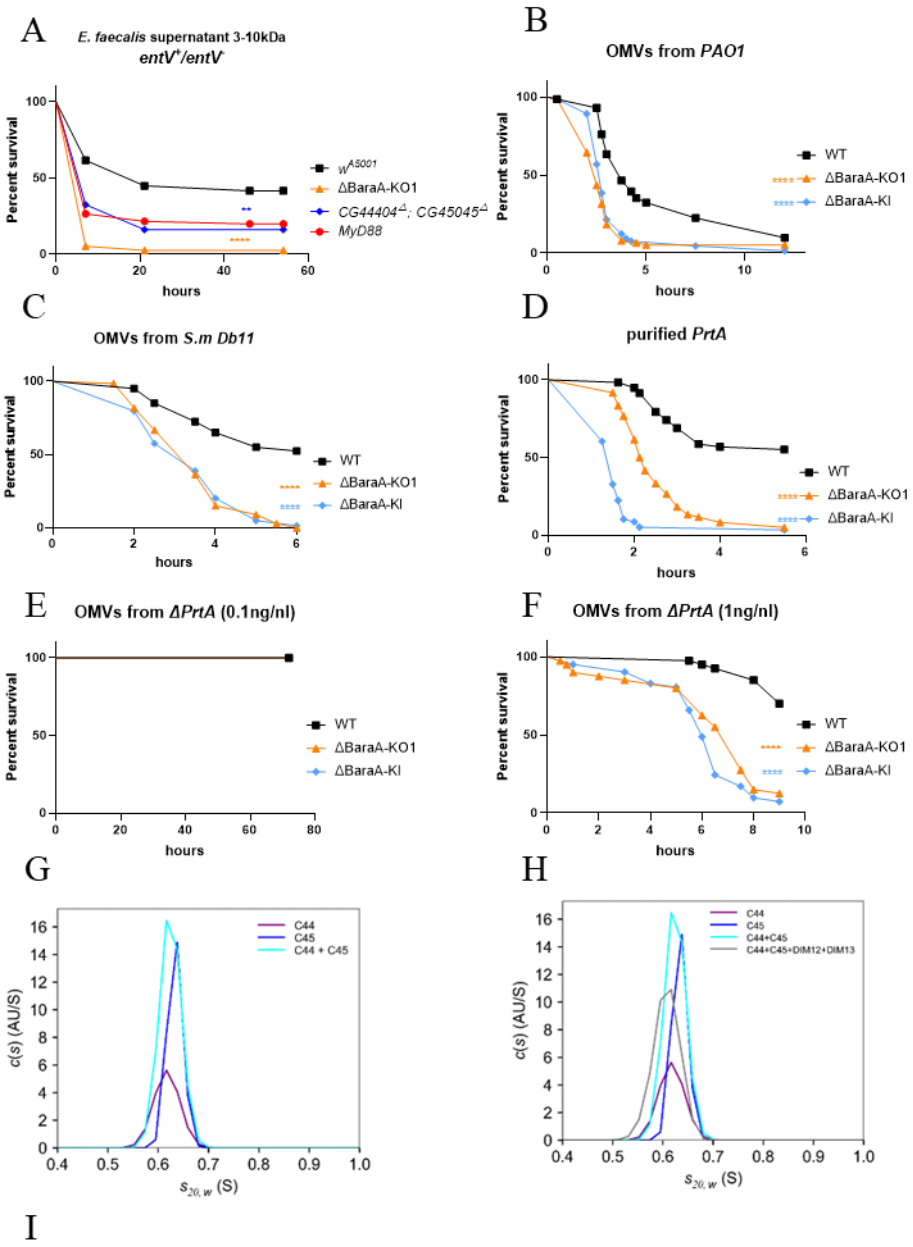


Figure 7. Perméabilité de la barrière hémato-encéphalique des mouches après traitement à la Destruxine A. (A) Le taux de croissance du double mutant et de w^{iso} après un traitement à la Destruxine A 4,6nl, 8mM. Les données regroupées de trois expériences indépendantes ont été montrées. La corrélation non paramétrique de Sepearman a été réalisée entre le w^{iso} infecté et le double mutant pour l'analyse statistique. **(B) La** perméabilité de la barrière hémato-encéphalique a été surveillée en incubant le cerveau de larves wondering 3rd avec du Dextran 10 kDa, Texas Red™ ou **(C)** en injectant le 69nl Dextran 10 kDa, Texas Red™ directement dans les adultes. Les données regroupées de trois ou une expériences indépendantes ont été montrées. Le test de Kruskal-Wallis a été utilisé pour l'analyse statistique.

role in the host defense against DtxA and the EntV bacteriocin from *E. faecalis* [1]. Given the common susceptibility to DtxA and *M. robertsii*, we asked whether the *CG44404-CG45045* double mutant was also sensitive to EntV. Indeed, we found that the double mutants were more susceptible to a preparation of the wild-type *E. faecalis* supernatant fraction that contains EntV but not to a similar fraction prepared from an *entV*- *E. faecalis* strain (Fig. 8A; Fig. S6A). Thus, these data suggest that like *BaraA*, *CG44404* and *CG45045* may be required in the host defense against EntV.

Conversely, we asked whether *BaraA* might be required to counteract the action of bacterial OMVs. We found that *BaraA* mutants were more susceptible to injected OMV preparations from either *S. marcescens* Db11 or *P. aeruginosa* PAO1 (Fig. 8B-C). Furthermore, a *BaraA* null mutant was also more susceptible to injected PrtA (Fig. 8D). The susceptibility to *S.marcescens* OMVs was lost when the preparation was made



	C44 peptide	C45 peptide	Mixture C44+C45	Mixture C44+C45+ DIM12+DIM13
s_{exp} (S)	0.73	0.749	0.737	0.718
$s_{20°C,w}$ (S)*	0.618	0.634	0.624	0.607
vbar (ml/g) used in analysis	0.6979	0.7013	0.6979	0.6979
M_{exp} (kDa)	3.06	3.10	3.10	3.10

*s-value corrected to standard conditions—the density and viscosity of water at 20.0 °C

Figure 8. Taux de survie du mutant *Baramicin A* lors d'infections. (A) Le mutant *BaraA* KO et le double mutant *CG44404-CG45045* ont été injectés avec le surnageant concentré de 3 à 10 kDa filtré à partir d'*E. faecalis* en culture. Le mutant *MyD88* a été considéré comme un contrôle positif. **(B)** Les mutants BaraA KO et KI ont été injectés avec des OMV produites par *PAO1* (1ng/nl) et **(C)** *S. marcescens* (0,1ng/nl). **(D)** La PrtA purifiée de *S. marcescens* a été injectée dans des mutants *BaraA. (***E)** 0,1ng/nl et **(F)** 1ng/nl d'OMV produit par le mutant PrtA de *S. marcescens* ont été injectés dans les mutants *BaraA.* **(G) L'**analyse de l'ultracentrifugation a été effectuée sur le mélange de peptides synthétiques dérivés de CG44404 et CG45045 et **(H) le** mélange avec DIM12 et DIM13 synthétiques supplémentaires.

from a *PrtA* mutant strain (Fig. 8E). As reported above for the *CG44404-CG45045* double mutant, some virulence and associated enhanced sensitivity of *BaraA* was retrieved when 10x concentrated *PrtA* OMV preparations were injected into flies (Fig. 8F). In conclusion, *BaraA* and *CG44404-CG45045* mutants share a phenotype of sensitivity to DtxA, EntV, and OMVs/PrtA.

We next tested for possible direct interactions between synthetic CG44404, CG45045, BaraA-derived DIM12 and BaraA-derived DIM13 by analytical ultracentrifugation. This analysis failed to reveal a direct interaction between CG44404 and CG45045 on the one hand and between the four peptides mixed together on the other hand (Fig. 8G-I). Thus, any potential interactions between CG44404/CG45045 and BaraA may either be indirect or involve other BaraA-derived peptides.

CG44404 ***may interfere with the action of the complement factor TEP2 that promotes the pathogenicity of PrtA***

To further determine how CG44404 functions in the host defense against virulence factors, we attempted a co-immunoprecipitation approach. To this end, we used the two transgenes described in Fig. 1H that allow the production of either secreted mCherry (TsR44) or of a CG4404-mCherry protein (TtR44). We first made extracts of whole transgenic flies after a mixed challenge of *M. luteus* and *E. coli* that leads to the induction of both the IMD and Toll pathways and thus of the *CG44404-mCherry and mCherry* transgenes. An additional control was provided by nontransgenic A5001 flies. All extracts were immuno-precipitated with agarose beads coated with nanobodies that bind to mCherry epitopes. The composition of affinity-purified mCherry-containing complexes were then determined using liquid chromatography coupled to mass spectrometry (LC-MS/MS). We found a weak differential signal for the Thioester-containing protein 2 (TEP2), one of the members of the complement family in *Drosophila*. We also found limited evidence for an association with BaraA that map either to the DIM22 or the DIM24 BaraA-derived peptides and not to the BaraA motif-containing peptides such as DIM12 or DIM13 (Table 1 and Supplementary Table 1

online for primary data, Fig. S6B. To confirm this result, we repeated the procedure using this time collected hemolymph from transgenic flies instead of using a whole fly extract. Again, we noted a mild enrichment with TEP2 and specific BaraA-derived peptides (Table 2 and Supplementary Table 2 online for primary data, Fig. S6B).

Next, we asked whether TEP proteins might play a role in the host defense against secreted microbial virulence factors using either *Tep2*, *Tep4, Tep2-Tep3-Tep4*, or the Tep_q^{Δ} mutant in which the *Tep1, Tep2, Tep3*, and *Tep4* loci are deleted [112, 117]. We observed a mildly yet significantly enhanced susceptibility of the *Tep2, Tep3, Tep4* triple mutant to DtxA injection but note that the Tep_q^{Δ} mutant did not harbor a similar susceptibility (Fig. 9A). In contrast, the *Tep2* mutant displayed a strongly reduced sensitivity to injected PrtA, unlike *Tep4* which was more susceptible to PrtA than its wild-type control. Interestingly, all *Tep* compound mutants, in which both *Tep2* and *Tep4* are mutated, displayed a *Tep4*-like susceptibility to this challenge, suggesting that *Tep4* is epistatic to *Tep2* (Fig. 9B). We thus document unexpected opposite roles for complement proteins in the host defense against a pathogenic protease.

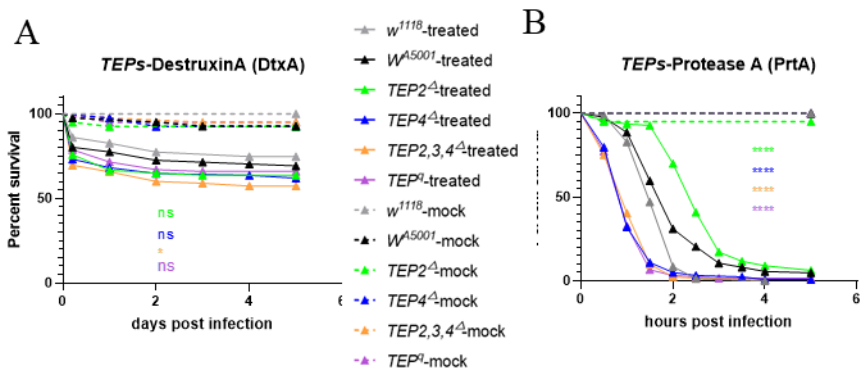


Figure 9. Taux de survie des mutants TEPs après traitement à la Destruxine A et à la Protéase A. (A) différents mutants TEP ont été traités avec la Destruxine A et **(B) la Protéase** A (PrtA). TEPq$^{\Delta}$ porte des mutations incluant TEP1, 2, 3, 4. Tous les mutants TEP sont sur w^{A5001} sauf TEPq sur w^{1118} . Les données regroupées de deux ou trois expériences indépendantes ont été montrées. Le log-rank a été utilisé pour l'analyse statistique

	BASIC Spectral Count (#spectra)						TsR vs TtR		
Accession and Description	CTRL1	CTRL2	TsR_1	TsR_2	TtR_1	TtR_2	LogFC	p.value	adjp
Accession and Description	Basic SC	Basic SC	Basic SC	Basic SC	Basic SC	Basic SC	LogFC	p.value	adjp
SignalP-mCherry	**0**	**0**	**4603**	**2275**	**0**	**0**	**15.0964**		
CG44404-mCherry	**0**	**0**	**0**	**0**	**3518**	**2483**	**-14.7230**		
|A0ZWT1| Tep2, isoform A	7	5	12	13	46	31	-1.6296	0.0787	0.9994
|Q8IPH4| Tep2, isoform D	7	4	12	14	48	35	-1.6830	0.0684	0.9994
|A0ZWQ1| Tep2, isoform A	7	4	12	12	48	30	-1.7037	0.0665	0.9994
|A0ZWQ6| Tep2, isoform A	7	4	12	13	49	32	-1.7086	0.0649	0.9994
|A0ZWP6| Tep2, isoform D	7	4	12	13	49	32	-1.7086	0.0649	0.9994
|A0ZWR1| Tep2, isoform A	7	4	12	13	51	33	-1.7629	0.0568	0.9994
|Q8ML70| BaramicinA	0	0	2	10	10	11	-0.9870	0.4171	0.9994
|A0A6H2EDS4| CG45045	0	0	0	1	0	1	0.0313	0.9892	0.9994
|Q95U97| Attacin A	1	2	1	5	7	6	-1.2978	0.4281	0.9994
|Q95NP8| Attacin A	2	2	1	5	9	8	-1.6724	0.2537	0.9994
|A7LFL7| Attacin A	1	2	0	5	6	5	-1.4323	0.4346	0.9994
|P45884| Attacin A	2	2	0	5	8	7	-1.8640	0.2522	0.9994
|Q9V751| Attacin B	**2**	**2**	**0**	**4**	**8**	**8**	**-2.2159**	**0.1807**	**0.9994**
|Q95NH6| Attacin C	0	1	4	9	15	10	-1.0903	0.3177	0.9994
|A0A5J6DIB5| Diptericin	1	0	2	4	7	5	-1.0872	0.5053	0.9994
|A1ZBF6| Diptericin B	0	0	0	2	0	2	0.0305	0.9880	0.9994
|P41964| Drosomycin	**0**	**0**	**0**	**0**	**4**	**3**	**-4.9710**	**0.0867**	**0.9994**
|C0HKQ7| Cecropin A1	0	0	0	1	0	0	2.2512	0.4248	0.9994
|P36192| Defensin	0	0	1	0	0	1	0.3086	0.9087	0.9994

Tableau 1. Immunoprécipitation à l'aide de billes anti-mCherry-Agarose- dans les échantillons dupliqués de mouches entières extraites des mouches TsR44, TtR44 et w^{45001} traitées avec un mélange d'*E. coli* et de *M. luteus*. Les gènes concernés sont indiqués dans le tableau.

	BASIC Spectral Count (#spectra)									TsR vs TtR		
Accession and Description	CTRL1	CTRL2	CTRL3	TsR_1	TsR_2	TsR_3	TtR_1	TtR_2	TtR_3	LogFC	p.value	adjp
Accession and Description	Basic SC	Basic SC	Basic SC	Basic SC	Basic SC	Basic SC	Basic SC F	Basic SC	Basic SC	LogFC	p.value	adjp
SignalP-mCherry	**0**	**0**	**0**	**5691**	**4255**	**2397**	**0**	**0**	**0**	**15.0472**		
CG44404-mCherry	**3**	**1**	**0**	**0**	**0**	**0**	**4187**	**4834**	**3752**	**-15.0010**		
|A0ZWS1| Tep2, isoform A	0	0	0	21	26	0	38	39	27	-1.1008	0.2999	0.4841
|A0ZWQ6| Tep2, isoform A	0	0	0	21	25	0	38	39	27	-1.1299	0.2883	0.4841
|A0ZWS6| Tep2, isoform A	0	0	0	21	25	0	38	39	27	-1.1299	0.2883	0.4841
|Q8IPH4| Tep2, isoform D	0	0	0	20	26	0	40	40	28	-1.1874	0.2629	0.4841
|A0ZWR1| Tep2, isoform A	0	0	0	20	26	0	33	35	22	-0.9231	0.3920	0.5391
|Q8ML70| Baramicin A	0	71	0	3	20	1	17	14	9	-0.7135	0.5715	0.7234
|P45884| Attacin A	0	13	0	3	3	1	5	4	3	-0.6589	0.6433	0.7868
|Q95U97| Attacin A	0	6	0	3	4	1	2	3	3	0.0628	0.9636	0.9886
|A7LFL7| Attacin A	0	6	0	3	4	1	1	2	3	0.4455	0.7454	0.8293
|Q95NP8| Attacin A	0	13	0	3	3	1	6	5	3	-0.8701	0.5486	0.6982
|Q9V751| Attacin B	**0**	**13**	**0**	**1**	**2**	**0**	**5**	**4**	**2**	**-1.6761**	**0.2343**	**0.4841**
|Q95NH6| Attacin C	0	12	0	10	17	4	16	16	14	-0.5084	0.6674	0.7897
|A1ZBF6| Diptericin B	0	0	0	0	0	0	1	1	0	-2.6007	0.2010	0.4841
|A0A5J6DIB5| Diptericin	1	4	0	1	4	0	4	2	2	-0.5944	0.6641	0.7897
|P41964| Drosomycin	**0**	**0**	**0**	**0**	**1**	**0**	**5**	**10**	**5**	**-3.8197**	**0.0274**	**0.4841**
|C0HKQ7| Cecropin A1	0	2	0	0	2	0	1	0	1	0.0232	0.9870	0.9899

Tableau 2. Immunoprécipitation à l'aide de billes d'agarose anti-mCherry dans l'hémolymphe d'échantillons triples extraits de mouches TsR44, TtR44 et w^{45001} traitées avec un mélange d'*E. coli* et de *M. luteus*. Les gènes concernés sont indiqués dans le tableau.

Discussion

We report here that two related secreted peptides CG44404 and CG45045 play a role in host defense against a variety of secreted virulence factors that include the circular hexadepsipeptide DtxA from the entomopathogen *M. robertsii*, the PrtA metalloprotease (and likely relatives thereof in other species) from *S. marcescens* that is carried by secreted OMVs, and the EntV bacteriocin secreted by *E. faecalis*. In terms

of sensitivity to infection, we found that the double mutant was sensitive to injected *M. robertsii* spores, to injected *P. aeruginosa* but not to injected *E. faecalis*. Strikingly, these phenotypes are related to those reported for the Toll pathway effector BaraA; *BaraA* null mutants have been previously shown to be susceptible to DtxA and to EntV and are now reported to be also sensitive to injected OMVs as well as to purified PrtA. Taken together, these results suggest that BaraA-derived peptides and CG44404/CG45045 act in concert and protect the host to some degree against the actions of these biochemically distinct classes of secreted virulence factors.

We establish in this work that the *CG44404* and *CG45045*, initially annotated as lncRNAs, are *bona fide* coding genes that correspond to short secreted peptides upon immune challenges, a rather common situation. Whereas CG45045 was directly observed by mass-spectrometry on collected hemolymph, we did not detect CG44404 at the expected size in this analysis and had to use a transgenic fusion gene to establish that it has the capacity to be secreted. Why CG44404 was not detected by mass-spectrometry may be ascribed to either post-translational modifications or strong interactions with another protein in the hemolymph. The CG44404 peptide was not detected by mass-spectrometry in the hemolymph of *BaraA* null mutants and it would be interesting to test whether it would become observable in *Tep2* mutants.

We have discovered that both *CG44404* and *CG45045* are required in the host defense against injected *M. robertsii* conidia and that against DtxA, suggesting that each fulfills a unique function. Whether both genes are required to protect against the EntV bacteriocin remains to be established. Further, we have established that the strong overexpression of *CG44404* is sufficient to confer a protection against *M. robertsii*, suggesting that the functions of CG44404 and CG45045 are overlapping, at least to some extent. Indeed, *CG44404* and *CG45045* appear to be functionally redundant as only the double mutant exhibited a significantly enhanced susceptibility to *P. aeruginosa*. This might reflect a distinctive mechanism of action against DtxA as compared to that against the metalloprotease PrtA, as further exemplified by the genetic requirement for *Tep2* only in the latter case. On an evolutionary scale, the two genes are

found in three *melanogaster* subgroup species, *D. melanogaster*, *D. erecta*, and *D. biarmipes*. In the other species of the group, only one of the two genes is present in their genomes. This might reflect either an exposure to different kinds of pathogens that do not include Dtx-like toxin-producing pathogens. Alternatively, the single gene might be induced much more strongly or be already expressed in a basal unchallenged state.

Another aspect of the relationship between the two genes is their dissimilar regulatory control at the level of transcription. Whereas *CG44404* can be induced by either the Toll or IMD pathways, *CG45045* appears to be induced only by the latter pathway. Thus, it is puzzling that *CG45045* is strictly required in the host defense against injected *M. robertsii* conidia that mostly induce the Toll pathway. In keeping with a lack of role of *CG45045* against *M. robertsii* natural infection, which does not elicit the IMD pathway, it is likely that the weak and short-lived induction of the IMD pathway that occurs upon wounding may induce *CG45045* expression in high enough amounts that, in conjunction with high levels of induction of *CG44404*, may prove sufficient to provide a degree of protection against injected conidia of this entomopathogenic fungus.

The classification of genes between coding and noncoding genes may be somewhat superficial and several instances have been described in the past 15 years of genes having effects both at the transcript and protein levels [226]. In keeping with a possible role as lncRNAs, it has been reported that the *CG44404* transcripts are detected both in nuclei and in the cytoplasm [225]. By running the LncTar tool [227], Joo Hyun Im observed that the transcripts from *CG44404* or *CG45045* were predicted to directly bind to mRNA of several AMP genes including some *Attacin, Diptericin*, and *Cecropin* genes. Such interactions might account for the decreased expression in the *CG44404-CG45045* double mutant of some IMD-pathway regulated genes observed in this study (Fig. 5, Fig. S5). However, this tool also predicted an interaction with *Drosocin*, *Metchnikowin*, or *CecA2* genes, the expression of which were not impacted in the double mutant. Thus, further studies will be required to determine whether the optimal induction of *Attacin A&B*, as well as *Attacin D, Defensin* and *Diptericin* requires the

function of either *CG44404* or *CG45045* through an interaction of these AMP transcripts with the transcripts from these two genes [228]. Of note, a direct effect of *CG44404* or *CG45045* on the induction of the Toll or IMD pathways is unlikely as several genes regulated by these pathways appear to be induced normally in the double-mutant (Fig. S5).

As for BaraA, it is striking that CG44404/CG45045 are involved in the host defense against biochemically very distinct categories of virulence factors. The molecular targets of EnterocinV on prokaryotic and eukaryotic cells remain to be delineated despite the elucidation of the structure of the bacteriocin. Recent work has shown that the antibacterial activity of EntV requires the whole protein whereas the antifungal activity is recapitulated by a 12 amino-acid peptide corresponding to helix α7 of the bacteriocin [229]. It is presently unknown whether the virulence function of EntV in *Drosophila* might be ascribed to this short peptide, which would make the study of this virulence factor more experimentally amenable.

A multiplicity of effector mechanisms has been proposed for the action of DtxA. It is insecticidal, cytotoxic, and phytotoxic. The initial tetanic paralysis has been ascribed to its action on muscles through the opening of calcium channels [230]. Its action on hemocytes has been proposed to neutralize to some extent the cellular arm of the insect innate immune defenses whereas it may also inhibit the *Drosophila* systemic innate immune response [231]. More recently, DtxA has been shown by electrophysiology on the *Drosophila* larval midgut at relatively high concentrations to act as a transient transmembrane channel that leads to the depletion of intracellular ions [232]. The finding that BaraA is required in glial cells to provide a degree of protection against DestruxinA and EntV-containing *E. faecalis* supernatant suggests that both toxins are able to act on the nervous system. We did not detect any expression of *CG44404* and *CG45045* in the whole mounts of brains of *E. coli*-challenged flies using reporter transgenes, although we cannot formally exclude their expression in a few brain cells if our read-outs are not sensitive enough. Both genes are however expressed in the cephalic fat body and it is thus possible that they may reach the brain, provided

they are able to cross the BBB. While we have been unable to construct *CG44404-BaraA* double mutants because they are lethal, it may be interesting to test whether knocking down *BaraA* expression in glial cells, or other cell types, in a *CG44404-CG45045* background would lead to an enhanced or similar phenotype as compared to the *CG44404-CG45045* or *BaraA* silenced mutant flies. The finding that some BaraA-derived peptides, especially DIM24 that has been proposed to act in the nervous system, are found in a complex with CG44404 opens the possibility that this complex may be required to help the flies recover from the initial paralysis induced by DtxA injection.

The association of DIM24 BaraA-derived peptide with CG44404 may help explain the discrepancy observed between the *BaraA* and *CG44404-CG45045* mutant phenotypes as regards the sensitivity to an *E. faecalis* challenge, only the former displaying an enhanced susceptibility in survival experiments [1]. We have previously reported that *BaraA* is also required in the resistance against *E. faecalis* as we observed a faster proliferation of the pathogen in the mutant. It is an open possibility that this function in resistance against this Gram-positive pathogen be mediated by the short BaraA-derived peptides (DIM10, DIM12, DIM13 and relatives thereof) whereas the resilience function against EntV might be mediated by DIM24 in conjunction with CG44404 and possibly CG45045. Thus, the sensitivity to *E. faecalis* of *BaraA* mutants might result from the impaired resistance function whereas the altered resilience function might yield a phenotype too subtle to be detected in a survival assay, as might be the case for the *CG44404-CG45045* that may be involved only in the resilience to EntV action.

The finding of Tep2 being co-immunoprecipitated with CG44404 was unexpected but remains however to be confirmed by a converse Tep2 co-immunoprecipitation experiment. The finding that *Tep2* mutants are protected from the action of PrtA to a large degree underscores the importance of the potential interaction. Importantly, it suggests that the action of CG44404 in the defense against DtxA and PrtA involves distinct effector mechanisms. From experiments performed in cultured S2 cells, Tep2 has been proposed to act as an opsonin against *E. coli* but not *Candida albicans* or

Staphylococcus aureus [115]. In systemic infection models with *Photorhabdus* species, it has been shown to promote the pathogenicity of these entomopathogenic bacteria, like Tep4 [233]. In *Tep2* and *Tep4* mutants, the melanization arm of the innate immune system is more strongly activated, providing a potential explanation for the decreased bacterial burden observed in these mutants [119, 233]. We have ourselves reported that Tep4 likely acts as an opsonin against ingested *P. aeruginosa* PA14 [234], whereas it promotes the pathogenicity of injected PA14. Finally, both *Tep2* and *Tep4* mutants exhibit increased metabolic stores of carbohydrates and triglycerides [118]. Here, *Tep4* mutants display an increased susceptibility to injected PrtA, which is epistatic to *Tep2*. We have shown that hemocytes are required to promote the pathogenicity of injected *S. marcescens* OMVs, a phenotype akin to that of *Tep2* and *eater* mutants. Eater is a proposed phagocytosis receptor [108, 235] but is also playing a role in maintaining hemocytes attached to tissues [107] (most adult hemocytes are sessile). Taken together, these findings open the possibility that *Tep2* mediates the action of hemocytes in the response to injected OMVs. Indeed, it has been reported that hemocytes are required for the induction of *Tep2* expression upon *Photorhabdus* challenge [119]. Why and how it opposes the action of Tep4 is an important issue. In this regard, we have already reported that their functions may differ, *Tep4* only being required in the host defense against ingested *P. aeruginosa* [234].

Tep2 and Tep4 belong to the thioester-containing family of proteins in *Drosophila*. This family includes complement-like proteins but is also related to alpha2-macroglobulins, suicidal inhibitors of proteases secreted by pathogens [112]. Thus, the finding that *Tep4* mutants are sensitive to injected PrtA might be accounted for if it were inhibiting to some degree the activity of the protease. One possible model is as follows: Tep2 would compete with Tep4 for binding to PrtA and may actually promote its activity by bringing it to its cellular targets or simply by preventing the inhibition of PrtA by Tep4. The strong induction of CG44404, and likely of CG45045 and some BaraA-derived peptides, would trap Tep2 and therefore allow Tep4 inhibitory function.

In conclusion, this study underscores the complexity of host defenses against

secreted microbial virulence factors. Whereas the Toll-regulated Bomanin peptide are involved in the resilience to *A. fumigatus* secreted mycotoxins [97], a mixed bag of virulence factors with strikingly distinct biochemical mode of actions is counteracted by Toll-regulated BaraA peptides and CG44404-CG45045. It will be important to determine which organs are critically targeted by EntV, DtxA, and PrtA. We have obtained evidence that the nervous system is targeted by the latter two whereas the mechanism of action *in vivo* on the host of EntV remains to be established. As exemplified by the involvement of Tep2 in a complex with CG44404 that may relevant only in the host defense against PrtA, we have to be open to the possibility of the formation of complexes that may vary in composition according to the tissue in which they function and that may be tuned to the specific virulence factor category to which flies are exposed. Finally, by injecting purified and likely enriched preparations of virulence factors, we reveal some unexplored facets of host defense. The challenge will be however to understand how the host deal with a multiplicity of virulence factors secreted by pathogens at low doses, simultaneously or at distinct step of the infection process.

The initial study of the *CG44404* locus relied on a genetic overexpression strategy that did not reveal the actual functions of *CG44404* in host defense. The finding that this locus functions redundantly or in conjunction with *CG45045* leads us to propose a name for these two genes that refers to Chinese mythology under the Eastern Han dynasty. Shenshu and Yulü are two guardian deities posted on twin doors of a ghost gate that are in charge of inspecting the transit of countless spirits of the dead and to neutralize the evil-deeded ones by feeding them to tigers. We thus propose the name *Shenshu* (*shen*) for *CG45045* and *Yulü* (*yul*) for *CG44404*.

Supplementary

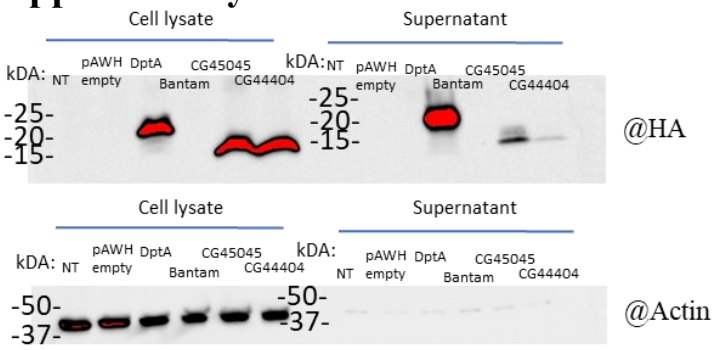


Figure S1. Le Western blot a été réalisé sur les échantillons de lysat de cellules S2 et sur le surnageant de la culture cellulaire. Les cellules S2 ont été transfectées avec un plasmide contenant le gène d'intérêt marqué avec 3xHA. La *diptérincine A (DptA)* a servi de contrôle positif tandis que *Bantam*, un lncRNA, a servi de contrôle négatif. NT : non transfecté ; pAWH empty : transfecter un plasmide vide. Les bandes dans les cases sont les peptides attendus.

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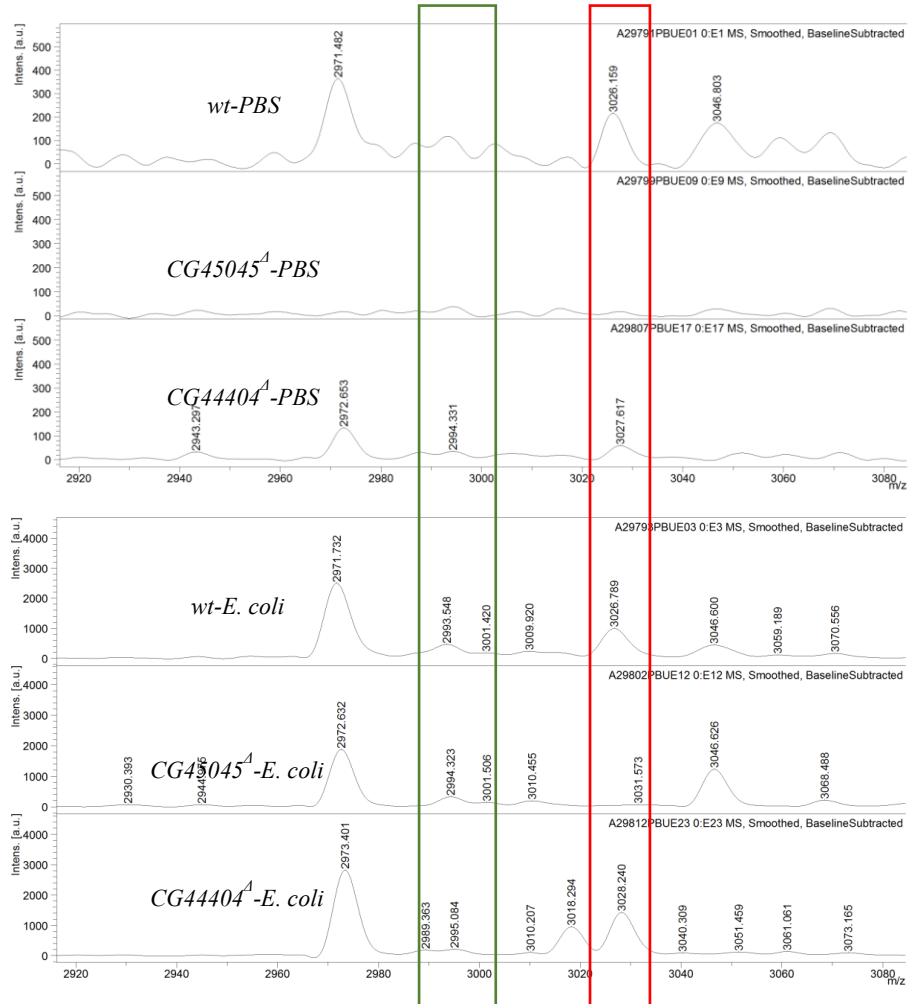

Figure S2. Analyse MALDI-TOF d'échantillons d'hémolymphe de mouches wt et mutantes avec et sans traitement par *E. coli. La* case en rouge est le pic attendu des peptides sécrétés CG45045. La case en vert aurait dû être le pic prévu des peptides sécrétés CG44404.

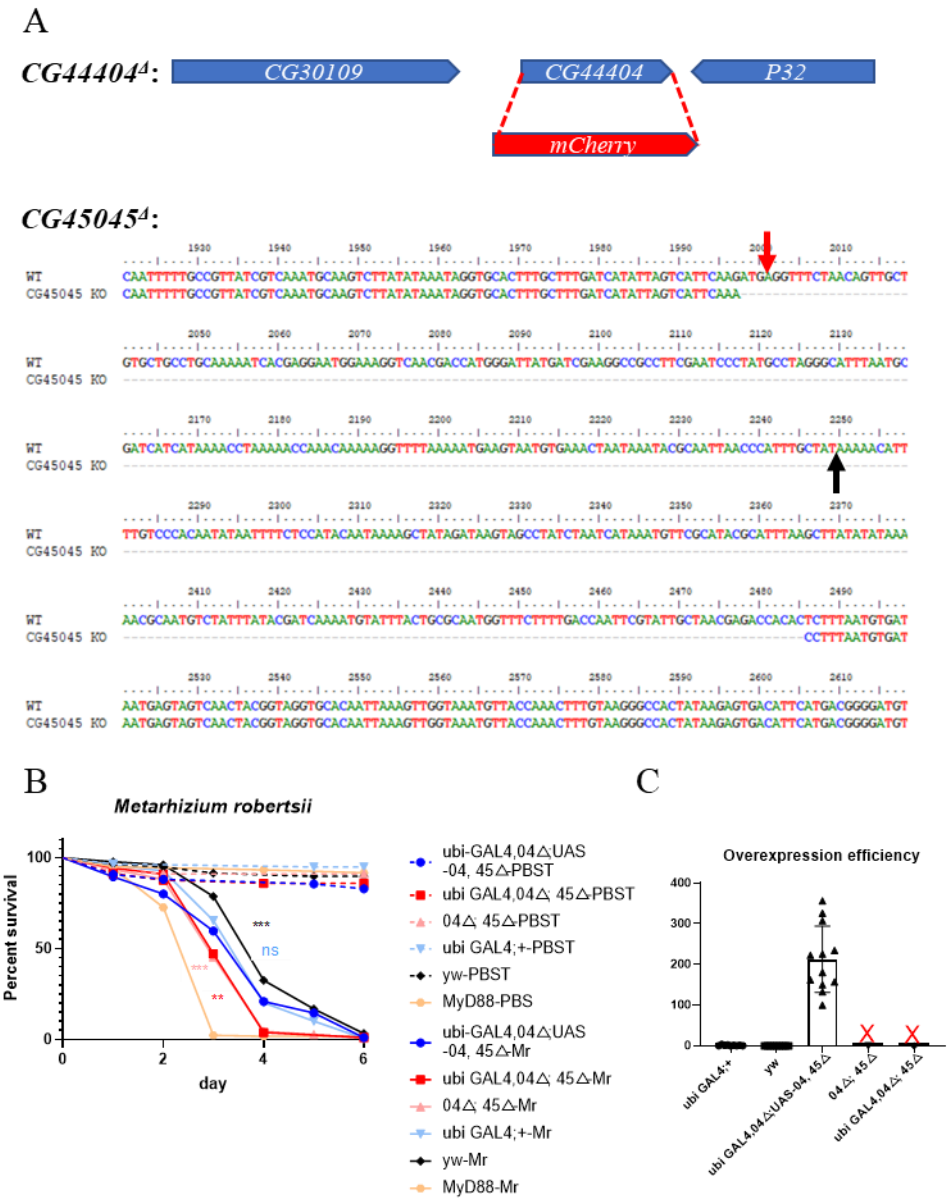

Figure S3. (A) Schéma de la construction du mutant *CG44404* (partie supérieure). L'alignement des séquences autour du gène *CG45045* entre les mouches de type sauvage et le mutant *CG45045*. La flèche rouge indique le début de *CG45045* et la flèche noire indique la fin de *CG45045* (partie inférieure). **(B) Des** mouches surexprimant *CG44404* dans un contexte de double mutant ont été injectées avec *M. robertsii*. **(C)** Le niveau d'expression de *CG44404* dans toutes les mouches utilisées dans l'expérience de sauvetage.

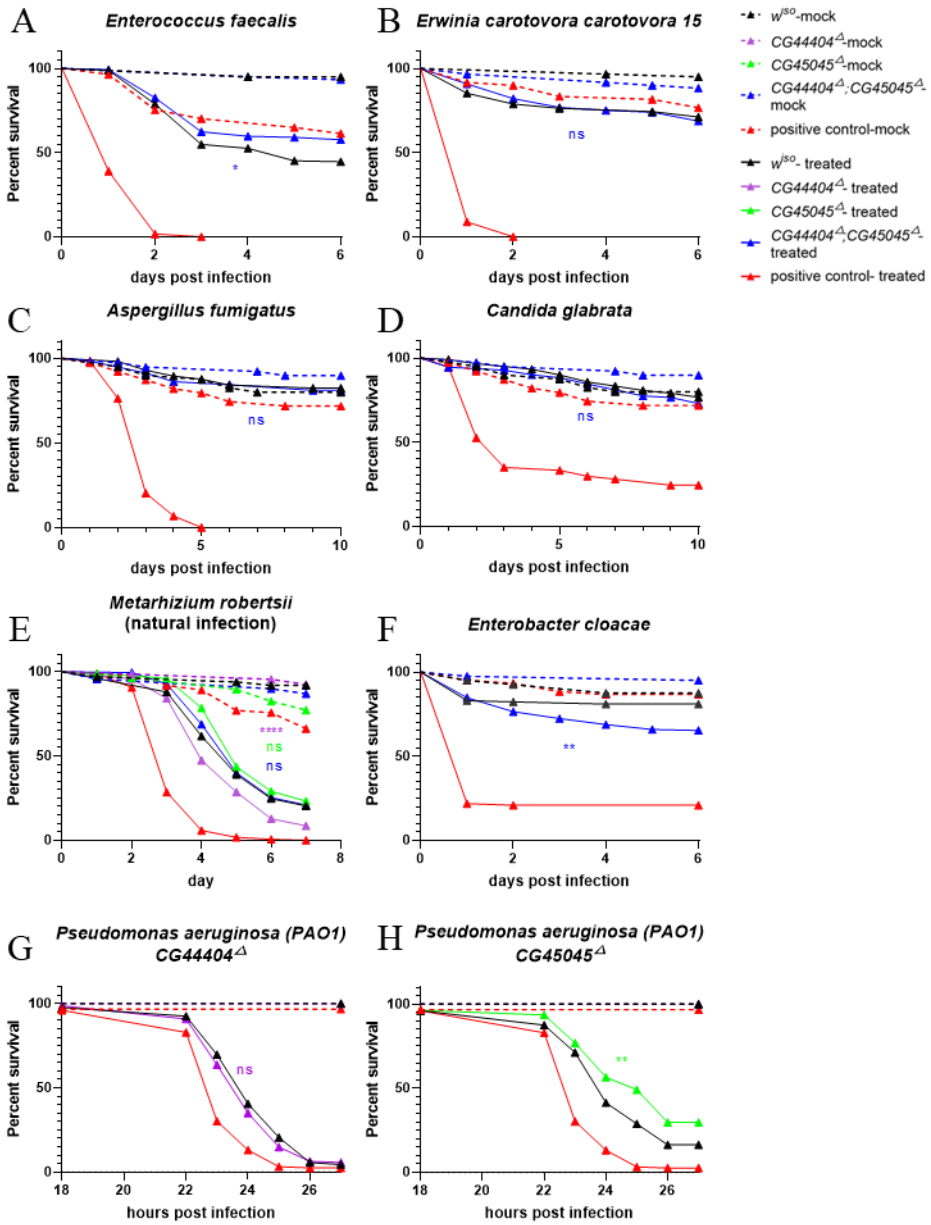

Figure S4. Taux de survie lors d'infections par différents pathogènes. Des mutants simples ou doubles de *CG44404* et *CG45054* ont été infectés par différents pathogènes. w^{iso} est le contrôle de type sauvage et *kenny* ou *MyD88* est le contrôle positif. Lors de l'infection des mouches par des champignons, des levures et des bactéries Gram (+), *MyD88* était le contrôle positif. Lorsque les mouches sont infectées par des bactéries Gram (-), *Kenny* est le contrôle positif.

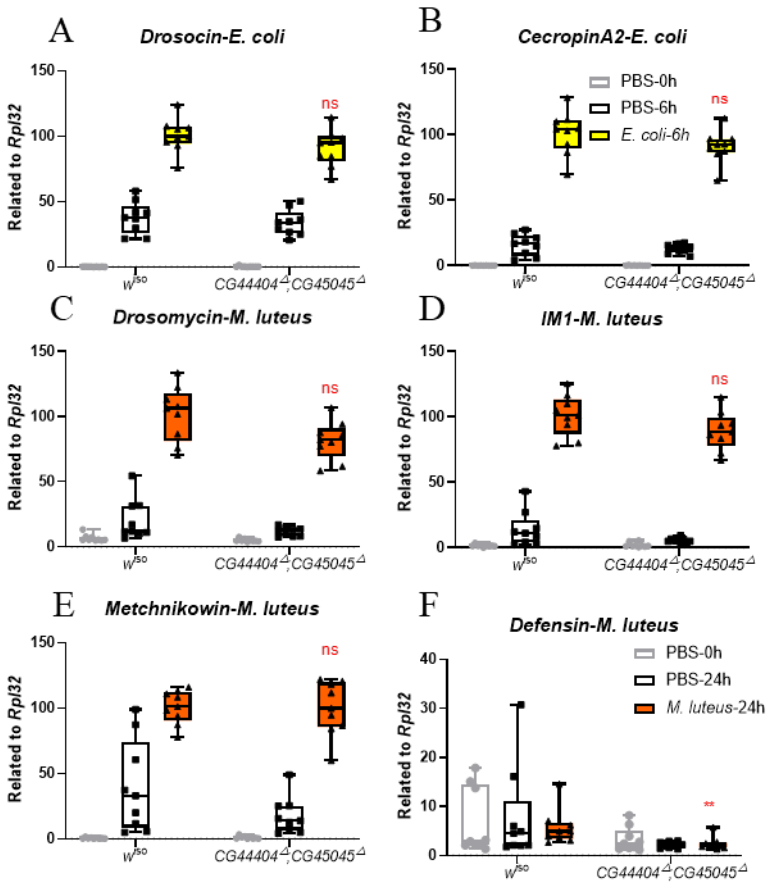

Figure S5. L'expression de certains gènes AMPs. (A) L'expression de la *Drosocine* et **(B) de la** *Cecropine A2 a* été contrôlée chez w^{iso} et les doubles mutants 6 heures après l'infection par *E. coli.* **(C) L'**expression de la *drosomycine,* **(D) de l'***IM1,* **(E) de la** *Metchnikowin* et **(F) de la** *Defensin* a été contrôlée chez w^{iso} et les doubles mutants 24 heures après l'infection par *M. luteus.* Toutes les données ont été normalisées sur le w^{iso} infecté tandis que le w^{iso} infecté a été considéré comme 100. Les données regroupées de trois expériences indépendantes ont été montrées. Le test de Mann-Whitney a été effectué entre le w^{iso} infecté et le double mutant.

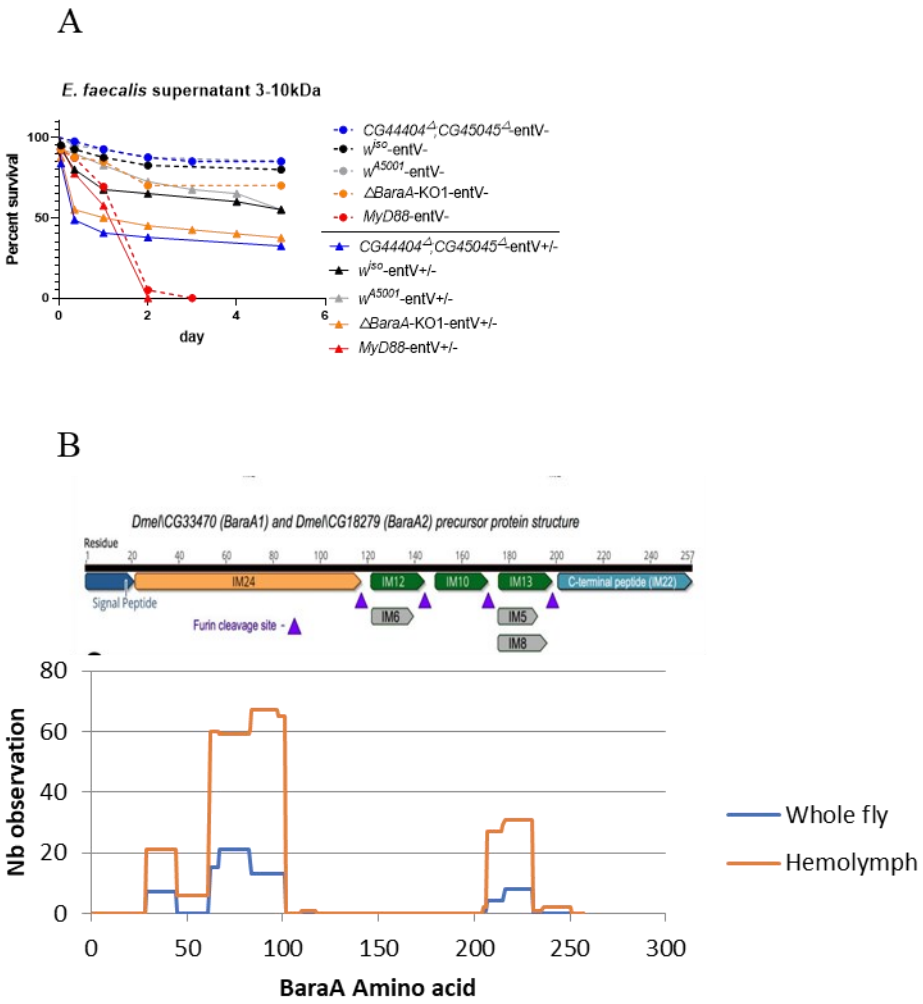

Figure S6 (A) Le surnageant d'*E. faecalis* OG1RF cultivé a été prélevé sur des souches *entV-* et *entV+/-*. Le surnageant a été filtré pour retenir les molécules de 3 à 10 kDa. Cette fraction a été injectée dans le double mutant *CG44404-CG45045*, le mutant *BaraA* KO et le mutant *MyD88* de contrôle positif. **(B)** Séquence détectée des peptides dérivés de *BaraA* dans l'immunoprécipitation à partir d'échantillons de mouches entières et d'hémolymphe.

Material and Methods

Fly strains and maintenance

Fly lines were raised on the media prepared for flies at 25 ℃ with 65% humidity. The receipt of 100 liter of fly medium is that 4.8 kg cornmeal (Priméal), 4.8 kg glucose (Tereos Syral), 6 kg yeast (Bio Springer), 360 g nipagin (VWR Chemicals) were diluted into 1400 mL ethanol (Sigma-Aldrich), 480 g agar (Sobigel) and distilled water were used to cook.

The mutant flies of *CG44404* and *CG45045* were generated by CRISPR/cas9 technique. All the transgenic reporter lines were generated by PhiC31 integrase system, while the UAS-*CG44404* is a kind gift from Prof. Mika RAMET. Where stated, mutant flies were isogenized in the w^{A5001} [236] background and the w^{iso} is respectably regarded as the wild-type control of mutants. The positive controls for infection assays for Gram-positive bacterial / fungal infections and Gram-negative bacterial infections were respectively *MyD88* and key^{1} on the w^{A5001} background.

For overexpressing gene of interest, flies after cross were raised at 25 degree for 2 days at the beginning then shifted to 18 degree until the adult offspring hatching at the sufficient number. Flies were then raised at 29 degree for 5 days before infection.

Generation of CRISPR/Cas9-mediated mutants

The mutant of *CG44404* was generated by replacing the endogenic whole gene region with *mCherry* sequence. PCRs were done with the Q5 Hot-start 2× master mix (New England BioLabs, NEB), and cloning was performed using the Gibson Assembly 2× Master Mix (NEB) following the manufacturer's instructions. The 20bp-long gRNAs for the target genes were devised using DRSC Find CRISPRs (www.flyrnai.org/crispr/). The pCFD5 plasmid vector was used. A cloning protocol to generate the pCFD5 plasmids encoding one to six tRNA-flanked sgRNAs was followed as described [18]. The primers used to generate the pCFD5 vector containing the gRNAs are shown in Table S3. We used a pSK vector as donor plasmid with the homology arms flanking the

mCherry: 3 a fragment 1000bp upstream of *CG44404* had been amplified as a left arm; a fragment 1000bp downstream of *CG44404* as a right arm. Left arm + *mCherry* + right arm have been assembled (Gilson Assembly) and the resulting fragment ligated to Pst1-Spe1 double-digested pSK and checked by sequencing. The plasmid mixture containing the two plasmids at a ration pCFD5:pSK=3:1, was injected into recipient y^1 M{Act5C-Cas9.P.RFP-}TIZH-2A w^{1118} *DNAlig4*^{169} (BDSC#58492) embryos.

Table S3. Primer used for generating mutant

CG44404_gRNA1_PCR-F	GCGGCCCGGGTTCGATTCCCGGCCGATGCA**GGCAAGTTAACTTTAGATTG**GTTTTAGAGCTAGAAATAGCAAG
CG44404_gRNA2_PCR-R	ATTTTAACTTGCTATTTCTAGCTCTAAAAC**GTGGGTTGAGTTTCGTGATC**TGCACCAGCCGGGAATCGAACCC
Donor Vector L-F	CTTGATATCGAATTCctgcaATCTCTGATAGCTTATCTGTAG
Donor Vector L-R	CCATGGTGGCGTGCTGCAGCTTTTAATG
Donor Vector m-F	GCTGCAGCACGCCACCATGGTGAGCAAG
Donor Vector m-R	AGTTTTTTTTTTTTTACTTGTACAGCTCGTCCATG
Donor Vector R-F	GTACAAGTAAAAAAAAAAAAAACTAGGGAATTAACGTTGTATTTGTTAAAAAC
Donor Vector R-R	CGGTGGCGGCCGCTCTAGAACCATGCCGCCGGTTACATAAC

Pathogen infections

Pathogens used in this study were described in the table S4. Flies were anesthetized with light CO2 and injected with pathogens into the thorax with a Nanoject II/III auto-nanoliter injector (Drummond). Natural infection with *M. robertsii* were initiated by shaking anesthetized flies in 5ml 0.01% tween-20 solution containing *M. robertsii* spores at a concentration of $5x10^4$/ml. All infected flies were subsequently maintained at 29°C.

Table S4. Information of pathogens used in this study

pathogen	medium	temperature (℃)	infection	concentration	injection volume
Metarhizium robertsii (ARSEF2575)	Potato Dextrose Agar (PDA)	25	injection	10^7 spores/ml	4.6nl
			natural infection	$5*10^4$ spores/ml	
DestruxinS1 mutant	Potato Dextrose Agar (PDA)	25	injection	10^7 spores/ml	4.6nl
Aspergillus fumigatus	Potato Dextrose Agar (PDA)	29	injection	$5*10^7$ spores/ml	4.6nl
Candida glabrata	Yeast extract- Peptone-Glucose Broth Agar (YPDA)	25	prick	clone	
Escherichia coli (DH5α)	Luria Broth (LB)	37	injection	OD_{600}=50	9.2nl
Erwinia carotovora carotovora 15	Luria Broth (LB)	30	injection	OD_{600}=50	4.6nl
Enterobacter cloacae	Luria Broth (LB)	30	injection	OD_{600}=50	4.6nl
Pseudomonas aeruginosa (PAO1)	Brain-Heart Infusion broth (BHB)	37	injection	OD_{600}=0.001	13.8nl
Micrococcus luteus	Luria Broth (LB)	30	injection	OD_{600}=50	9.2nl
Enterococcus faecalis (NCTC 775 / ATCC 19433)	Luria Broth (LB)	37	injection	OD_{600}=0.1	4.6nl
Serratia marcescens (RM66262)	Luria Broth (LB)	30	for producing OMVs		

Survival tests

Survival tests were performed using 20 flies per vial in biological triplicates. Female adult flies used for survival tests were 3–6-day old. Unless stated otherwise, each experiment shown is representative of at least two independent experiments. Log-rank test was used for the statistics of survival test.

Quantification of the pathogen burden in infected flies

To characterize the dynamics of within-host microbial loads or BLUDs or FLUDs, live flies were taken at each time point post-injection for pathogen load or flies were infected with *P. aeruginosa* or *M. robertsii* and vials were monitored every 30 minutes for newly dead flies (PLUD). These flies were then individually homogenized with a bead in 100 µl PBS with 0.01% tween20 (PBST) or PBS. Homogenates were diluted serially (ten-fold dilutions) in PBST or PBS and spread on LB (*P. aeruginosa*) or PDA (*M. robertsii*) plates for incubation at 37°C (*P. aeruginosa*) or 25°C (*M. robertsii*) until the clone units are visible to count. Colonies were counted manually. Data were obtained from three independent experiments and pooled.

Collection and preparation of* bacterial *supernatants

Filter-sterilized supernatants phases were obtained from 35-40 ml overnight *P. aeruginosa* cultures grown in BHB medium or 10ml overnight *E. faecalis* OG1RF (ATCC 47077) cultures grown in LB medium that were collected by centrifugation at 4,000 rpm for 10min. The sterilized supernatants were centrifuged through a 15mL Amicon Centricon™ filter to separately collect the molecules larger or lower than 100kDa (*P. aeruginosa*) or 10kDa (*E. faecalis*). The fraction of *P. aeruginosa* retained on the filter was diluted with PBS corresponding to the original concentration. This fraction and the flow through fraction were then injected into flies with a volume of 69nl. For processing the preparation of supernatant 3-10kDa from *E. faecalis*, 1.5mL Eppendorf tubes were used to collect the supernatant lower than 10kDa, which were vacuum freeze-dried for 24 hours. The powder was resuspended with H_2O and thus

concentrated 10 to 20-fold. The solution was filtered on 3kDa Amicon Centricon™ filter by centrifugation at 10000rpm for 30min. The nonfiltered fraction was then injected into flies with a volume optimized according to the batch (16 to 69 nL) and the same volume of buffer was used for the controls.

Purification of outer membrane vesicles (OMVs)

A saturated culture of *S. marcescens* RM66262, *prtA* mutant or *PAO1* were diluted 1:500 in fresh LB and placed at 30ºC or 37ºC with agitation for a maximum of 16 hours. About 600 mL of cultures were centrifuged at 8,000 g for 15 min. The pellets were discarded, and the supernatants were filtered using 0.45 µm filter (Thermo scientific, cat# 126-0045) to remove all remaining cells. The filtered supernatants were further precipitated with ammonium sulfate (129g/250mL of supernatant) overnight at 4ºC. Then, the supernatants were centrifuged for 20 min at 10,000 g. The supernatants were discarded, and the pellets were re-suspended in 10 mL of 50 mM hepes buffer (pH 7.5). The samples were dialyzed against hepes (50 mM) overnight at 4ºC, then concentrated using Amicon® Ultra 15 mL (Millipore, UFC901024) by centrifugation at 5,000 g. Further, 4 mg from the samples were loaded on the top of a 30% to 60% sucrose/hepes density gradient and centrifuged at 100,000 g at 4ºC for 16 hours. Fractions of 1 mL were collected from each gradient and checked on Coomassie gel. Only the 6 first fractions were selected and dialyzed against hepes 50mM overnight at 4ºC. Finally, the samples were centrifuged at 100 000 g for 3 hours at 4ºC, the supernatants were discarded and the pellets that contain OMVs were re-suspended in 400 µL of fresh 50 mM Hepes (pH 7.5). The OMVs were stored at -80ºC.

Purification of PrtA

Strain *slpE*$^{-}$ culture was grown at 30 °C (major expression of *PrtA*) for 16 h in SLB (SLB is LB broth medium without salt) since the molecular weight and isoelectric point of this metalloprotease (*SlpE*) encoded by our *Serratia* is similar and could interfere with the purification of native PrtA. The cells were centrifuged and the supernatant was

filtered and concentrated with a centricon. The supernatant was applied to an anion exchange column (Mono Q) and elution was carried out by a linear NaCl gradient. The active fractions were collected, and concentration and proteolytic activity were measured (using azocasein as substrate), to ensure that the protease is catalytically active after purification.

Toxin injection

Destruxin A (MCE) was resuspended in high-quality grade DMSO and was diluted with PBS to 8mM concentration. 4.6 nl of the solution or of control DMSO diluted in PBS at the same concentration was injected into flies using the Nanoject II/III auto-nanoliter microinjector (Drummond).

Molecular mass fingerprints by MALDI MS

Each individual hemolymph sample was analyzed with the Bruker AutoFlex™ III based on Bruker Daltonics' smartbeam laser technology. The molecular mass fingerprints (MFP) were acquired using a sandwich sample preparation on a MALDI MTP 384 polished ground steel plate (Bruker Daltonics Inc., Germany). Briefly, the hemolymph samples were 10-fold diluted in acidified water (0.1% trifluoroacetic acid - 0.1% TFA, Sigma Aldrich, France), 0.6µL was deposited on a thin layer of an air-dried saturated solution (0.6µL) of the matrix alpha-cyano-4-hydroxycinnamic Acid (4-HCCA, Sigma Aldrich, France) in pure acetone. Then 0.4 µL of a saturated solution of 4-HCCA prepared in 50% acetonitrile acidified with 0.1% TFA was mixed with the *Drosophila* hemolymph. Following co-crystallization of the hemolymph spots with the second matrix droplet and evaporation under mild vacuum, MALDI MS spectra were recorded in a linear positive mode and in an automatic data acquisition using FlexControl 4.0 software (Bruker Daltonics Inc.). The following instrument settings were used: the pseudo-molecular ions desorbed from the hemolymph were accelerated under 1.3kV, dynamic range of detection of 600 to 18,000 Da, between 50-60% of laser power, a global attenuator offset of 60% with 200Hz laser frequency, and 2,000 accumulated

laser shots per hemolymph spectrum. The linear detector gain was setup at 1,906V with a suppression mass gate up to *m/z* 600 to prevent detector saturation by clusters of the 4-HCCA matrix. An external calibration of the mass spectrometer was performed using a standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonik) covering the dynamic range of analysis. All of the recorded spectra were processed with a baseline subtraction and spectral smoothing using FlexAnalysis 4.0 software (Bruker Daltonics Inc.).

Sedimentation velocity analytical ultracentrifugation (SV-AUC)

Sedimentation velocity experiments were conducted in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) at 29°C. The peptides diluted at 80 µM in PBS buffer were loaded into AUC cell assemblies with 12 charcoal-filled Epon double-sector centerpieces and quartz windows. The sample cells were loaded into a four-hole An-60 Ti rotor for temperature equilibration for 2–3 h, followed by acceleration to full speed at 60 000 RPM. Absorbance data at 240 nm were collected at 3 min intervals for 20 h. The partial specific volume of the peptides, buffer density and viscosity were calculated using the software SEDNTERP. Sedimentation data were time corrected and modeled with diffusion-deconvoluted sedimentation coefficient distributions c(s) in SEDFIT 16.1c, with signal-average frictional ratio and meniscus position refined with nonlinear regression [237]. Maximum entropy regularization was applied at a confidence level of 68%. Sedimentation coefficient distributions were corrected to standard conditions of 20°C in water ($s_{20,W}$). The plot was created in GUSSI [238].

Gene expression quantitation

Four to five whole flies were homogenized into 100µl of Trizol. Samples were filled with 900µl of Trizol and mixed with 200µl of chloroform. Samples were centrifuged at 10, 000g for 10 min at 4ºC. The 400µl liquid at the upper phase of the samples was collected carefully without disruption of other phase into a new 1.5ml Eppendorf tube containing 400µL of isopropanol. The samples were vortexed well and incubated at

room temperature for 5 mins and then centrifuged at 12,000 g for 15 min at 4ºC. The pellet was washed in 1ml of 75% ethanol and dried. RNAs were then re-suspended in DEPC water. A volume of 20 µL was used to generate cDNA by reverse transcription, using the Transcript II all in one first strand synthesis supermix for qPCR (one step gDNA removal) synthesis kit (transgen biotech #AT341-02). The quantitative Polymerase Chain Reaction (qPCR) was performed with the same kit on cDNA diluted 20 times. The program-used was the following: 30 sec at 98ºC; 34 cycles of 5 sec at 95ºC, 30 sec at 98ºC and finally 30 sec at 65ºC. The data were analyzed using the CFX384 software (Bio-Rad). The Ct (Cycle threshold) values of the genes were normalized with the Ct values of Rpl32 (housekeeping gene that codes for a ribosomal protein). Furthermore, the normalized values of treated conditions were normalized with the normalized values of untreated conditions (delta delta Ct). All primers used in this report are listed in Table S5.

Table S5. Primers used in this study

genes	primers	sequence
Rpl32	Fw	GACGCTTCAAGGGACAGTATCTG
	Rv	AAACGCGGTTCTGCATGAG
CG44404	Fw	CAACTGCTGCCAATCCTCG
	Rv	GCCTGGGATCGTAGTCACTT
CG45045	Fw	TGCCTGCAAAAATCACGAGG
	Rv	GGCATAGGGATTCGAAGGCG
Drsomycin	Fw	TACTTGTTCGCCCTCTTCG
	Rv	GAGCGTCCCTCCTCCTTGC
IM1	Fw	CTCGGTCTGCTGGCTGTGGC
	Rv	CCGTGGACATTGCACACCC
Metchnikowin	Fw	GCTACATCAGTGCTGGCAGA
	Rv	TTAGGATTGAAGGGCGACGG
Defensin	Fw	AGTTCTTCGTTCTCGTGGCTA
	Rv	CCACATCGGAAACTGGCTGA
Drosocin	Fw	CACCCATGGCAAAAACGC
	Rv	TGAAGTTCACCATCGTTTTCCTG
CecropinA2	Fw	CATTCTGGCCATCACCATTGGACA
	Rv	GTGTGCTGACCAACACGTTCGATT
AttacinD	Fw	ATGGAATGTCAGGCTTCAGGA
	Rv	CCTGGAGTGGAGGCGAATAC
AttacinA&B	Fw	GGCCCATGCCAATTTATTCA
	Rv	AGCAAAGACCTTGGCATCCA
DeptericinA	Fw	TGCCGTCGCCTTACTTTGCT
	Rv	GCCGCCTCCCTGAAGATTGA

Immunoprecipitation

Samples were collected from whole flies or hemolymph of TsR44, TtR44, and w^{A5001} at

24 hours after treatment with a mixture of *E. coli* and *M. luteus.* Whole fly samples were collected from 40 flies each in buffer A (0.1% NP-40, 50Mm Tris pH=8, 100mM NaCl, 5mM $MgCl_2$, protease inhibitor cocktail [Roche cOmplete™ EDTA-free protease inhibitor cocktail]), while hemolymph samples were collected from 100 flies in total for each in buffer B (50mM Tris pH=8, protease inhibitor). To collect hemolymph in bulk, 20 wounded flies with an incision spanning the thorax made using a capillary were put in a PCR microcentrifuge tube perforated at its bottom with a needle. This tube was then placed above an intact 1.5mL microcentrifuge tube that contained 25 µl Buffer B (50mM Tris pH=8, protease inhibitor). The assembly was spun at 5,000g for ten minutes at 4°C. Then, 20µL of supernatant were collected, taking care not to include hemocytes that are retrieved by this method along the hemolymph. Samples were then immediately frozen on dry ice.

ChromoTek RFP-Trap Agarose™ (Cat No. rta) was used to perform the immunoprecipitation following the protocol provided by the supplier but using buffer A as a washing buffer. 25 µl of beads were used for whole-fly samples and 10 µl for hemolymph samples. At the last step, we eluted the sample with two times 60µl elution buffer (Miltenyi Biotech).

LC-MS/MS analyses

Proteins were prepared as described in a previous study [239]. Each sample was washed by 2 sequential overnight precipitations with glacial 0.1 M ammonium acetate in 100% methanol (5 volumes) followed by 3 washes with glacial 0.1 M ammonium acetate in 80% methanol. Proteins were then solubilized in 50 mM ammonium bicarbonate for a reduction-alkylation step (dithiothreitol 5 mM – iodoacetamide 10 mM) and an overnight digestion with 300ng of sequencing-grade porcine trypsin (Promega, Fitchburg, MA, USA). Digested peptides were resuspended in 0.1% formic acid (solvent A) and injected on an Easy-nanoLC-1000 system coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Germany). One fourth of each sample was loaded on a C-18 precolumn (75 µm ID × 20 mm nanoViper, 3µm Acclaim PepMap;

Thermo-Fisher Scientific) and separated on an analytical C18 analytical column (75 µm ID × 25 cm nanoViper, 3µm Acclaim PepMap) with a 160 minutes gradient of solvent B (0.1% of formic acid in acetonitrile). Data from the Qexactive will be deposited to the ProteomeXchange Consortium via the PRIDE partner repository with a dataset identifier.

Database search and mass-spectrometry data post-processing

Qexactive data were searched with Mascot algorithm (version 2.8, Matrix Science) against the UniProtKB database *D.melanogaster* taxonomy(42,818 sequences, release 2021_02) and the 2 sequences of TSR and TtR. The resulting .dat Mascot files were then imported into Proline v2.1 package [240] to align the identified proteins. Proteins were then validated on Mascot pretty rank equal to 1, 1% FDR on both peptide spectrum matches (PSM) and protein sets (based on Mascot score).

For statistical analyses, raw Spectral Count values were imported into R (v. 4.0.3) where the number of spectra were first normalization using the DESeq2 median of ratio normalization method. A negative-binomial test using an edgeR GLM regression generated for each identified protein a p-value and a protein fold-change (FC). The R script used to process the dataset is published on Github [241].

For the BaraA sequence coverage analysis, the Mascot search was carried out on the BaraA sequence with no enzyme specification in order to observe all the different cleaved peptides. The diagramm represents the occurance of observation of each amino acid in the peptides identified in both whole-fly and hemolymph experiments.

Quantification of mCherry intensity in hemolymph

The hemolymph from 10 flies were collected as the method described in immunoprecipitation in a 110 µl PBS containing 1.5 ml Eppendorf tube. Samples were spun at 5,000g at 4 degree for 10 mins. 100 µl Supernatant was then collected in a 96 well plate in black. Measurement of mCherry intensity was using fluorometer (varioskan) with the excitation at 580 nm, emission at 606 nm, excitation bandwidth of

12 nm and 1s of measurement time. Triplicates of samples were performed each time and result was shown with pooled data of three independent experiments.

Measurement of blood brain barrier permeability and brain imaging

In larvae, the wondering 3rd instar larvae were collected and injected with 4.6nl 8mM Destruxin A (DtxA) using Nanoject II/III auto-nanoliter injector (Drummond). Two hours post infection, larvae were wash with PBS and dissected always in PBS on ice. Larvae were cut into two parts from middle and flipped out without touching brain but exposing the organs in PBS. The tissues were then incubated in 25 mg/ml 10kDa Dextran-Texas Red™ (ThermoFisher Scientific D1863) for 30mins at room temperature and continuously incubated in 4% paraformaldehyde (PFA) for 2 hours at room temperature. The processing brain dissection was performed after washing the tissue in PBS.

In adults, 69 nl of 25 mg/ml 10kDa Dextran-Texas Red™ and 1.3 Mm DtxA was injected in the thorax of adult females by Nanoject II/III auto-nanoliter injector (Drummond). When flies recovered from DtxA but still paralyzed, they were injected with 69nl 8% PFA and incubated in 4% PFA for 90 minutes after removing the wings. Flies were washed in 0.1% PBST before brain dissection [242].

Brains were imaged using a spinning disk. Average intensity was measured from the central plane of the brain at two regions using Fiji.

Quantification of protease activity

1% Azocasein was dissolved in 50mM Tris-HCl, 0.5mM $CaCl_2$ at pH 8.0-8.5. 0.4mM of PrtA was incubated with 1% Azocasein at 30 degree for 1 hour. Reaction was stopped by 3 times volume of 10% Trichloroacetic acid (TCA) more that 15mins at room temperature. Sample was then centrifuged at maximum speed for appropriate time. 100µl of supernatant was then mixed with 100µl 0.5M NAOH. Wavelength of 440 was measured as the readout of proteas activity.

Western Blots

For western blots, hemolymph samples were collected from 40 flies in a protease inhibitor cocktails solution. Protein concentration of the samples was determined by Bradford assay. 30 μg protein was separated on an 8% (PPO1) or 10% (mCherry) gel by SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% bovine serum albumin in PBST for 1 h at room temperature, samples were incubated at 4 °C overnight with rabbit antibodies against *Drosophila* PPO1 at a 1: 10,000 dilution (a kind gift from Prof. Erjun Ling) or antibodies against mCherry (NOVUS NBP1-96752) at 1: 5,000 dilution. After washed, a goat anti-rabbit/mouse-horseradish peroxidase (HRP) secondary antibody at a 1: 20,000 dilution was applied for 1 h at room temperature. Enhanced chemiluminescence substrate (ECL, General Electric Healthcare) was used according to the manufacturer's instructions to reveal the blot.

Statistical tests

Statistical analyses were performed using GraphPad software Prism 8. Data were expressed as means ± SEM. RT-qPCR data were analyzed by Mann-Whitney test or ANOVA (one-way) with Dunnett's multiple comparisons test, with a significance threshold of P=0.05. Log-rank tests were used to determine whether survival curves of female flies were significantly different from each other. Experiment measuring the recovery rate was analyzed using Linear models (lm). Details are included in the legend of each figure. * $p < 0.05$; ** $p < 0.01$; *** $p< 0.001$; **** $p<0.0001$.

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General discussion

The initial goal of my Ph. D work was to determine whether lncRNAs identified in an RNAseq experiment as being induced upon *M. robertsii* infections may be involved in the host defense against infections. I have initially focused on two lncRNAs that had distinctive properties. *CR45601* is located antisense to the major codon of *Yp1* and displayed an expression pattern that paralleled that of *Yp1*, with a striking decreased expression upon *M. robertsii* infections. In contrast, *CR44404* was the top induced lncRNA and appeared to be simpler to study experimentally as it did not overlap any other gene. As described in Chapter I, the initial study of *CR45601* led to the conclusion that further investigations were not very promising since mutations that removed either the whole locus or affected only the *Yp1* open reading frame failed to reveal any consistent phenotype when challenged with several representative pathogens. Thus, even though I had developed genetic tools to address the question as to whether *CR45601* is responsible for the decreased expression of *Yp1* upon *M. robertsii* challenge, I did not further pursue this project as the study of what had become *CG44404* appeared much more interesting. Indeed, we had found the likely coding capacity of this locus as well as the homology to CG45045, which was actually being studied by **Adrian Acker** in the team of **Prof. Nicolas Matt**, a project that had been started in cultured S2 cells with the similar ambition of understanding the function of lncRNAs in the IMD-dependent systemic immune response. Thus, we decided to join forces to study these two loci in concert, which turned out to be a highly relevant decision since it allowed us to discover the sensitivity to injected *P. aeruginosa* PAO1, the first step toward discovering their function in the host defense against OMVs secreted either by PAO1 or *S. marcescens*, a topic that had been under study for many years in the host team. Thus, our work opens the possibility that the degree of protection afforded by the IMD pathway against OMVs may largely be mediated by the Shenshu/Yulü couple.

Two other lines of investigation opened by the work on *A. fumigatus* and a Toll pathway effector, BaraA, in *Drosophila* by the host team at the Sino-French Hoffmann

laboratory provided the conceptual background to understand the functions of *shen* and *yul*. Indeed, it had been found that *A. fumigatus* killed Toll pathway immunodeficient hosts solely through secreted microbial virulence factors, mostly secondary metabolites and also a protein ribotoxin [97]. The work on BaraA was the first to describe a dual function against toxins secreted by two strikingly different pathogens, a bacterium and a eukaryotic fungus [1].

Pathogens secrete many types of effectors that are not solely targeting potential hosts. Indeed, many toxins are secreted and allow the microorganism to survive among a strong competition in microbe-rich environments. For instance, yeasts secrete ethanol that enables them to kill bacterial competitors. Conversely, *E. faecalis* secretes EntV, a bacteriocin effective against *Lactobacilli* species but also against *Candida albicans* by acting on hyphae and preventing biofilm formation [243]. It should also be noted that microorganisms have developed through selection effectors that allow them to respond to predation by amoebae or like-organisms. This has been proposed to constitute one of the evolutionary pressures that allowed pathogens such as *Cryptococcus neoformans* to survive macrophage attacks [244]. Secreted toxins thus constitute an important armament used by pathogens to survive both outside and inside the host. As regards bacteria, a major category of virulence factors is represented by pore-forming toxins (PFTs) that directly attack host membranes, such as the cytoplasmic membrane or that of organelles (mitochondria) or phagosome. PFTs actually make up to 30% of secreted bacterial toxins [245]. Toxins present varied biochemical activities such as those of proteases, DNases, lipases… They are however not limited to proteins and secondary metabolites constitute an important category of virulence factors or effectors against microbial competition, *e.g.*, antibiotics.

Fungi have developed sophisticated strategies to invade their hosts and have selected during evolution hundreds of effectors. One good example is provided by fungal entomopathogens such as *M. robertsii* and *B. bassiana*, the sequenced genome of which revealed the existence of hundreds of secreted proteins that are likely virulence effectors [160]. Such a picture has also been gained from the study of plant fungal

pathogens. For instance, a recent RNAseq study documenting the expression of fungal genes at different steps of infection revealed the existence of 10 modules of temporally co-expressed genes. Thus, 863 genes predicted to encode secreted proteins are differentially expressed and 546 of them are likely effectors [246]. Such studies reveal a high level of complexity in the strategies implemented by the pathogens during the invasion into the host [247] but need to be complemented by parallel investigations on the release of secondary metabolites that is likely regulated with a similar precision. *A. fumigatus*, like many fungi, is known to produce an extensive array of secondary metabolites that are effective against hosts but also other microbes [248].

As regards *Drosophila* host defenses against microbial toxins, the host team in Strasbourg has revealed evolutionarily original responses of the intestinal epithelium to the exposure to PFT whereby the apical cytoplasm of enterocytes is expelled thereby purging the cell of virulence factors, invading bacteria, and damaged organelles [249]. This response however does not appear to involve known immune response pathways. In contrast, the Toll pathway appears to be involved in both resistance and resilience, the latter function allowing to effectively counteract the action of several mycotoxins. It should however be noted that similar defenses against other microbial toxins may have been selected during evolution and might involve other pathways. For instance, it is likely that larvae, which are feeding on rotting fruits, have been constantly exposed to “alimentary” mycotoxins produced by fungi that also affect human crops. Our preliminary attempts have failed to reveal a sensitivity of wild-type or Toll pathway mutant flies to three of the most common mycotoxins that contaminate food produces but there are many more to test.

Our studies so far allow us to categorize virulence factors that are somewhat counteracted with by Toll pathway effectors in three categories. On the one hand, Bomanins encoded by the 55C locus appear to mediate most of the protection against two families of *A. fumigatus* mycotoxins that display strikingly distinct properties. One is a protein that cleaves the host 28S RNA and blocks translation of most genes whereas the other belongs to a family of secondary metabolites that target the nervous system.

How Bomanins mediate such a protection remains to be elucidated and is the focus of intensive work. A second category corresponds to the toxins we have identified in this work as well as in BaraA studies, DtxA, a hexadepsipeptide, EntV, a bacteriocin, and OMVs/PrtA, the latter being a protease. The protection is afforded by at least three types of effectors, BaraA-derived peptides, Shenshu and/or Yulü, and likely Teps. Finally, a third category corresponds to toxins that are counteracted through the Toll pathway but independently of 55C Bomanins and BaraA. This is the case for Beauvericin, another cyclic hexadepsipeptide secreted by *Beauveria bassiana*, an entomopathogenic fungus highly related to *Metarhizium*. It appears also to be the case of ergot alkaloids, some of which are secreted by *A. fumigatus* (fumigaclavines). At SFHI, the host team has performed a large-scale RNAi mutagenesis screen in which the survival of mutant lines to *A. fumigatus* challenge was monitored. Some 50 lines display a highly reproducible phenotype. In a secondary screen, it was found that most of them are required for host defense against one or several mycotoxins that were tested, restrictocin, verruculogen, and an ergot alkaloid, bromocriptine. A similar survival screen has been performed in parallel on *M. robertsii*, to which I contributed, and has revealed a lower number of hits. One difficulty associated with this screen is that wild-type flies do succumb to *M. robertsii*, making the reliable identification of mutants more difficult as the phenotypes are usually much less pronounced. A systematic retest of these lines with DtxA has not been undertaken yet. However, some hits that are under current investigations failed to reveal an enhanced sensitivity to DtxA. As the screen was performed using a "natural" infection model, it is an open possibility that DtxA does not play an important role in this model and therefore that the hits we have found may not be related to host defense against DtxA. Indeed, both *BaraA* and *shen/yul* mutants are sensitive to a *M. robertsii* challenge in the septic injury model and not the "natural" infection model [1]. This is in keeping with the doctoral work of Dr. Wenhui Wang who documented differences both in the fungal virulence strategy and involved host defenses depending on the route of infection of this fungus [250].

Presently, the underlying logic as to why a given toxin is counteracted by

Bomanins, BaraA/Shenshu/Yulü/Teps or other Toll-dependent or independent effectors eludes us. Thus, a thorough work on the action of toxins needs to be undertaken. It will be especially important to identify the molecular targets of each toxin and also to identify in which cell type/tissue/ organ it is critically important that they act. This program is currently being undertaken for restrictocin and verruculogen, for which biochemical or behavioral assays are available, provided they are sensitive enough.

An important issue has to be kept in mind. The virulence strategies of a pathogen do not rely on single factors but a whole array of secreted proteins and secondary metabolites that are likely expressed coordinately in distinct waves at different steps of the infectious process. It is likely that some of the virulence factors might act redundantly and it is not surprising that for instance *DtxS1* mutants display only a weak phenotype at best, in the silkworm as in *Drosophila* ([159] and unpublished work from the laboratory). Thus, the strategy of overexpression of a given virulence factor reveals the potentialities of this factor while also sometimes allowing to identify the relevant host defenses. It is actually striking that each tested virulence factor may actually be very potent, as exemplified by virulence factors from *Drechmeria coniospora* that are able to kill the *Caenorhabditis elegans* host when expressed from transgenes [251]. A question arises then: why has evolution selected so many distinct virulence factors in the pathogen when one might be sufficient. The answer likely lies in the co-evolution between host and pathogen in an endless arms race. Also, one has to take other factors into consideration. One is that the pathogen must be able to protect itself from its own toxins and that its own defenses may be effective against only limited amounts of the toxins. A second one has to deal with the different steps of the infection and it may be actually counter-productive to express virulence factors at the wrong moment [247] or to kill the host too fast. This is well-exemplified by pathogens that manipulate host behavior to optimize the dissemination of the spores to the next host, a strategy developed by several categories of “zombie” fungi [148] or parasites such as *Toxoplasma gondi*. Finally, it is likely that virulence factors may act in concert, that is synergistically for a cocktail effect, as has been found the case for some AMPs on the

host side [91].

The article placed in annex documents investigations to understand how OMVs act in the fly and has systematically addressed whether the known host defenses had any impact on their pathogenicity. Whereas this work has not yet been undertaken for the *A. fumigatus* mycotoxins, it had been performed for the fungus itself and had revealed a role for melanization in preventing the colonization of the host but no apparent involvement of the cellular immune response. Of note, the study of a line in which most *Teps* have been genetically removed did not reveal a role for them in the host defense against *A. fumigatus* [117]. However, this analysis may need to be reiterated for the injected toxins as this approach might reveal phenotypes that are too subtle when using the whole fungus that secretes only limited amounts of each virulence factor.

More generally, we will need to also test the effects of DtxA and EntV on the following host defenses: humoral systemic immune response, cellular immune response, melanization, ROS, and Teps. Of note, this work would be difficult for EntV as, so far, we have to compare supernatants of wild-type *vs*. *EntV* mutant *E. faecalis*. Hopefully, a short EntV-derived peptide that mediates its activity against *C. albicans* will prove to recapitulate the virulence properties of EntV in *Drosophila*. In the case of DtxA, some analyses have been already performed as regards the systemic immune response, although it was not reported whether the IMD pathway is really important in the host defense against *M. robertsii* [231]. My own data suggest that *kenny* mutants are actually more susceptible to DtxA, although a comparison of the behavior of injected wild-type *vs. DtxS1 M. robertsii* should be performed.

Finally, it is striking that the IMD and Toll pathways regulate the expression of hundreds of genes in response to microbial infections. Many of them likely correspond to effectors, the function of which remains unknown to this stage. The flies are exposed to the pressure of multiple pathogens and thus to a high complexity of potentially noxious biochemical activities. It is likely that these effectors have been selected during evolution. Thus, studies presented in this work provide a modest first step to resolve

such a major challenge and it is likely that the strategies of loss-of-function and overexpression are going to be useful only when the right virulence factor from the "right" pathogens against which they are effective have been identified. It remains nevertheless perplexing that the 55C Bomanin cluster appears to play such a major role in the Toll-mediated host defenses. Thus, we shall also have to understand how the protection afforded by other Toll-pathway regulated genes act in concert with this category of Toll effectors.

Reference

[1] J. Huang *et al.*, "A Toll pathway effector protects *Drosophila* specifically from distinct toxins secreted by a fungus or a bacterium," *Proc Natl Acad Sci U S A,* vol. 120, no. 12, p. e2205140120, Mar 21 2023, doi: 10.1073/pnas.2205140120.

[2] T. H. Morgan, "SEX LIMITED INHERITANCE IN *DROSOPHILA*," (in eng), *Science,* vol. 32, no. 812, pp. 120-2, Jul 22 1910, doi: 10.1126/science.32.812.120.

[3] M. D. Adams *et al.*, "The genome sequence of *Drosophila melanogaster*," (in eng), *Science,* vol. 287, no. 5461, pp. 2185-95, Mar 24 2000, doi: 10.1126/science.287.5461.2185.

[4] B. Lemaitre and J. Hoffmann, "The host defense of *Drosophila melanogaster*," *Annu Rev Immunol,* vol. 25, pp. 697-743, 2007, doi: 10.1146/annurev.immunol.25.022106.141615.

[5] U. B. Pandey and C. D. Nichols, "Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery," *Pharmacol Rev,* vol. 63, no. 2, pp. 411-36, Jun 2011, doi: 10.1124/pr.110.003293.

[6] A. M. Celotto and M. J. Palladino, "*Drosophila*: a "model" model system to study neurodegeneration," (in eng), *Mol Interv,* vol. 5, no. 5, pp. 292-303, Oct 2005, doi: 10.1124/mi.5.5.9.

[7] P. Bergman, S. Seyedoleslami Esfahani, and Y. Engström, "*Drosophila* as a Model for Human Diseases-Focus on Innate Immunity in Barrier Epithelia," (in eng), *Curr Top Dev Biol,* vol. 121, pp. 29-81, 2017, doi: 10.1016/bs.ctdb.2016.07.002.

[8] A. Souidi and K. Jagla, "*Drosophila* Heart as a Model for Cardiac Development and Diseases," (in eng), *Cells,* vol. 10, no. 11, Nov 8 2021, doi: 10.3390/cells10113078.

[9] D. Chatterjee and W. M. Deng, "*Drosophila* Model in Cancer: An Introduction," (in eng), *Adv Exp Med Biol,* vol. 1167, pp. 1-14, 2019, doi: 10.1007/978-3-030-23629-8_1.

[10] J. Hoffmann, R. Romey, C. Fink, and T. Roeder, "*Drosophila* as a model to study metabolic disorders," (in eng), *Adv Biochem Eng Biotechnol,* vol. 135, pp. 41-61, 2013, doi: 10.1007/10_2013_196.

[11] B. K. Barik and M. Mishra, "Nanoparticles as a potential teratogen: a lesson learnt from fruit fly," *Nanotoxicology,* vol. 13, no. 2, pp. 258-284, Mar 2019, doi: 10.1080/17435390.2018.1530393.

[12] Y. Ishino, M. Krupovic, and P. Forterre, "History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology," (in eng), *J Bacteriol,* vol. 200, no. 7, Apr 1 2018, doi: 10.1128/jb.00580-17.

[13] R. Barrangou *et al.*, "CRISPR provides acquired resistance against viruses in prokaryotes," (in eng), *Science,* vol. 315, no. 5819, pp. 1709-12, Mar 23 2007, doi: 10.1126/science.1138140.

[14] F. Jiang and J. A. Doudna, "CRISPR-Cas9 Structures and Mechanisms," *Annu Rev Biophys,* vol. 46, pp. 505-529, May 22 2017, doi: 10.1146/annurev-biophys-062215-010822.

[15] H. H. Y. Chang, N. R. Pannunzio, N. Adachi, and M. R. Lieber, "Non-homologous DNA end joining and alternative pathways to double-strand break repair," *Nat Rev Mol Cell Biol,* vol. 18, no. 8, pp. 495-506, Aug 2017, doi: 10.1038/nrm.2017.48.

[16] B. Cai *et al.*, "Application of CRISPR/Cas9 technologies combined with iPSCs in the study and treatment of retinal degenerative diseases," (in eng), *Hum Genet,* vol. 137, no. 9, pp. 679-688, Sep 2018, doi: 10.1007/s00439-018-1933-9.

[17] X. Zhang, W. H. Koolhaas, and F. Schnorrer, "A versatile two-step CRISPR- and RMCE-based strategy for efficient genome engineering in *Drosophila*," *G3 (Bethesda),* vol. 4, no. 12, pp. 2409-18, Oct 15 2014, doi: 10.1534/g3.114.013979.

[18] F. Port and S. L. Bullock, "Augmenting CRISPR applications in *Drosophila* with tRNA-flanked sgRNAs," *Nat Methods,* vol. 13, no. 10, pp. 852-4, Oct 2016, doi: 10.1038/nmeth.3972.

[19] A. C. Spradling and G. M. Rubin, "The effect of chromosomal position on the expression of the *Drosophila* xanthine dehydrogenase gene," (in eng), *Cell,* vol. 34, no. 1, pp. 47-57, Aug 1983, doi: 10.1016/0092-8674(83)90135-6.

[20] Y. H. Gray, M. M. Tanaka, and J. A. Sved, "P-element-induced recombination in *Drosophila melanogaster*: hybrid element insertion," (in eng), *Genetics,* vol. 144, no. 4, pp. 1601-10, Dec 1996, doi: 10.1093/genetics/144.4.1601.

[21] F. J. Olorunniji, M. Lawson-Williams, A. L. McPherson, J. E. Paget, W. M. Stark, and S. J. Rosser, "Control of ϕC31 integrase-mediated site-specific recombination by protein trans-splicing," (in eng), *Nucleic Acids Res,* vol. 47, no. 21, pp. 11452-11460, Dec 2 2019, doi: 10.1093/nar/gkz936.

[22] K. J. Venken, Y. He, R. A. Hoskins, and H. J. Bellen, "P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. *melanogaster*," (in eng), *Science,* vol. 314, no. 5806, pp. 1747-51, Dec 15 2006, doi: 10.1126/science.1134426.

[23] R. T. Hillman and M. P. Calos, "Site-specific integration with bacteriophage ΦC31 integrase," (in eng), *Cold Spring Harb Protoc,* vol. 2012, no. 5, May 1 2012, doi: 10.1101/pdb.prot069211.

[24] J. R. Bateman, A. M. Lee, and C. T. Wu, "Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange," *Genetics,* vol. 173, no. 2, pp. 769-77, Jun 2006, doi: 10.1534/genetics.106.056945.

[25] J. B. Duffy, "GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife," *Genesis,* vol. 34, no. 1-2, pp. 1-15, Sep-Oct 2002, doi: 10.1002/gene.10150.

[26] J. A. Fischer, E. Giniger, T. Maniatis, and M. Ptashne, "GAL4 activates transcription in *Drosophila*," (in eng), *Nature,* vol. 332, no. 6167, pp. 853-6, Apr 28 1988, doi: 10.1038/332853a0.

[27] E. E. Caygill and A. H. Brand, "The GAL4 System: A Versatile System for the Manipulation and Analysis of Gene Expression," (in eng), *Methods Mol Biol,* vol. 1478, pp. 33-52, 2016, doi: 10.1007/978-1-4939-6371-3_2.

[28] C. B. Phelps and A. H. Brand, "Ectopic gene expression in *Drosophila* using GAL4 system," (in eng), *Methods,* vol. 14, no. 4, pp. 367-79, Apr 1998, doi: 10.1006/meth.1998.0592.

[29] V. Pilauri, M. Bewley, C. Diep, and J. Hopper, "Gal80 dimerization and the yeast GAL gene switch," (in eng), *Genetics,* vol. 169, no. 4, pp. 1903-14, Apr 2005, doi: 10.1534/genetics.104.036723.

[30] F. Henkler *et al.*, "Caspase-mediated cleavage converts the tumor necrosis factor (TNF) receptor-associated factor (TRAF)-1 from a selective modulator of TNF receptor signaling to a general inhibitor of NF-kappaB activation," *J Biol Chem,* vol. 278, no. 31, pp. 29216-30, Aug 1 2003, doi: 10.1074/jbc.M211090200.

[31] L. M. Sedger and M. F. McDermott, "TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future," *Cytokine Growth Factor Rev,* vol. 25, no. 4, pp. 453-72, Aug 2014, doi: 10.1016/j.cytogfr.2014.07.016.

[32] C. A. Janeway, Jr., "Approaching the asymptote? Evolution and revolution in immunology," (in

eng), *Cold Spring Harb Symp Quant Biol,* vol. 54 Pt 1, pp. 1-13, 1989, doi: 10.1101/sqb.1989.054.01.003.

[33] W. Vollmer, D. Blanot, and M. A. de Pedro, "Peptidoglycan structure and architecture," *FEMS Microbiol Rev,* vol. 32, no. 2, pp. 149-67, Mar 2008, doi: 10.1111/j.1574-6976.2007.00094.x.

[34] V. Bischoff, C. Vignal, B. Duvic, I. G. Boneca, J. A. Hoffmann, and J. Royet, "Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2," *PLoS Pathog,* vol. 2, no. 2, p. e14, Feb 2006, doi: 10.1371/journal.ppat.0020014.

[35] A. Zaidman-Remy *et al.*, "*Drosophila* immunity: analysis of PGRP-SB1 expression, enzymatic activity and function," *PLoS One,* vol. 6, no. 2, p. e17231, Feb 18 2011, doi: 10.1371/journal.pone.0017231.

[36] L. Guo, J. Karpac, S. L. Tran, and H. Jasper, "PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan," *Cell,* vol. 156, no. 1-2, pp. 109-22, Jan 16 2014, doi: 10.1016/j.cell.2013.12.018.

[37] M. Gottar *et al.*, "The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein," (in eng), *Nature,* vol. 416, no. 6881, pp. 640-4, Apr 11 2002, doi: 10.1038/nature734.

[38] K. M. Choe, T. Werner, S. Stöven, D. Hultmark, and K. V. Anderson, "Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*," (in eng), *Science,* vol. 296, no. 5566, pp. 359-62, Apr 12 2002, doi: 10.1126/science.1070216.

[39] M. Rämet, P. Manfruelli, A. Pearson, B. Mathey-Prevot, and R. A. Ezekowitz, "Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for E. coli," (in eng), *Nature,* vol. 416, no. 6881, pp. 644-8, Apr 11 2002, doi: 10.1038/nature735.

[40] C. Neyen, M. Poidevin, A. Roussel, and B. Lemaitre, "Tissue- and ligand-specific sensing of gram-negative infection in *Drosophila* by PGRP-LC isoforms and PGRP-LE," *J Immunol,* vol. 189, no. 4, pp. 1886-97, Aug 15 2012, doi: 10.4049/jimmunol.1201022.

[41] J.-H. Lim *et al.*, "Structural Basis for Preferential Recognition of Diaminopimelic Acid-type Peptidoglycan by a Subset of Peptidoglycan Recognition Proteins," *Journal of Biological Chemistry,* vol. 281, no. 12, pp. 8286-8295, 2006, doi: 10.1074/jbc.M513030200.

[42] M. Gendrin *et al.*, "Functional analysis of PGRP-LA in *Drosophila* immunity," *PLoS One,* vol. 8, no. 7, p. e69742, 2013, doi: 10.1371/journal.pone.0069742.

[43] I. Iatsenko, S. Kondo, D. Mengin-Lecreulx, and B. Lemaitre, "PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the *Drosophila* Imd Pathway," *Immunity,* vol. 45, no. 5, pp. 1013-1023, Nov 15 2016, doi: 10.1016/j.immuni.2016.10.029.

[44] D. Ferrandon, J. L. Imler, C. Hetru, and J. A. Hoffmann, "The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections," *Nat Rev Immunol,* vol. 7, no. 11, pp. 862-74, Nov 2007, doi: 10.1038/nri2194.

[45] A. Meinander *et al.*, "Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling," *EMBO J,* vol. 31, no. 12, pp. 2770-83, Jun 13 2012, doi: 10.1038/emboj.2012.121.

[46] R. Zhou *et al.*, "The role of ubiquitination in *Drosophila* innate immunity," *J Biol Chem,* vol. 280, no. 40, pp. 34048-55, Oct 7 2005, doi: 10.1074/jbc.M506655200.

[47] A. Kleino *et al.*, "Inhibitor of apoptosis 2 and TAK1-binding protein are components of the

Drosophila Imd pathway," *EMBO J,* vol. 24, no. 19, pp. 3423-34, Oct 5 2005, doi: 10.1038/sj.emboj.7600807.

[48] N. Silverman *et al.*, "Immune activation of NF-kappaB and JNK requires *Drosophila* TAK1," *J Biol Chem,* vol. 278, no. 49, pp. 48928-34, Dec 5 2003, doi: 10.1074/jbc.M304802200.

[49] S. Vidal, R. S. Khush, F. Leulier, P. Tzou, M. Nakamura, and B. Lemaitre, "Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses," *Genes Dev,* vol. 15, no. 15, pp. 1900-12, Aug 1 2001, doi: 10.1101/gad.203301.

[50] S. Rutschmann, A. C. Jung, R. Zhou, N. Silverman, J. A. Hoffmann, and D. Ferrandon, "Role of *Drosophila* IKK gamma in a toll-independent antibacterial immune response," (in eng), *Nat Immunol,* vol. 1, no. 4, pp. 342-7, Oct 2000, doi: 10.1038/79801.

[51] S. Stoven *et al.*, "Caspase-mediated processing of the *Drosophila* NF-kappaB factor Relish," (in eng), *Proc Natl Acad Sci U S A,* vol. 100, no. 10, pp. 5991-6, May 13 2003, doi: 10.1073/pnas.1035902100.

[52] C. Hetru and J. A. Hoffmann, "NF-kappaB in the immune response of *Drosophila*," *Cold Spring Harb Perspect Biol,* vol. 1, no. 6, p. a000232, Dec 2009, doi: 10.1101/cshperspect.a000232.

[53] J. Royet and R. Dziarski, "Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences," (in eng), *Nat Rev Microbiol,* vol. 5, no. 4, pp. 264-77, Apr 2007, doi: 10.1038/nrmicro1620.

[54] M. S. Busse, C. P. Arnold, P. Towb, J. Katrivesis, and S. A. Wasserman, "A kappaB sequence code for pathway-specific innate immune responses," *EMBO J,* vol. 26, no. 16, pp. 3826-35, Aug 22 2007, doi: 10.1038/sj.emboj.7601798.

[55] G. W. Lau *et al.*, "The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen Pseudomonas aeruginosa," *Infect Immun,* vol. 71, no. 7, pp. 4059-66, Jul 2003, doi: 10.1128/IAI.71.7.4059-4066.2003.

[56] T. Tanji, E. Y. Yun, and Y. T. Ip, "Heterodimers of NF-kappaB transcription factors DIF and Relish regulate antimicrobial peptide genes in *Drosophila*," *Proc Natl Acad Sci U S A,* vol. 107, no. 33, pp. 14715-20, Aug 17 2010, doi: 10.1073/pnas.1009473107.

[57] A. Goto *et al.*, "Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in *Drosophila* and mice," *Nat Immunol,* vol. 9, no. 1, pp. 97-104, Jan 2008, doi: 10.1038/ni1543.

[58] F. Bonnay *et al.*, "Akirin specifies NF-kappaB selectivity of *Drosophila* innate immune response via chromatin remodeling," *EMBO J,* vol. 33, no. 20, pp. 2349-62, Oct 16 2014, doi: 10.15252/embj.201488456.

[59] C. Nusslein-Volhard, "The Toll gene in *Drosophila* pattern formation," *Trends Genet,* Oct 11 2021, doi: 10.1016/j.tig.2021.09.006.

[60] M. Ochiai and M. Ashida, "A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, Bombyx mori," *J Biol Chem,* vol. 275, no. 7, pp. 4995-5002, Feb 18 2000, doi: 10.1074/jbc.275.7.4995.

[61] V. Gobert *et al.*, "Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors," *Science,* vol. 302, no. 5653, pp. 2126-30, Dec 19 2003, doi: 10.1126/science.1085432.

[62] L. Wang, A. N. Weber, M. L. Atilano, S. R. Filipe, N. J. Gay, and P. Ligoxygakis, "Sensing of

Gram-positive bacteria in *Drosophila*: GNBP1 is needed to process and present peptidoglycan to PGRP-SA," (in eng), *Embo j,* vol. 25, no. 20, pp. 5005-14, Oct 18 2006, doi: 10.1038/sj.emboj.7601363.

[63] V. Bischoff, C. Vignal, I. G. Boneca, T. Michel, J. A. Hoffmann, and J. Royet, "Function of the *Drosophila* pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria," *Nat Immunol,* vol. 5, no. 11, pp. 1175-80, Nov 2004, doi: 10.1038/ni1123.

[64] P. Leone, V. Bischoff, C. Kellenberger, C. Hetru, J. Royet, and A. Roussel, "Crystal structure of *Drosophila* PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan," *Mol Immunol,* vol. 45, no. 9, pp. 2521-30, May 2008, doi: 10.1016/j.molimm.2008.01.015.

[65] M. Gottar *et al.*, "Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors," *Cell,* vol. 127, no. 7, pp. 1425-37, Dec 29 2006, doi: 10.1016/j.cell.2006.10.046.

[66] Y. Mishima *et al.*, "The N-terminal domain of *Drosophila* Gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors," (in eng), *J Biol Chem,* vol. 284, no. 42, pp. 28687-97, Oct 16 2009, doi: 10.1074/jbc.M109.034587.

[67] N. Buchon *et al.*, "A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway," (in eng), *Proc Natl Acad Sci U S A,* vol. 106, no. 30, pp. 12442-7, Jul 28 2009, doi: 10.1073/pnas.0901924106.

[68] Z. Kambris *et al.*, "*Drosophila* immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation," *Curr Biol,* vol. 16, no. 8, pp. 808-13, Apr 18 2006, doi: 10.1016/j.cub.2006.03.020.

[69] L. El Chamy, V. Leclerc, I. Caldelari, and J. M. Reichhart, "Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll," *Nat Immunol,* vol. 9, no. 10, pp. 1165-70, Oct 2008, doi: 10.1038/ni.1643.

[70] J. P. Dudzic, M. A. Hanson, I. Iatsenko, S. Kondo, and B. Lemaitre, "More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in *Drosophila*," (in eng), *Cell Rep,* vol. 27, no. 4, pp. 1050-1061.e3, Apr 23 2019, doi: 10.1016/j.celrep.2019.03.101.

[71] N. Issa *et al.*, "The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway," *Mol Cell,* vol. 69, no. 4, pp. 539-550 e6, Feb 15 2018, doi: 10.1016/j.molcel.2018.01.029.

[72] A. N. Weber *et al.*, "Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling," (in eng), *Nat Immunol,* vol. 4, no. 8, pp. 794-800, Aug 2003, doi: 10.1038/ni955.

[73] S. Tauszig, E. Jouanguy, J. A. Hoffmann, and J. L. Imler, "Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*," (in eng), *Proc Natl Acad Sci U S A,* vol. 97, no. 19, pp. 10520-5, Sep 12 2000, doi: 10.1073/pnas.180130797.

[74] J. L. Imler, D. Ferrandon, J. Royet, J. M. Reichhart, C. Hetru, and J. A. Hoffmann, "Toll-dependent and Toll-independent immune responses in *Drosophila*," *J Endotoxin Res,* vol. 10, no. 4, pp. 241-6, 2004, doi: 10.1179/096805104225005887.

[75] R. Bettencourt, T. Tanji, Y. Yagi, and Y. T. Ip, "Toll and Toll-9 in *Drosophila* innate immune response," *J Endotoxin Res,* vol. 10, no. 4, pp. 261-8, 2004, doi: 10.1179/096805104225004897.

[76] S. Tauszig-Delamasure, H. Bilak, M. Capovilla, J. A. Hoffmann, and J. L. Imler, "*Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections," *Nat Immunol,* vol. 3, no. 1, pp. 91-7, Jan 2002, doi: 10.1038/ni747.

[77] M. C. Moncrieffe, J. G. Grossmann, and N. J. Gay, "Assembly of oligomeric death domain

complexes during Toll receptor signaling," *J Biol Chem,* vol. 283, no. 48, pp. 33447-54, Nov 28 2008, doi: 10.1074/jbc.M805427200.

[78] H. Sun, B. N. Bristow, G. Qu, and S. A. Wasserman, "A heterotrimeric death domain complex in Toll signaling," (in eng), *Proc Natl Acad Sci U S A,* vol. 99, no. 20, pp. 12871-6, Oct 1 2002, doi: 10.1073/pnas.202396399.

[79] J. Daigneault, L. Klemetsaune, and S. A. Wasserman, "The IRAK homolog Pelle is the functional counterpart of IkappaB kinase in the *Drosophila* Toll pathway," *PLoS One,* vol. 8, no. 9, p. e75150, 2013, doi: 10.1371/journal.pone.0075150.

[80] P. Manfruelli, J. M. Reichhart, R. Steward, J. A. Hoffmann, and B. Lemaitre, "A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF," (in eng), *Embo j,* vol. 18, no. 12, pp. 3380-91, Jun 15 1999, doi: 10.1093/emboj/18.12.3380.

[81] S. Rutschmann, A. C. Jung, C. Hetru, J. M. Reichhart, J. A. Hoffmann, and D. Ferrandon, "The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*," (in eng), *Immunity,* vol. 12, no. 5, pp. 569-80, May 2000, doi: 10.1016/s1074-7613(00)80208-3.

[82] A. Meng, B. Moore, H. Tang, B. Yuan, and S. Lin, "A *Drosophila* doublesex-related gene, terra, is involved in somitogenesis in vertebrates," (in eng), *Development,* vol. 126, no. 6, pp. 1259-68, Mar 1999, doi: 10.1242/dev.126.6.1259.

[83] M. A. Hanson and B. Lemaitre, "New insights on *Drosophila* antimicrobial peptide function in host defense and beyond," *Curr Opin Immunol,* vol. 62, pp. 22-30, Feb 2020, doi: 10.1016/j.coi.2019.11.008.

[84] Y. Lai and R. L. Gallo, "AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense," (in eng), *Trends Immunol,* vol. 30, no. 3, pp. 131-41, Mar 2009, doi: 10.1016/j.it.2008.12.003.

[85] K. A. Brogden, "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?," (in eng), *Nat Rev Microbiol,* vol. 3, no. 3, pp. 238-50, Mar 2005, doi: 10.1038/nrmicro1098.

[86] P. Bulet, C. Hetru, J. L. Dimarcq, and D. Hoffmann, "Antimicrobial peptides in insects; structure and function," (in eng), *Dev Comp Immunol,* vol. 23, no. 4-5, pp. 329-44, Jun-Jul 1999, doi: 10.1016/s0145-305x(99)00015-4.

[87] C. Wicker, J. M. Reichhart, D. Hoffmann, D. Hultmark, C. Samakovlis, and J. A. Hoffmann, "Insect immunity. Characterization of a *Drosophila* cDNA encoding a novel member of the diptericin family of immune peptides," *Journal of Biological Chemistry,* vol. 265, no. 36, pp. 22493-22498, 1990, doi: 10.1016/s0021-9258(18)45732-8.

[88] P. Fehlbaum *et al.*, "Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides," *Journal of Biological Chemistry,* vol. 269, no. 52, pp. 33159-33163, 1994, doi: 10.1016/s0021-9258(20)30111-3.

[89] E. A. Levashina, S. Ohresser, P. Bulet, J. M. Reichhart, C. Hetru, and J. A. Hoffmann, "Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila* with antibacterial and antifungal properties," (in eng), *Eur J Biochem,* vol. 233, no. 2, pp. 694-700, Oct 15 1995, doi: 10.1111/j.1432-1033.1995.694_2.x.

[90] R. L. Unckless, V. M. Howick, and B. P. Lazzaro, "Convergent Balancing Selection on an Antimicrobial Peptide in *Drosophila*," (in eng), *Curr Biol,* vol. 26, no. 2, pp. 257-262, Jan 25

2016, doi: 10.1016/j.cub.2015.11.063.

[91] M. A. Hanson, A. Dostalova, C. Ceroni, M. Poidevin, S. Kondo, and B. Lemaitre, "Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach," *Elife,* vol. 8, Feb 26 2019, doi: 10.7554/eLife.44341.

[92] J. L. Imler and P. Bulet, "Antimicrobial peptides in *Drosophila*: structures, activities and gene regulation," (in eng), *Chem Immunol Allergy,* vol. 86, pp. 1-21, 2005, doi: 10.1159/000086648.

[93] S. Uttenweiler-Joseph, M. Moniatte, M. Lagueux, A. Van Dorsselaer, J. A. Hoffmann, and P. Bulet, "Differential display of peptides induced during the immune response of *Drosophila*: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study," (in eng), *Proc Natl Acad Sci U S A,* vol. 95, no. 19, pp. 11342-7, Sep 15 1998, doi: 10.1073/pnas.95.19.11342.

[94] A. W. Clemmons, S. A. Lindsay, and S. A. Wasserman, "An effector Peptide family required for *Drosophila* toll-mediated immunity," *PLoS Pathog,* vol. 11, no. 4, p. e1004876, Apr 2015, doi: 10.1371/journal.ppat.1004876.

[95] S. J. H. Lin, A. Fulzele, L. B. Cohen, E. J. Bennett, and S. A. Wasserman, "Bombardier Enables Delivery of Short-Form Bomanins in the *Drosophila* Toll Response," (in eng), *Front Immunol,* vol. 10, p. 3040, 2019, doi: 10.3389/fimmu.2019.03040.

[96] S. A. Lindsay, S. J. H. Lin, and S. A. Wasserman, "Short-Form Bomanins Mediate Humoral Immunity in *Drosophila*," *J Innate Immun,* vol. 10, no. 4, pp. 306-314, 2018, doi: 10.1159/000489831.

[97] R. Xu *et al.*, "The Toll pathway mediates *Drosophila* resilience to Aspergillus mycotoxins through specific Bomanins," (in eng), *EMBO Rep,* vol. 24, no. 1, p. e56036, Jan 9 2023, doi: 10.15252/embr.202256036.

[98] L. B. Cohen, S. A. Lindsay, Y. Xu, S. J. H. Lin, and S. A. Wasserman, "The Daisho Peptides Mediate *Drosophila* Defense Against a Subset of Filamentous Fungi," *Front Immunol,* vol. 11, p. 9, 2020, doi: 10.3389/fimmu.2020.00009.

[99] F. Levy, D. Rabel, M. Charlet, P. Bulet, J. A. Hoffmann, and L. Ehret-Sabatier, "Peptidomic and proteomic analyses of the systemic immune response of *Drosophila*," *Biochimie,* vol. 86, no. 9-10, pp. 607-16, Sep-Oct 2004, doi: 10.1016/j.biochi.2004.07.007.

[100] M. A. Hanson, L. B. Cohen, A. Marra, I. Iatsenko, S. A. Wasserman, and B. Lemaitre, "The *Drosophila* Baramicin polypeptide gene protects against fungal infection," *PLoS Pathog,* vol. 17, no. 8, p. e1009846, Aug 2021, doi: 10.1371/journal.ppat.1009846.

[101] S. Kurata, S. Ariki, and S. Kawabata, "Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity," (in eng), *Immunobiology,* vol. 211, no. 4, pp. 237-49, 2006, doi: 10.1016/j.imbio.2005.10.016.

[102] L. M. Stuart and R. A. Ezekowitz, "Phagocytosis: elegant complexity," (in eng), *Immunity,* vol. 22, no. 5, pp. 539-50, May 2005, doi: 10.1016/j.immuni.2005.05.002.

[103] C. Melcarne, B. Lemaitre, and E. Kurant, "Phagocytosis in *Drosophila*: From molecules and cellular machinery to physiology," *Insect Biochem Mol Biol,* vol. 109, pp. 1-12, Jun 2019, doi: 10.1016/j.ibmb.2019.04.002.

[104] H. G. Roddie, E. L. Armitage, J. A. Coates, S. A. Johnston, and I. R. Evans, "Simu-dependent clearance of dying cells regulates macrophage function and inflammation resolution," (in eng), *PLoS Biol,* vol. 17, no. 5, p. e2006741, May 2019, doi: 10.1371/journal.pbio.2006741.

[105] E. Kurant, S. Axelrod, D. Leaman, and U. Gaul, "Six-microns-under acts upstream of Draper in

the glial phagocytosis of apoptotic neurons," (in eng), *Cell,* vol. 133, no. 3, pp. 498-509, May 2 2008, doi: 10.1016/j.cell.2008.02.052.

[106] C. Melcarne *et al.*, "Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in *Drosophila melanogaster*," (in eng), *Febs j,* vol. 286, no. 14, pp. 2670-2691, Jul 2019, doi: 10.1111/febs.14857.

[107] A. J. Bretscher *et al.*, "The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in *Drosophila melanogaster*," (in eng), *Biol Open,* vol. 4, no. 3, pp. 355-63, Feb 13 2015, doi: 10.1242/bio.201410595.

[108] C. Kocks *et al.*, "Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*," (in eng), *Cell,* vol. 123, no. 2, pp. 335-46, Oct 21 2005, doi: 10.1016/j.cell.2005.08.034.

[109] S. Limmer *et al.*, "Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model," (in eng), *Proc Natl Acad Sci U S A,* vol. 108, no. 42, pp. 17378-83, Oct 18 2011, doi: 10.1073/pnas.1114907108.

[110] S. Nonaka, K. Nagaosa, T. Mori, A. Shiratsuchi, and Y. Nakanishi, "Integrin αPS3/βν-mediated phagocytosis of apoptotic cells and bacteria in *Drosophila*," (in eng), *J Biol Chem,* vol. 288, no. 15, pp. 10374-80, Apr 12 2013, doi: 10.1074/jbc.M113.451427.

[111] U. Shokal and I. Eleftherianos, "Evolution and Function of Thioester-Containing Proteins and the Complement System in the Innate Immune Response," (in eng), *Front Immunol,* vol. 8, p. 759, 2017, doi: 10.3389/fimmu.2017.00759.

[112] R. Bou Aoun, C. Hetru, L. Troxler, D. Doucet, D. Ferrandon, and N. Matt, "Analysis of thioester-containing proteins during the innate immune response of *Drosophila melanogaster*," *J Innate Immun,* vol. 3, no. 1, pp. 52-64, 2011, doi: 10.1159/000321554.

[113] J. Pompon and E. A. Levashina, "A New Role of the Mosquito Complement-like Cascade in Male Fertility in Anopheles gambiae," (in eng), *PLoS Biol,* vol. 13, no. 9, p. e1002255, 2015, doi: 10.1371/journal.pbio.1002255.

[114] T. Batz, D. Forster, and S. Luschnig, "The transmembrane protein Macroglobulin complement-related is essential for septate junction formation and epithelial barrier function in *Drosophila*," *Development,* vol. 141, no. 4, pp. 899-908, Feb 2014, doi: 10.1242/dev.102160.

[115] S. L. Stroschein-Stevenson, E. Foley, P. H. O'Farrell, and A. D. Johnson, "Identification of *Drosophila* gene products required for phagocytosis of Candida albicans," (in eng), *PLoS Biol,* vol. 4, no. 1, p. e4, Jan 2006, doi: 10.1371/journal.pbio.0040004.

[116] B. Arefin *et al.*, "Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins," (in eng), *J Innate Immun,* vol. 6, no. 2, pp. 192-204, 2014, doi: 10.1159/000353734.

[117] A. Dostalova, S. Rommelaere, M. Poidevin, and B. Lemaitre, "Thioester-containing proteins regulate the Toll pathway and play a role in *Drosophila* defence against microbial pathogens and parasitoid wasps," *BMC Biol,* vol. 15, no. 1, p. 79, Sep 5 2017, doi: 10.1186/s12915-017-0408-0.

[118] U. Shokal, H. Kopydlowski, S. Harsh, and I. Eleftherianos, "Thioester-Containing Proteins 2 and 4 Affect the Metabolic Activity and Inflammation Response in *Drosophila*," (in eng), *Infect Immun,* vol. 86, no. 5, May 2018, doi: 10.1128/iai.00810-17.

[119] U. Shokal, H. Kopydlowski, and I. Eleftherianos, "The distinct function of Tep2 and Tep6 in the

immune defense of *Drosophila melanogaster* against the pathogen Photorhabdus," *Virulence,* vol. 8, no. 8, pp. 1668-1682, Nov 17 2017, doi: 10.1080/21505594.2017.1330240.

[120] W. J. Lee and M. Miura, "Mechanisms of systemic wound response in *Drosophila*," (in eng), *Curr Top Dev Biol,* vol. 108, pp. 153-83, 2014, doi: 10.1016/b978-0-12-391498-9.00001-2.

[121] A. Takehana, T. Yano, S. Mita, A. Kotani, Y. Oshima, and S. Kurata, "Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity," (in eng), *Embo j,* vol. 23, no. 23, pp. 4690-700, Nov 24 2004, doi: 10.1038/sj.emboj.7600466.

[122] R. L. Schmidt, T. R. Trejo, T. B. Plummer, J. L. Platt, and A. H. Tang, "Infection-induced proteolysis of PGRP-LC controls the IMD activation and melanization cascades in *Drosophila*," (in eng), *Faseb j,* vol. 22, no. 3, pp. 918-29, Mar 2008, doi: 10.1096/fj.06-7907com.

[123] A. A. Matskevich, J. Quintin, and D. Ferrandon, "The *Drosophila* PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function," (in eng), *Eur J Immunol,* vol. 40, no. 5, pp. 1244-54, May 2010, doi: 10.1002/eji.200940164.

[124] C. An, M. Zhang, Y. Chu, and Z. Zhao, "Serine protease MP2 activates prophenoloxidase in the melanization immune response of *Drosophila melanogaster*," (in eng), *PLoS One,* vol. 8, no. 11, p. e79533, 2013, doi: 10.1371/journal.pone.0079533.

[125] J. P. Dudzic, S. Kondo, R. Ueda, C. M. Bergman, and B. Lemaitre, "*Drosophila* innate immunity: regional and functional specialization of prophenoloxidases," (in eng), *BMC Biol,* vol. 13, p. 81, Oct 1 2015, doi: 10.1186/s12915-015-0193-6.

[126] O. Binggeli, C. Neyen, M. Poidevin, and B. Lemaitre, "Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*," (in eng), *PLoS Pathog,* vol. 10, no. 5, p. e1004067, May 2014, doi: 10.1371/journal.ppat.1004067.

[127] S. Liegeois and D. Ferrandon, "Sensing microbial infections in the *Drosophila melanogaster* genetic model organism," (in eng), *Immunogenetics,* vol. 74, no. 1, pp. 35-62, Feb 2022, doi: 10.1007/s00251-021-01239-0.

[128] H. Tang, "Regulation and function of the melanization reaction in *Drosophila*," *Fly (Austin),* vol. 3, no. 1, pp. 105-11, Jan-Mar 2009, doi: 10.4161/fly.3.1.7747.

[129] F. Serras, "The sooner, the better: ROS, kinases and nutrients at the onset of the damage response in *Drosophila*," (in eng), *Front Cell Dev Biol,* vol. 10, p. 1047823, 2022, doi: 10.3389/fcell.2022.1047823.

[130] T. L. Leto and M. Geiszt, "Role of Nox family NADPH oxidases in host defense," (in eng), *Antioxid Redox Signal,* vol. 8, no. 9-10, pp. 1549-61, Sep-Oct 2006, doi: 10.1089/ars.2006.8.1549.

[131] E. M. Ha, C. T. Oh, Y. S. Bae, and W. J. Lee, "A direct role for dual oxidase in *Drosophila* gut immunity," (in eng), *Science,* vol. 310, no. 5749, pp. 847-50, Nov 4 2005, doi: 10.1126/science.1117311.

[132] E. M. Ha *et al.*, "Regulation of DUOX by the Galphaq-phospholipase Cbeta-Ca2+ pathway in *Drosophila* gut immunity," *Dev Cell,* vol. 16, no. 3, pp. 386-97, Mar 2009, doi: 10.1016/j.devcel.2008.12.015.

[133] K. A. Lee *et al.*, "Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in *Drosophila*," *Cell,* vol. 153, no. 4, pp. 797-811, May 9 2013, doi: 10.1016/j.cell.2013.04.009.

[134] R. M. Jones *et al.*, "Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-

mediated generation of reactive oxygen species," (in eng), *Embo j,* vol. 32, no. 23, pp. 3017-28, Nov 27 2013, doi: 10.1038/emboj.2013.224.

[135] I. Iatsenko, J. P. Boquete, and B. Lemaitre, "Microbiota-Derived Lactate Activates Production of Reactive Oxygen Species by the Intestinal NADPH Oxidase Nox and Shortens *Drosophila* Lifespan," (in eng), *Immunity,* vol. 49, no. 5, pp. 929-942.e5, Nov 20 2018, doi: 10.1016/j.immuni.2018.09.017.

[136] S. Noselli, "JNK signaling and morphogenesis in *Drosophila*," (in eng), *Trends Genet,* vol. 14, no. 1, pp. 33-8, Jan 1998, doi: 10.1016/s0168-9525(97)01320-6.

[137] S. Noselli and F. Agnès, "Roles of the JNK signaling pathway in *Drosophila* morphogenesis," (in eng), *Curr Opin Genet Dev,* vol. 9, no. 4, pp. 466-72, Aug 1999, doi: 10.1016/s0959-437x(99)80071-9.

[138] L. D. Rios-Barrera and J. R. Riesgo-Escovar, "Regulating cell morphogenesis: the *Drosophila* Jun N-terminal kinase pathway," *Genesis,* vol. 51, no. 3, pp. 147-62, Mar 2013, doi: 10.1002/dvg.22354.

[139] B. Biteau, J. Karpac, D. Hwangbo, and H. Jasper, "Regulation of *Drosophila* lifespan by JNK signaling," *Exp Gerontol,* vol. 46, no. 5, pp. 349-54, May 2011, doi: 10.1016/j.exger.2010.11.003.

[140] B. E. Stronach and N. Perrimon, "Stress signaling in *Drosophila*," (in eng), *Oncogene,* vol. 18, no. 45, pp. 6172-82, Nov 1 1999, doi: 10.1038/sj.onc.1203125.

[141] E. Kuranaga and M. Miura, "Molecular genetic control of caspases and JNK-mediated neural cell death," (in eng), *Arch Histol Cytol,* vol. 65, no. 4, pp. 291-300, Oct 2002, doi: 10.1679/aohc.65.291.

[142] O. P. Ryabinina, E. Subbian, and M. S. Iordanov, "D-MEKK1, the *Drosophila* orthologue of mammalian MEKK4/MTK1, and Hemipterous/D-MKK7 mediate the activation of D-JNK by cadmium and arsenite in Schneider cells," *BMC Cell Biol,* vol. 7, p. 7, Feb 1 2006, doi: 10.1186/1471-2121-7-7.

[143] B. Glise, H. Bourbon, and S. Noselli, "hemipterous encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement," (in eng), *Cell,* vol. 83, no. 3, pp. 451-61, Nov 3 1995, doi: 10.1016/0092-8674(95)90123-x.

[144] P. Sathyanarayana *et al.*, "*Drosophila* mixed lineage kinase/slipper, a missing biochemical link in *Drosophila* JNK signaling," *Biochim Biophys Acta,* vol. 1640, no. 1, pp. 77-84, Apr 7 2003, doi: 10.1016/s0167-4889(03)00022-3.

[145] M. C. Wang, D. Bohmann, and H. Jasper, "JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling," *Cell,* vol. 121, no. 1, pp. 115-25, Apr 8 2005, doi: 10.1016/j.cell.2005.02.030.

[146] G. P. Copenhaver *et al.*, "ROS-Induced JNK and p38 Signaling Is Required for Unpaired Cytokine Activation during *Drosophila* Regeneration," *PLOS Genetics,* vol. 11, no. 10, 2015, doi: 10.1371/journal.pgen.1005595.

[147] E. Martín-Blanco *et al.*, "puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*," (in eng), *Genes Dev,* vol. 12, no. 4, pp. 557-70, Feb 15 1998, doi: 10.1101/gad.12.4.557.

[148] C. de Bekker, W. C. Beckerson, and C. Elya, "Mechanisms behind the Madness: How Do Zombie-Making Fungal Entomopathogens Affect Host Behavior To Increase Transmission?," (in eng), *mBio,* vol. 12, no. 5, p. e0187221, Oct 26 2021, doi: 10.1128/mBio.01872-21.

[149] H. L. Lu and R. J. St Leger, "Insect Immunity to Entomopathogenic Fungi," (in eng), *Adv Genet,* vol. 94, pp. 251-85, 2016, doi: 10.1016/bs.adgen.2015.11.002.

[150] C. Wang and R. J. St Leger, "A collagenous protective coat enables Metarhizium anisopliae to evade insect immune responses," (in eng), *Proc Natl Acad Sci U S A,* vol. 103, no. 17, pp. 6647-52, Apr 25 2006, doi: 10.1073/pnas.0601951103.

[151] X. Hu *et al.*, "Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation," (in eng), *Proc Natl Acad Sci U S A,* vol. 111, no. 47, pp. 16796-801, Nov 25 2014, doi: 10.1073/pnas.1412662111.

[152] P. Feng, Y. Shang, K. Cen, and C. Wang, "Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity," (in eng), *Proc Natl Acad Sci U S A,* vol. 112, no. 36, pp. 11365-70, Sep 8 2015, doi: 10.1073/pnas.1503200112.

[153] Q. Gao *et al.*, "Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi Metarhizium anisopliae and M. acridum," (in eng), *PLoS Genet,* vol. 7, no. 1, p. e1001264, Jan 6 2011, doi: 10.1371/journal.pgen.1001264.

[154] M. S. Pedras, L. Irina Zaharia, and D. E. Ward, "The destruxins: synthesis, biosynthesis, biotransformation, and biological activity," (in eng), *Phytochemistry,* vol. 59, no. 6, pp. 579-96, Mar 2002, doi: 10.1016/s0031-9422(02)00016-x.

[155] M. Hinaje, M. Ford, L. Banting, S. Arkle, and B. Khambay, "An investigation of the ionophoric characteristics of destruxin A," (in eng), *Arch Biochem Biophys,* vol. 405, no. 1, pp. 73-77, Sep 1 2002, doi: 10.1016/s0003-9861(02)00275-8.

[156] P. S. Gôlo, C. Angelo Ida, M. G. Camargo, W. M. Perinotto, and V. R. Bittencourt, "Effects of destruxin A on Rhipicephalus (Boophilus) microplus ticks (Acari: Ixodidae)," (in eng), *Rev Bras Parasitol Vet,* vol. 20, no. 4, pp. 338-41, Oct-Dec 2011, doi: 10.1590/s1984-29612011000400015.

[157] P. Götz, V. Matha, and A. Vilcinskas, "Effects of the entomopathogenic fungus Metarhizium anisopliae and its secondary metabolites on morphology and cytoskeleton of plasmatocytes isolated from the greater wax moth, Galleria mellonella," (in eng), *J Insect Physiol,* vol. 43, no. 12, pp. 1149-1159, Nov 1997, doi: 10.1016/s0022-1910(97)00066-8.

[158] C. Dumas, V. Matha, J. M. Quiot, and A. Vey, "Effects of destruxins, cyclic depsipeptide mycotoxins, on calcium balance and phosphorylation of intracellular proteins in lepidopteran cell lines," (in eng), *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol,* vol. 114, no. 3, pp. 213-9, Jul 1996, doi: 10.1016/0742-8413(96)00041-2.

[159] B. Wang, Q. Kang, Y. Lu, L. Bai, and C. Wang, "Unveiling the biosynthetic puzzle of destruxins in Metarhizium species," (in eng), *Proc Natl Acad Sci U S A,* vol. 109, no. 4, pp. 1287-92, Jan 24 2012, doi: 10.1073/pnas.1115983109.

[160] C. Wang and S. Wang, "Insect Pathogenic Fungi: Genomics, Molecular Interactions, and Genetic Improvements," (in eng), *Annu Rev Entomol,* vol. 62, pp. 73-90, Jan 31 2017, doi: 10.1146/annurev-ento-031616-035509.

[161] P. A. Grimont and F. Grimont, "The genus Serratia," (in eng), *Annu Rev Microbiol,* vol. 32, pp. 221-48, 1978, doi: 10.1146/annurev.mi.32.100178.001253.

[162] E. Iosifidis *et al.*, "Outbreak of bloodstream infections because of Serratia marcescens in a pediatric department," *Am J Infect Control,* vol. 40, no. 1, pp. 11-5, Feb 2012, doi: 10.1016/j.ajic.2011.03.020.

[163] H. C. Maltezou *et al.*, "Consecutive Serratia marcescens multiclone outbreaks in a neonatal

intensive care unit," *Am J Infect Control,* vol. 40, no. 7, pp. 637-42, Sep 2012, doi: 10.1016/j.ajic.2011.08.019.

[164] R. Marre, J. Hacker, and V. Braun, "The cell-bound hemolysin of Serratia marcescens contributes to uropathogenicity," (in eng), *Microb Pathog,* vol. 7, no. 2, pp. 153-6, Aug 1989, doi: 10.1016/0882-4010(89)90034-x.

[165] M. J. Albers, J. W. Mouton, and D. Tibboel, "Colonization and infection by Serratia species in a paediatric surgical intensive care unit," *J Hosp Infect,* vol. 48, no. 1, pp. 7-12, May 2001, doi: 10.1053/jhin.2001.0939.

[166] C. L. Kurz *et al.*, "Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening," (in eng), *Embo j,* vol. 22, no. 7, pp. 1451-60, Apr 1 2003, doi: 10.1093/emboj/cdg159.

[167] S. J. Cronin *et al.*, "Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection," (in eng), *Science,* vol. 325, no. 5938, pp. 340-3, Jul 17 2009, doi: 10.1126/science.1173164.

[168] N. T. Nehme *et al.*, "A model of bacterial intestinal infections in *Drosophila melanogaster*," (in eng), *PLoS Pathog,* vol. 3, no. 11, p. e173, Nov 2007, doi: 10.1371/journal.ppat.0030173.

[169] Y. Arakawa *et al.*, "Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds," (in eng), *J Clin Microbiol,* vol. 38, no. 1, pp. 40-3, Jan 2000, doi: 10.1128/jcm.38.1.40-43.2000.

[170] S. Knowles *et al.*, "An outbreak of multiply resistant Serratia marcescens: the importance of persistent carriage," (in eng), *Bone Marrow Transplant,* vol. 25, no. 8, pp. 873-7, Apr 2000, doi: 10.1038/sj.bmt.1702218.

[171] M. J. Kuehn and N. C. Kesty, "Bacterial outer membrane vesicles and the host-pathogen interaction," *Genes Dev,* vol. 19, no. 22, pp. 2645-55, Nov 15 2005, doi: 10.1101/gad.1299905.

[172] K. J. McMahon, M. E. Castelli, E. Garcia Vescovi, and M. F. Feldman, "Biogenesis of outer membrane vesicles in Serratia marcescens is thermoregulated and can be induced by activation of the Rcs phosphorelay system," *J Bacteriol,* vol. 194, no. 12, pp. 3241-9, Jun 2012, doi: 10.1128/JB.00016-12.

[173] A. M. Spagnolo, M. Sartini, and M. L. Cristina, "Pseudomonas aeruginosa in the healthcare facility setting," *Reviews in Medical Microbiology,* vol. 32, no. 3, pp. 169-175, 2021, doi: 10.1097/mrm.0000000000000271.

[174] S. Limmer, J. Quintin, C. Hetru, and D. Ferrandon, "Virulence on the fly: *Drosophila melanogaster* as a model genetic organism to decipher host-pathogen interactions," (in eng), *Curr Drug Targets,* vol. 12, no. 7, pp. 978-99, Jun 2011, doi: 10.2174/138945011795677818.

[175] S. H. Kim, S. Y. Park, Y. J. Heo, and Y. H. Cho, "*Drosophila melanogaster*-based screening for multihost virulence factors of Pseudomonas aeruginosa PA14 and identification of a virulence-attenuating factor, HudA," *Infect Immun,* vol. 76, no. 9, pp. 4152-62, Sep 2008, doi: 10.1128/IAI.01637-07.

[176] S. A. Lee *et al.*, "Insecticidal activity of the metalloprotease AprA occurs through suppression of host cellular and humoral immunity," *Dev Comp Immunol,* vol. 81, pp. 116-126, Apr 2018, doi: 10.1016/j.dci.2017.11.014.

[177] K. Koeppen, R. Barnaby, A. A. Jackson, S. A. Gerber, D. A. Hogan, and B. A. Stanton, "Tobramycin reduces key virulence determinants in the proteome of Pseudomonas aeruginosa outer membrane vesicles," *PLoS One,* vol. 14, no. 1, p. e0211290, 2019, doi:

10.1371/journal.pone.0211290.

[178] M. Kaparakis-Liaskos and R. L. Ferrero, "Immune modulation by bacterial outer membrane vesicles," *Nat Rev Immunol,* vol. 15, no. 6, pp. 375-87, Jun 2015, doi: 10.1038/nri3837.

[179] A. Kulp and M. J. Kuehn, "Biological functions and biogenesis of secreted bacterial outer membrane vesicles," *Annu Rev Microbiol,* vol. 64, pp. 163-84, 2010, doi: 10.1146/annurev.micro.091208.073413.

[180] S. R. Schooling and T. J. Beveridge, "Membrane vesicles: an overlooked component of the matrices of biofilms," *J Bacteriol,* vol. 188, no. 16, pp. 5945-57, Aug 2006, doi: 10.1128/JB.00257-06.

[181] C. Schwechheimer and M. J. Kuehn, "Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions," *Nat Rev Microbiol,* vol. 13, no. 10, pp. 605-19, Oct 2015, doi: 10.1038/nrmicro3525.

[182] B. L. Deatherage, J. C. Lara, T. Bergsbaken, S. L. Rassoulian Barrett, S. Lara, and B. T. Cookson, "Biogenesis of bacterial membrane vesicles," *Mol Microbiol,* vol. 72, no. 6, pp. 1395-407, Jun 2009, doi: 10.1111/j.1365-2958.2009.06731.x.

[183] T. Song *et al.*, "A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles," *Mol Microbiol,* vol. 70, no. 1, pp. 100-11, Oct 2008, doi: 10.1111/j.1365-2958.2008.06392.x.

[184] A. J. McBroom, A. P. Johnson, S. Vemulapalli, and M. J. Kuehn, "Outer membrane vesicle production by Escherichia coli is independent of membrane instability," *J Bacteriol,* vol. 188, no. 15, pp. 5385-92, Aug 2006, doi: 10.1128/JB.00498-06.

[185] A. J. McBroom and M. J. Kuehn, "Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response," *Mol Microbiol,* vol. 63, no. 2, pp. 545-58, Jan 2007, doi: 10.1111/j.1365-2958.2006.05522.x.

[186] N. C. Kesty, K. M. Mason, M. Reedy, S. E. Miller, and M. J. Kuehn, "Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells," *EMBO J,* vol. 23, no. 23, pp. 4538-49, Nov 24 2004, doi: 10.1038/sj.emboj.7600471.

[187] L. Mashburn-Warren, R. J. McLean, and M. Whiteley, "Gram-negative outer membrane vesicles: beyond the cell surface," *Geobiology,* vol. 6, no. 3, pp. 214-9, Jun 2008, doi: 10.1111/j.1472-4669.2008.00157.x.

[188] J. F. Dubern and S. P. Diggle, "Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species," *Mol Biosyst,* vol. 4, no. 9, pp. 882-8, Sep 2008, doi: 10.1039/b803796p.

[189] L. M. Mashburn and M. Whiteley, "Membrane vesicles traffic signals and facilitate group activities in a prokaryote," *Nature,* vol. 437, no. 7057, pp. 422-5, Sep 15 2005, doi: 10.1038/nature03925.

[190] S. J. Bauman and M. J. Kuehn, "Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response," *Microbes Infect,* vol. 8, no. 9-10, pp. 2400-8, Aug 2006, doi: 10.1016/j.micinf.2006.05.001.

[191] N. V. Vasilyeva *et al.*, "Lytic peptidase L5 of Lysobacter sp. XL1 with broad antimicrobial spectrum," *J Mol Microbiol Biotechnol,* vol. 24, no. 1, pp. 59-66, 2014, doi: 10.1159/000356838.

[192] J. L. Kadurugamuwa and T. J. Beveridge, "Bacteriolytic effect of membrane vesicles from Pseudomonas aeruginosa on other bacteria including pathogens: conceptually new antibiotics," (in eng), *J Bacteriol,* vol. 178, no. 10, pp. 2767-74, May 1996, doi: 10.1128/jb.178.10.2767-

2774.1996.

[193] H. Yonezawa *et al.*, "Outer membrane vesicles of Helicobacter pylori TK1402 are involved in biofilm formation," *BMC Microbiol,* vol. 9, p. 197, Sep 15 2009, doi: 10.1186/1471-2180-9-197.

[194] A. L. Horstman and M. J. Kuehn, "Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles," *J Biol Chem,* vol. 275, no. 17, pp. 12489-96, Apr 28 2000, doi: 10.1074/jbc.275.17.12489.

[195] F. Galka *et al.*, "Proteomic characterization of the whole secretome of Legionella pneumophila and functional analysis of outer membrane vesicles," *Infect Immun,* vol. 76, no. 5, pp. 1825-36, May 2008, doi: 10.1128/IAI.01396-07.

[196] R. D. Pathirana and M. Kaparakis-Liaskos, "Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis," *Cell Microbiol,* vol. 18, no. 11, pp. 1518-1524, Nov 2016, doi: 10.1111/cmi.12658.

[197] R. C. Laughlin, M. Mickum, K. Rowin, L. G. Adams, and R. C. Alaniz, "Altered host immune responses to membrane vesicles from Salmonella and Gram-negative pathogens," *Vaccine,* vol. 33, no. 38, pp. 5012-9, Sep 11 2015, doi: 10.1016/j.vaccine.2015.05.014.

[198] M. Kaparakis *et al.*, "Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells," *Cell Microbiol,* vol. 12, no. 3, pp. 372-85, Mar 2010, doi: 10.1111/j.1462-5822.2009.01404.x.

[199] S. Schild, E. J. Nelson, and A. Camilli, "Immunization with Vibrio cholerae outer membrane vesicles induces protective immunity in mice," *Infect Immun,* vol. 76, no. 10, pp. 4554-63, Oct 2008, doi: 10.1128/IAI.00532-08.

[200] V. Davenport *et al.*, "Mucosal immunity in healthy adults after parenteral vaccination with outer-membrane vesicles from Neisseria meningitidis serogroup B," *J Infect Dis,* vol. 198, no. 5, pp. 731-40, Sep 1 2008, doi: 10.1086/590669.

[201] T. N. Ellis and M. J. Kuehn, "Virulence and immunomodulatory roles of bacterial outer membrane vesicles," *Microbiol Mol Biol Rev,* vol. 74, no. 1, pp. 81-94, Mar 2010, doi: 10.1128/MMBR.00031-09.

[202] J. Winter, D. Letley, J. Rhead, J. Atherton, and K. Robinson, "Helicobacter pylori membrane vesicles stimulate innate pro- and anti-inflammatory responses and induce apoptosis in Jurkat T cells," *Infect Immun,* vol. 82, no. 4, pp. 1372-81, Apr 2014, doi: 10.1128/IAI.01443-13.

[203] K. Koeppen *et al.*, "A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles," *PLoS Pathog,* vol. 12, no. 6, p. e1005672, Jun 2016, doi: 10.1371/journal.ppat.1005672.

[204] D. G. Fisk *et al.*, "Saccharomyces cerevisiae S288C genome annotation: a working hypothesis," (in eng), *Yeast,* vol. 23, no. 12, pp. 857-65, Sep 2006, doi: 10.1002/yea.1400.

[205] A. Frankish *et al.*, "Gencode 2021," *Nucleic Acids Res,* vol. 49, no. D1, pp. D916-D923, Jan 8 2021, doi: 10.1093/nar/gkaa1087.

[206] K. C. Wang and H. Y. Chang, "Molecular mechanisms of long noncoding RNAs," *Mol Cell,* vol. 43, no. 6, pp. 904-14, Sep 16 2011, doi: 10.1016/j.molcel.2011.08.018.

[207] J. J. Quinn and H. Y. Chang, "Unique features of long non-coding RNA biogenesis and function," *Nat Rev Genet,* vol. 17, no. 1, pp. 47-62, Jan 2016, doi: 10.1038/nrg.2015.10.

[208] J. J. Quinn, Q. C. Zhang, P. Georgiev, I. A. Ilik, A. Akhtar, and H. Y. Chang, "Rapid evolutionary turnover underlies conserved lncRNA-genome interactions," *Genes Dev,* vol. 30, no. 2, pp. 191-

207, Jan 15 2016, doi: 10.1101/gad.272187.115.

[209] L. Statello, C. J. Guo, L. L. Chen, and M. Huarte, "Gene regulation by long non-coding RNAs and its biological functions," *Nat Rev Mol Cell Biol,* vol. 22, no. 2, pp. 96-118, Feb 2021, doi: 10.1038/s41580-020-00315-9.

[210] I. Ulitsky and D. P. Bartel, "lincRNAs: genomics, evolution, and mechanisms," *Cell,* vol. 154, no. 1, pp. 26-46, Jul 3 2013, doi: 10.1016/j.cell.2013.06.020.

[211] J. L. Rinn and H. Y. Chang, "Long Noncoding RNAs: Molecular Modalities to Organismal Functions," *Annu Rev Biochem,* vol. 89, pp. 283-308, Jun 20 2020, doi: 10.1146/annurev-biochem-062917-012708.

[212] J. L. Rinn and H. Y. Chang, "Genome regulation by long noncoding RNAs," *Annu Rev Biochem,* vol. 81, pp. 145-66, 2012, doi: 10.1146/annurev-biochem-051410-092902.

[213] J. Z. Huang *et al.*, "A Peptide Encoded by a Putative lncRNA HOXB-AS3 Suppresses Colon Cancer Growth," (in eng), *Mol Cell,* vol. 68, no. 1, pp. 171-184.e6, Oct 5 2017, doi: 10.1016/j.molcel.2017.09.015.

[214] M. F. Lin, I. Jungreis, and M. Kellis, "PhyloCSF: a comparative genomics method to distinguish protein coding and non-coding regions," *Bioinformatics,* vol. 27, no. 13, pp. i275-82, Jul 1 2011, doi: 10.1093/bioinformatics/btr209.

[215] M. K. Atianand, D. R. Caffrey, and K. A. Fitzgerald, "Immunobiology of Long Noncoding RNAs," *Annu Rev Immunol,* vol. 35, pp. 177-198, Apr 26 2017, doi: 10.1146/annurev-immunol-041015-055459.

[216] V. E. Villegas and P. G. Zaphiropoulos, "Neighboring gene regulation by antisense long non-coding RNAs," (in eng), *Int J Mol Sci,* vol. 16, no. 2, pp. 3251-66, Feb 3 2015, doi: 10.3390/ijms16023251.

[217] F. C. Beckedorff *et al.*, "The intronic long noncoding RNA ANRASSF1 recruits PRC2 to the RASSF1A promoter, reducing the expression of RASSF1A and increasing cell proliferation," (in eng), *PLoS Genet,* vol. 9, no. 8, p. e1003705, 2013, doi: 10.1371/journal.pgen.1003705.

[218] T. W. Sappington and A. S. Raikhel, "Molecular characteristics of insect vitellogenins and vitellogenin receptors," (in eng), *Insect Biochem Mol Biol,* vol. 28, no. 5-6, pp. 277-300, May-Jun 1998, doi: 10.1016/s0965-1748(97)00110-0.

[219] M. D. Brennan, A. J. Weiner, T. J. Goralski, and A. P. Mahowald, "The follicle cells are a major site of vitellogenin synthesis in *Drosophila melanogaster*," (in eng), *Dev Biol,* vol. 89, no. 1, pp. 225-36, Jan 1982, doi: 10.1016/0012-1606(82)90309-8.

[220] M. Bownes, K. Lineruth, and D. Mauchline, "Egg production and fertility in *Drosophila* depend upon the number of yolk-protein gene copies," (in eng), *Mol Gen Genet,* vol. 228, no. 1-2, pp. 324-7, Aug 1991, doi: 10.1007/bf00282485.

[221] C. L. Kurz, B. Charroux, D. Chaduli, A. Viallat-Lieutaud, and J. Royet, "Peptidoglycan sensing by octopaminergic neurons modulates *Drosophila* oviposition," *Elife,* vol. 6, Mar 7 2017, doi: 10.7554/eLife.21937.

[222] J. K. Herren, J. C. Paredes, F. Schüpfer, and B. Lemaitre, "Vertical transmission of a *Drosophila* endosymbiont via cooption of the yolk transport and internalization machinery," (in eng), *mBio,* vol. 4, no. 2, Mar 5 2013, doi: 10.1128/mBio.00532-12.

[223] N. T. Ingolia, J. A. Hussmann, and J. S. Weissman, "Ribosome Profiling: Global Views of Translation," (in eng), *Cold Spring Harb Perspect Biol,* vol. 11, no. 5, May 1 2019, doi: 10.1101/cshperspect.a032698.

[224] R. Vitorino, S. Guedes, F. Amado, M. Santos, and N. Akimitsu, "The role of micropeptides in biology," (in eng), *Cell Mol Life Sci,* vol. 78, no. 7, pp. 3285-3298, Apr 2021, doi: 10.1007/s00018-020-03740-3.

[225] S. Valanne, T. S. Salminen, M. Jarvela-Stolting, L. Vesala, and M. Ramet, "Immune-inducible non-coding RNA molecule lincRNA-IBIN connects immunity and metabolism in *Drosophila melanogaster*," *PLoS Pathog,* vol. 15, no. 1, p. e1007504, Jan 2019, doi: 10.1371/journal.ppat.1007504.

[226] B. Bogard, C. Francastel, and F. Hubé, "Multiple information carried by RNAs: total eclipse or a light at the end of the tunnel?," (in eng), *RNA Biol,* vol. 17, no. 12, pp. 1707-1720, Dec 2020, doi: 10.1080/15476286.2020.1783868.

[227] J. Li *et al.*, "LncTar: a tool for predicting the RNA targets of long noncoding RNAs," (in eng), *Brief Bioinform,* vol. 16, no. 5, pp. 806-12, Sep 2015, doi: 10.1093/bib/bbu048.

[228] J. H. Im, "Functional and population genetics of *Drosophila* innate immunity," The Graduate School of Cornell University, 2018, May.

[229] A. O. Brown *et al.*, "Antifungal Activity of the Enterococcus faecalis Peptide EntV Requires Protease Cleavage and Disulfide Bond Formation," (in eng), *mBio,* vol. 10, no. 4, Jul 2 2019, doi: 10.1128/mBio.01334-19.

[230] R. I. S. S. E. R. a. A. K. Charnley, "Calcium channel activation of insect muscle by Destruxins, insecticidal compounds produced by the entomopathogenic fungus Metarhizium anisopliae," *Camp.E iochemP. hysiol,* vol. V ol. 9OC,N o. 2, pp. 403-412,1 988, 1988.

[231] S. Pal, R. J. St Leger, and L. P. Wu, "Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*," (in eng), *J Biol Chem,* vol. 282, no. 12, pp. 8969-77, Mar 23 2007, doi: 10.1074/jbc.M605927200.

[232] S. R. Shanbhag, A. T. Vazhappilly, A. Sane, N. M. D'Silva, and S. Tripathi, "Electrolyte transport pathways induced in the midgut epithelium of *Drosophila melanogaster* larvae by commensal gut microbiota and pathogens," *J Physiol,* vol. 595, no. 2, pp. 523-539, Jan 15 2017, doi: 10.1113/JP272617.

[233] U. Shokal and I. Eleftherianos, "The *Drosophila* Thioester containing Protein-4 participates in the induction of the cellular immune response to the pathogen Photorhabdus," (in eng), *Dev Comp Immunol,* vol. 76, pp. 200-208, Nov 2017, doi: 10.1016/j.dci.2017.06.008.

[234] S. Haller *et al.*, "Quorum-sensing regulator RhlR but not its autoinducer RhlI enables Pseudomonas to evade opsonization," *EMBO Rep,* vol. 19, no. 5, May 2018, doi: 10.15252/embr.201744880.

[235] N. T. Nehme *et al.*, "Relative roles of the cellular and humoral responses in the *Drosophila* host defense against three gram-positive bacterial infections," (in eng), *PLoS One,* vol. 6, no. 3, p. e14743, Mar 3 2011, doi: 10.1371/journal.pone.0014743.

[236] S. T. Thibault *et al.*, "A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac," (in eng), *Nat Genet,* vol. 36, no. 3, pp. 283-7, Mar 2004, doi: 10.1038/ng1314.

[237] P. Schuck, "Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling," (in eng), *Biophys J,* vol. 78, no. 3, pp. 1606-19, Mar 2000, doi: 10.1016/s0006-3495(00)76713-0.

[238] C. A. Brautigam, "Calculations and Publication-Quality Illustrations for Analytical Ultracentrifugation Data," (in eng), *Methods Enzymol,* vol. 562, pp. 109-33, 2015, doi: 10.1016/bs.mie.2015.05.001.

[239] M. Schiaffini *et al.*, "A NYN domain protein directly interacts with DECAPPING1 and is required for phyllotactic pattern," (in eng), *Plant Physiol,* vol. 188, no. 2, pp. 1174-1188, Feb 4 2022, doi: 10.1093/plphys/kiab529.

[240] D. Bouyssié *et al.*, "Proline: an efficient and user-friendly software suite for large-scale proteomics," (in eng), *Bioinformatics,* vol. 36, no. 10, pp. 3148-3155, May 1 2020, doi: 10.1093/bioinformatics/btaa118.

[241] L. Kuhn, T. Vincent, P. Hammann, and H. Zuber, "Exploring Protein Interactome Data with IPinquiry: Statistical Analysis and Data Visualization by Spectral Counts," (in eng), *Methods Mol Biol,* vol. 2426, pp. 243-265, 2023, doi: 10.1007/978-1-0716-1967-4_11.

[242] J. Kim *et al.*, "Tumor-induced disruption of the blood-brain barrier promotes host death," (in eng), *Dev Cell,* vol. 56, no. 19, pp. 2712-2721.e4, Oct 11 2021, doi: 10.1016/j.devcel.2021.08.010.

[243] M. R. Cruz *et al.*, "Structural and functional analysis of EntV reveals a 12 amino acid fragment protective against fungal infections," (in eng), *Nat Commun,* vol. 13, no. 1, p. 6047, Oct 13 2022, doi: 10.1038/s41467-022-33613-1.

[244] M. S. Fu *et al.*, "Amoeba Predation of Cryptococcus neoformans Results in Pleiotropic Changes to Traits Associated with Virulence," (in eng), *mBio,* vol. 12, no. 2, Apr 27 2021, doi: 10.1128/mBio.00567-21.

[245] F. C. Los, T. M. Randis, R. V. Aroian, and A. J. Ratner, "Role of pore-forming toxins in bacterial infectious diseases," (in eng), *Microbiol Mol Biol Rev,* vol. 77, no. 2, pp. 173-207, Jun 2013, doi: 10.1128/mmbr.00052-12.

[246] X. Yan *et al.*, "The transcriptional landscape of plant infection by the rice blast fungus Magnaporthe oryzae reveals distinct families of temporally co-regulated and structurally conserved effectors," (in eng), *Plant Cell,* vol. 35, no. 5, pp. 1360-1385, Apr 20 2023, doi: 10.1093/plcell/koad036.

[247] B. Tang *et al.*, "Rgs1 is a regulator of effector gene expression during plant infection by the rice blast fungus Magnaporthe oryzae," (in eng), *Proc Natl Acad Sci U S A,* vol. 120, no. 12, p. e2301358120, Mar 21 2023, doi: 10.1073/pnas.2301358120.

[248] N. Raffa and N. P. Keller, "A call to arms: Mustering secondary metabolites for success and survival of an opportunistic pathogen," (in eng), *PLoS Pathog,* vol. 15, no. 4, p. e1007606, Apr 2019, doi: 10.1371/journal.ppat.1007606.

[249] K. Z. Lee *et al.*, "Enterocyte Purge and Rapid Recovery Is a Resilience Reaction of the Gut Epithelium to Pore-Forming Toxin Attack," (in eng), *Cell Host Microbe,* vol. 20, no. 6, pp. 716-730, Dec 14 2016, doi: 10.1016/j.chom.2016.10.010.

[250] W. Wang, "Host-pathogen interactions between *Drosophila melanogaster* and Metarhizium robertsii in two infection models," Université de Strasbourg and Guangzhou Medical University, 2020.

[251] X. Zhang *et al.*, "Antagonistic fungal enterotoxins intersect at multiple levels with host innate immune defences," (in eng), *PLoS Genet,* vol. 17, no. 6, p. e1009600, Jun 2021, doi: 10.1371/journal.pgen.1009600.

Annex I

Serratia marcescens Outer Membrane Vesicles are pathogenic to *Drosophila melanogaster* through paralysis resulting from the host-mediated induction of apoptosis in the nervous system

Foreword/Contributions

This project has been started more than ten years ago from the collaboration between our team and the microbiology laboratory led by Prof. GARCIA-VESCOVI in Rosario Argentina. The OMV project was initiated by Dr. Roberto BRUNA when he was a summer intern in the Strasbourg laboratory and then pursued when he came the following year for a longer 6-month stay. He had been working in collaboration with Dr. Bechara SINA RAHME who pursued the project, with the help of other interns from Argentina. Next, Dr. Marion DRAHEIM in our team at SFHI took over the project and developed it further. I myself finally took over and accomplished some parts of the project which include the study of *eiger*, the link between apoptosis and ROS, and the host response to the purified PrtA. I have also taken over the data reorganization from many contributors to this project and preparation of figures of the present manuscript, for which I wrote a first draft. Of note, many of the data have been generated independently both in Strasbourg and Guangzhou, which provides a higher degree of confidence in our results.

This manuscript, in a first version, provides the background knowledge required to fully understand my doctoral work (Chapter II).

Introduction

Serratia marcescens, a Gram-negative bacterium, belonging to Enterobacteriaceae, is found in diverse environments such as soil, water and air. *S. marcescens* has the capacity to infect plants, insects, and humans [1]. As an opportunistic pathogen in humans, *S. marcescens* mainly causes nosocomial infections and is able to infect several human tissues such as the urinary [2], respiratory epithelia or the digestive tract [3]. It can be critical for immune-deficient patients. One of the biggest problems in terms of health care is the ability of *S. marcescens* to resist to different antibiotics. Generally, the pathogenicity of *S. marcescens* competing bacteria or to the host cells, is mainly mediated by the quorum sensing, the secretion of several virulence factors, phospholipase, DNase, metalloprotease PrtA / Serralysin A…, and the formation of outer membrane vesicles.

Extracellular vesicles are produced and secreted by most living cells. In bacteria, they act as cargo for delivering virulence factors or are involved in cell-cell communication. For Gram-negative bacteria, these vesicles originate by a mechanism involving the pinching off of the outer membrane hence their name, outer membrane vesicles (OMVs). Besides incorporating outer membrane associated proteins OMVs may contain enzymes and virulence factors secreted in the periplasmic space through the cytoplasmic membrane, DNA and RNA as well as peptidoglycan fragments. Their secretion helps bacteria to communicate with each other and mediate some of their interactions with the host [4].. It has been demonstrated that OMVs participate in virulence by increasing bacteria communication and biofilm formation. In addition, compared to releasing the cargo in the supernatant directly, OMVs provide a beneficial option for pathogens. OMVs can protect virulence factors from host proteases, concentrate them for delivering to host cell, and contribute to a long-distance delivery. A study of McMahon et al., reported that *S. marcescens RM66262* (isolated from Human urinary tract infection [3]) produced OMVs in a thermoregulated manner. Indeed, a decrease of the temperature (from 37 to 30 degres) increased OMVs

production without affecting virulence since *G. mellonella* larvae succumbed at the same rate to OMVs from both temperatures [5]. A mass spectrometry analysis showed that virulence factors such as Serralysin A (PrtA) are present in OMV and not in the outer membrane compartment.

S. marcescens is well-studied in two *Drosophila* infection models: include a septic injury and oral infection. Among different *S. marcescens* strains, the *Drosophila* bacterium (DB strain) Db11 has been isolated from flies after death and is the most common strain used in *Drosophila* studies. It was found that a few bacteria are sufficient to kill within 24h flies by bacteremia [6]. It is reported that constantly feeding the flies with a bacteria-containing sucrose solution resulted in the bacteria crossing the gut to the hemocoel and in the body cavity, which is found to be controlled by phagocytosis. Notably, the bacteria trigger an *IMD*-dependent local immune response in parts of the gut epithelium but not a systemic immune response, even though the bacteria were found in the hemolymph, although in low numbers.

Since *Drosophila melanogaster* appears to be an ideal model for studying the interaction between host and pathogens, here we decided to investigate the pathogenesis of OMVs from *S. marcescens* in the *Drosophila melanogaster*.

Results

The OMVs from *Serratia marcescens* are virulent to *Drosophila*

We have injected wild-type flies with different concentrations of OMVs ranging from 0.01 to 0.1 ng/nL (Fig. 1A) and observed the demise of injected flies within hours, in a dose-dependent manner. The injection of 0.1 ng/nL OMVs led to most of the flies apparently succumbing in two hours, and lower concentrations led to a slower lethality until 0.01 ng/nL, a concentration for which OMVs exhibited no virulence. Here, we usually used a 0.1 ng/nL concentration for most experiments, although as there were batch to batch variations, the concentration had to be optimized for each preparation, and also according to the length of storage at -80°C.

Upon more careful scrutiny, we noticed that flies that had fallen to the bottom of vials were not dead but paralyzed (Fig.1B/Movie 1). Indeed, we observed pulsations of the dorsal vessel (Fig. 1C/Movie 2). As the flies never recovered, OMVs ultimately kill the flies.

In conclusion, we have shown that injected OMVs from *S. marcescens* display a high virulence to flies and lead to a neurological phenotype.

The IMD but not the Toll pathway is required for the host defense against OMVs

The *Drosophila* systemic humoral immune response is highly effective to fight off microorganisms, except for highly pathogenic strains such as *Serratia marcescens* [6]. The IMD and Toll pathways mediate the humoral immune response but their roles in the host defense against OMVs remains to be established. Here, we have monitored the survival rate of two mutans strains affecting the IMD pathway, *kenny* and *imd*, and found that these mutants are more sensitive to the injection of OMVs (Fig. 2A, Fig. S1A). In keeping with this function, the expression of the IMD-dependent *Diptericin* gene was induced already by one-hour post-injection (Fig. 2B). The situation for the Toll pathway appeared more complex. First, we observed a detectable induction of *Drosomycin*, used here as a Toll pathway activation read-out, only at a six-hour time

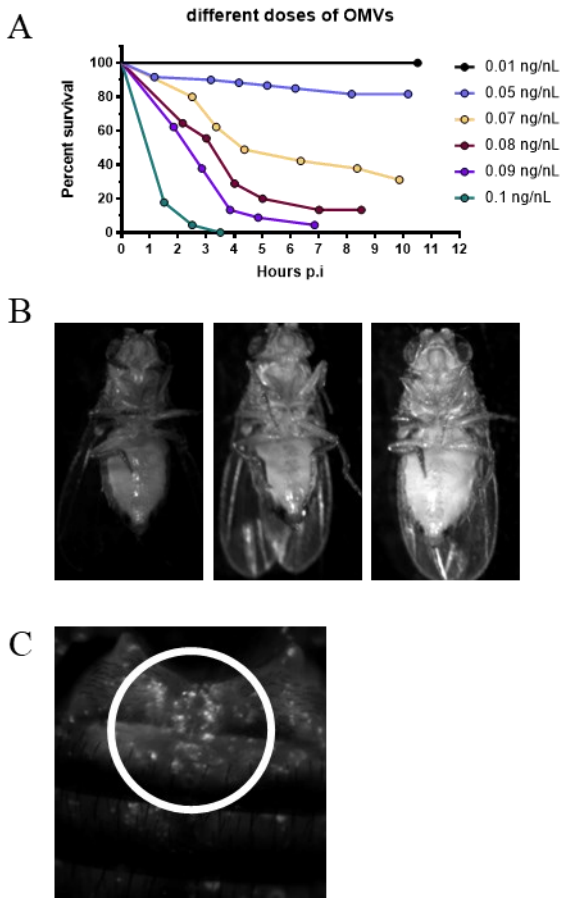

Figure 1. (A) La survie des mouches de type sauvage à différentes concentrations d'OMVs. La concentration d'OMVs a été déterminée par le kit BCA. **(B)** Vidéo montrant les mouches paralysées après l'injection de PMVs. **(C) La** vidéo montre le cœur qui bat dans la mouche même si la mouche s'arrête de bouger.

point, that is when flies have already been paralyzed (Fig. 2C). Next, the "survival" of mutants affecting distinct genes of the Toll pathway yielded inconsistent results. Most mutants, *MyD88*, *Toll, spätzle, GNBP3*^{hades} and *Persephone-GNBP3*^{hades} displayed a relatively late, mildly enhanced resistance to injected OMVs, *grass* also showing such a trend (Fig. S1B-G). *tube* and *pelle* "died" as controls whereas unexpectedly *Spätzle-processing enzyme* (*SPE*) mutants appeared to be more susceptible (Fig. S1H-J). That *GNBP3* mutants exhibit a phenotype was unexpected as it is thought to encode a sensor for ß-(1−3)-glucans, which are produced by fungi and usually not by bacteria. However, there are reports of such compounds being made by bacteria and for instance cyclic ß-(1-3)-glucans in the periplasm allow adaptation to osmotic conditions [7]. Thus, it is an open possibility that OMVs may carry such compounds that would be sensed by the

GNBP3 sensor. The opposite phenotypes displayed by *SPE* as compared to most Toll pathway mutants might suggest a noncanonical activation of the Toll pathway that deserves more in-depth studies.

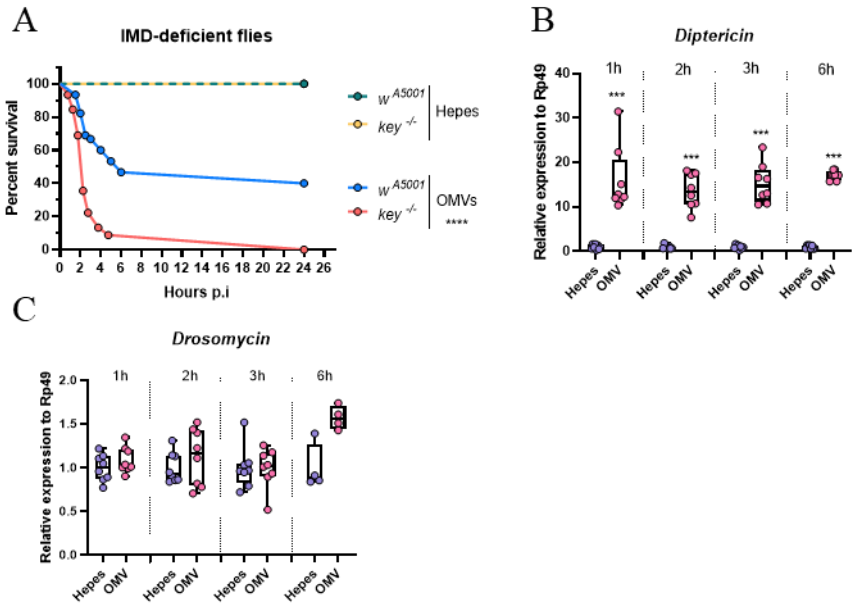

Figure 2. La réponse humorale et les OMV. (A) Le mutant de la voie IMD (*kenny*) a été injecté avec des OMV de *Serratia marcescens*. Le log-rank a été utilisé pour les statistiques. **(B)** Le niveau d'expression transcriptionnelle de la *diptéricine* à différents moments après l'injection d'OMVs par rapport au contrôle hepes. **(C)** Le niveau d'expression transcriptionnelle de la *Drosomycine* à différents moments après l'injection des OMVs par rapport au contrôle de l'hepes.

Phagocytosis is required for the pathogenesis of OMVs

We next asked whether the other arms of the innate immune response might be involved in the host defense against OMVs and first assessed the cellular immune response using three independent approaches.

First, we generated “hemoless” flies by inducing apoptosis in *hemolectin* expressing cells, which removes many but not all hemocytes [8, 9]. Unexpectedly, these flies appeared to be more resistant than control flies to OMV challenge (Fig. 3A). Next, we saturated the phagocytic apparatus by the prior injection of nondegradable “latex” beads and observed a similar, albeit somewhat weaker, phenotype (Fig. 3B). Finally, we tested two null mutants for *eater*, which encodes a prospective phagocytosis receptor that also plays a role in the adhesion of hemocytes to tissues [10, 11]. Again, a

lesser susceptibility to injected OMVs was observed. Taken together, these observations suggest that surprisingly the cellular immune response promotes the pathogenicity of injected OMVs (Fig. 3C).

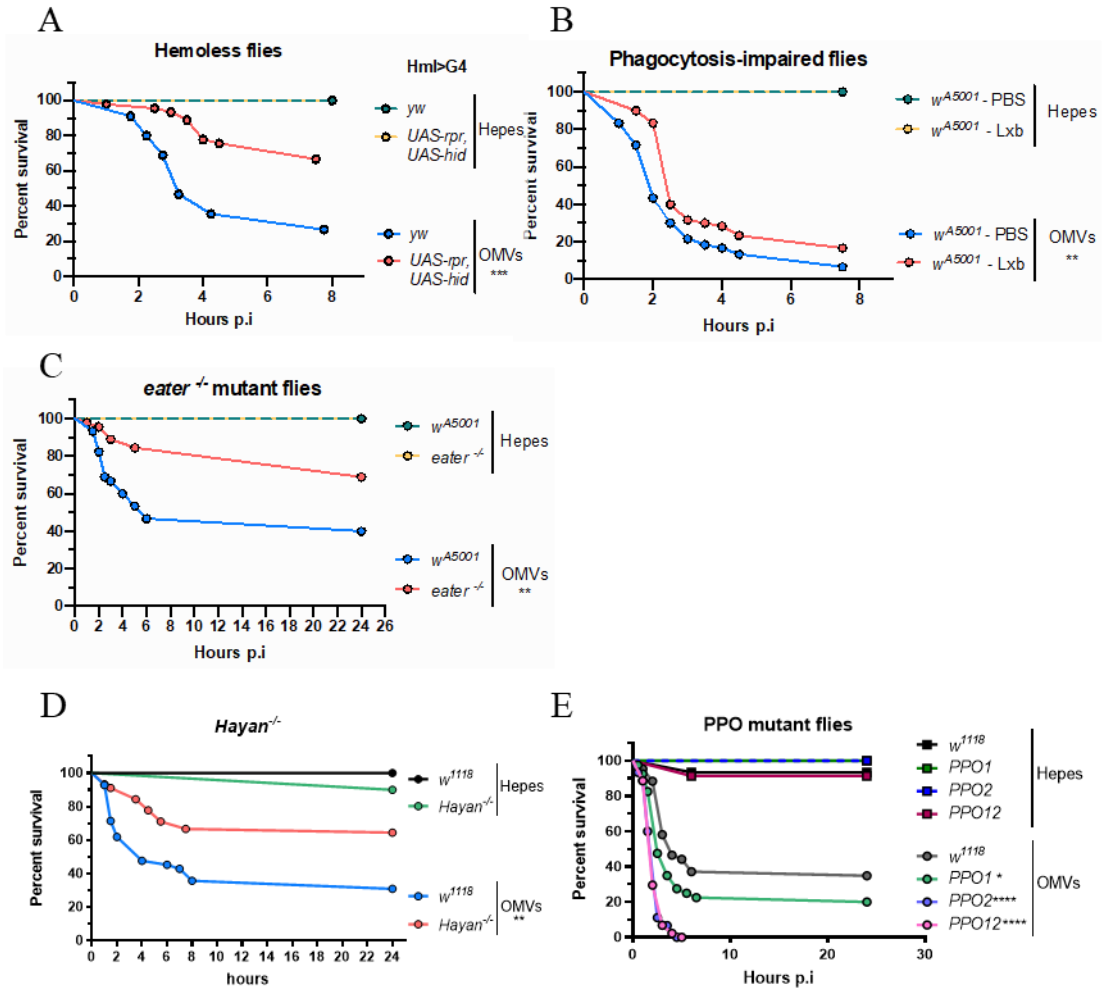

Figure 3. Réponse cellulaire et mélanisation après traitement aux OMVs. (A) hml-GAL4>UAS-rpr,hid représente des mouches sans hémoglobine. Test de survie comparant les mouches sans hémoglobine aux mouches de contrôle. **(B)** Lxb : billes de latex. Des mouches de type sauvage ont été infectées par des OMVs 24 heures après l'injection de Lxb. Test de survie comparant les mouches avec et sans injection de Lxb. **(C) Le** taux de survie des mutants *mangeurs* a été comparé à celui des mouches témoins. **(D)** Test de survie comparant les mutants *Hayan* et les mouches de type sauvage lors de l'infection par OMVs. **(E) Les** mutants simples *PPO1*, *PPO2* et les doubles mutants *PPO1,2* ont été injectés avec des OMVs par rapport au contrôle de type sauvage.

Hayan contributes to the pathogenesis of OMVs while PPOs are required in the host defense against OMVs

Next, we tested melanization, which is catalytically mediated by activated phenol

oxidases that lead to the formation of melanin and also to a microbicidal activity [12]. It has been reported that a serine protease named Hayan, which gets activated by the proteolytic cleavage of its N-terminal CLIP domain, is required for the cleavage of PPO to PO in *Drosophila* [13]. In the model of trauma developed by Nam et al., the cleavage of PPO into PO by Hayan during the melanization process led to a PO-dependent production of ROS and triggered a c-Jun N terminal kinase (JNK)-dependent stress response in some neurons, which resulted in a cytoprotective response against a large wound/ trauma [13]. We therefore monitored the survival rate of *Hayan* and *PPOs* deficient mutants upon OMVs infection. The loss of *Hayan* resulted in a protection against OMVs (Fig. 3D, Fig. S2A-B). Surprisingly, *PPO1* and/or *PPO2* mutants were more susceptible to OMVs (Fig. 3E).

To our knowledge, it is the first time that opposite phenotypes are reported for *Hayan* and *PPO1/PPO2* mutants. In the Nam *et al.* model, it had been proposed that ROS are generated by POs, which is clearly not the case here as POs participate in the defense against OMVs whereas Hayan promotes their pathogenicity.

ROS mediate the pathogenesis of OMVs

Because the data obtained on mutants affecting melanization yielded data that are not easily interpretable at this stage, we directly assessed whether ROS may play a role in the response to injected OMVs. We determined that there is an induction of H2O2 already one hour after the injection of OMVs as compared to the injection of buffer (Fig. 4A). We next co-injected antioxidants (N-acetyl cysteine, DTT, vitaminC) alongside OMVs. In the case of vitamin C, we added an additional control by pre-incubating OMVs with vitamin C and removing the antioxidant by filtration using Amicon 10K filters. As shown in Fig. 4B, the co-exposure to vitamin C totally protected the flies from the action of OMVs whereas the prior exposure of OMVs to the antioxidant did not alter their virulence, suggesting that an important part of the pathogenicity is mediated by host-generated ROS.

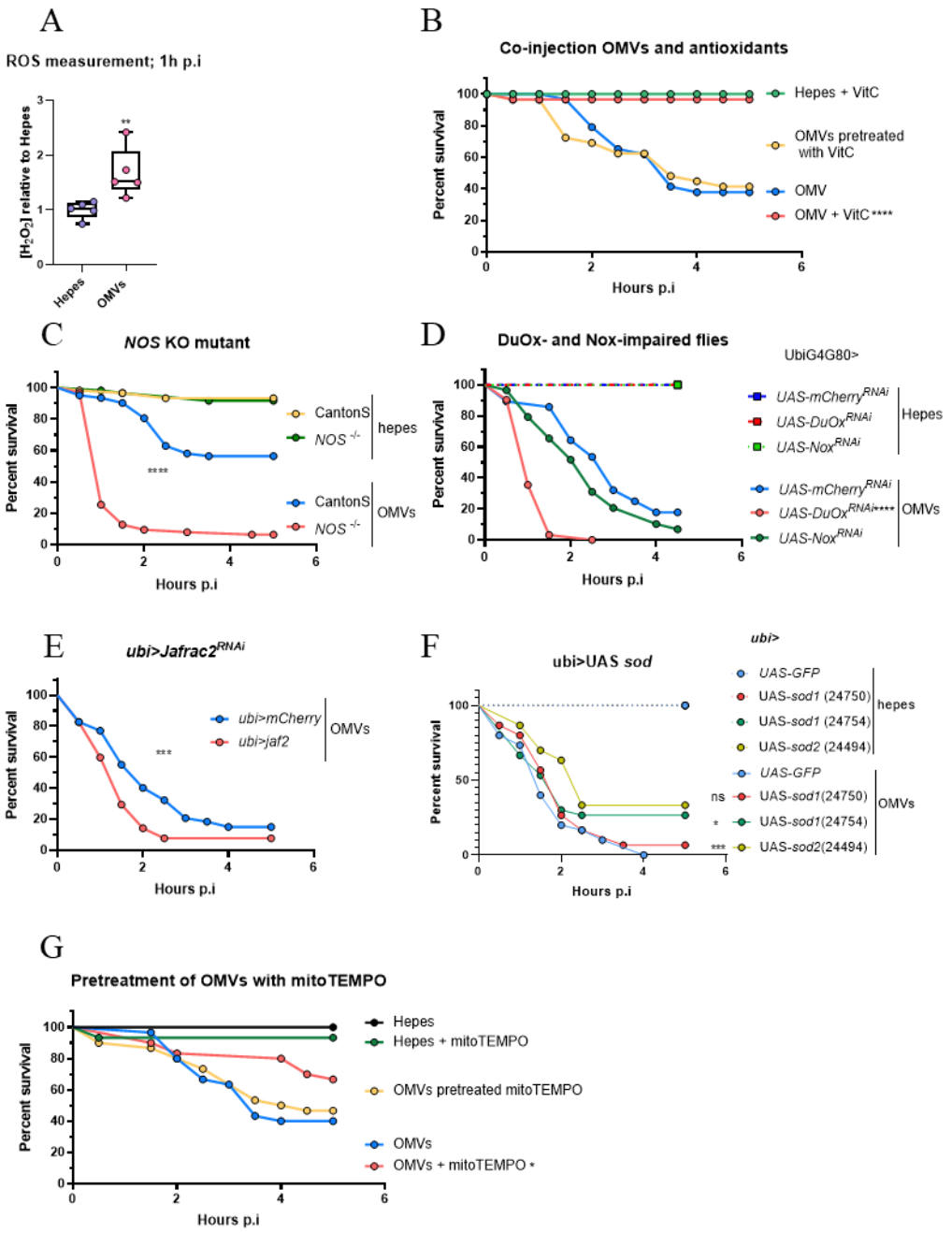

Figure 4. ROS et OMVs. (A) Le niveau de H O_{22} après traitement des OMVs. **(B)** Le taux de survie des mouches de type sauvage après traitement. Les OMVs ont été prétraitées avec de la vitamine C (VitC) sur de la glace pendant 30 minutes, puis injectées dans les mouches après filtrage de la vitamine C. La VitC n'a pas d'impact sur les OMVs. La VitC n'a pas d'impact sur les OMVs. La courbe rouge montre la co-injection d'OMVs et de VitC. **(C) Les** mutants *NOS* ont été traités avec des OMVs tandis que CantonS a été considéré comme le contrôle de type sauvage. **(D)** Réduction de l'expression de *Nox* et *DuOx de* manière ubiquitaire par ARNi. Les mouches KD ont été injectées avec des OMVs tandis que le *mCherryRNAi* a été généré comme les mouches de contrôle comme les mouches TRiP RNAi. **(E) Les** mouches dont l'expression ubiquitaire de *Jafrac2* **(F)** et de *Sods a* été supprimée ont été infectées par des OMV. **(G)** Les OMVs ont été prétraitées avec du mito-TEMPO sur de la glace pendant 30 minutes, puis injectées dans les mouches après avoir filtré le mito-TEMPO. Les mito-TEMPO n'ont pas d'impact sur les OMVs. La courbe rouge montre la co-injection d'OMVs et de mito-TEMPO.

We next attempted to pinpoint the source of these host-generated ROS using a genetic approach. A nitric oxide synthase null mutant or the ubiquitous silencing of the *Nox* and *Duox* genes revealed that *NOS* and *Duox* are involved in the defense against OMVs and not in promoting their pathogenicity (Fig. 4C-D). JAFRAC2 encodes a peroxiredoxin located in the endoplasmic reticulum. *Jafrac2* silencing in all tissues also revealed an involvement in the host defense against OMVs (Fig. 4E).

Another potential source of intracellular ROS is constituted by mitochondria. The overexpression of a mitochondrial superoxide dismutase gene, *Sod2*, yielded a phenotype of enhanced resistance to injected OMVs, suggesting that mitochondria may mediate the noxious effects of ROS upon OMV injection. In contrast, the overexpression of a cytoplasmic superoxide dismutase gene, *Sod1*, provided a much milder degree of protection (Fig. 4F). To independently confirm these results, we co-injected a mitochondrially targeted strong anti-oxidant, Mito-TEMPO [14], alongside OMVs. Results similar to those obtained with Vitamin C were obtained, namely that the co-injection protected the host from the harmful effects of mitochondria-generated ROS since the mito-TEMPO pre-incubated OMVs retained their virulence (Fig. 4G).

We conclude that mitochondria-generated ROS mediate much of the pathogenicity of injected OMVs whereas other ROS, likely secreted, play an opposite role and defend against OMVs.

JNK pathway activation in neurons is detrimental to the host upon OMVs challenge

The JNK pathway is known to be induced upon ROS stress and also to activate apoptosis. We first determined whether the JNK pathway might become activated upon OMV injection. Fig. 5A shows that *puckered*, which encodes a phosphatase that acts as a negative regulator of the JNK pathway and that is often used as a JNK pathway activation read-out, is induced one hour after OMV challenge (Fig. 5A). We therefore first ubiquitously silenced *kayak*, which encodes the cFos transcription factor, and observed an increased resistance to OMVs in “survival” experiments (Fig. S3A). Given

the paralysis phenotype, we next asked whether JNK activation in neurons might promote the pathogenicity of OMVs. Upon silencing *kayak* in neural cells using an *elav-Gal4* driver, we observed a strong, reproducible protection against injected OMVs (Fig. 5B). In contrast, silencing *kayak* in *hml*-positive hemocytes provided a slight susceptibility to injected OMVs (Fig. 5C). Of note, we observed an induction of the JNK pathway in samples consisting of the head and thorax, which contain most of the fly nervous system and only the cephalic fat body (Fig. S3B).

Apoptosis contributes to the pathogenesis of OMVs in *Drosophila*

As JNK pathway activation is known to induce apoptosis through the up-regulation of the inducer of apoptosis (IAP) antagonists Reaper (*rpr*) and Head Involution Defective (*hid*) genes, we monitored their induction using RTqPCR. Interestingly, their expression was also induced one hour after OMV challenge (Fig. 5D-E and Fig. S3C-D). As the JNK pathway can be induced by the TNF receptor Wengen upon binding to its TNF

ligand Eiger, we asked whether *eiger* might be involved in mediating the effects of injected OMVs. As shown in Fig. S3E, silencing *eiger* in various tissues did not yield any phenotype. Thus, we next assessed whether ROS might be required for the induction of *rpr* and *hid* expression. Upon vitamin C co-injection of OMVs, the induction of these genes was abolished in vitamin C-treated flies (Fig. 5F-G). Unexpectedly, *puc* was still induced under these conditions (Fig. S3F).

In keeping with these results, we next overexpressed *Sod* genes in neurons and observed that *Sod2* ectopic expression in neurons did protect the flies from injected OMV pathogenicity (Fig. 5H-I).

The apoptosis program involves executioner caspases that can be inhibited by the baculovirus p35 effector. We therefore ectopically expressed p35 in neurons using either an *elav-Gal4* driver or an *elav-GS (*Gene Switch) driver, which can be activated upon administration of RU486 to flies. In both cases, the p35 overexpressing flies displayed an enhanced resistance phenotype, which suggests that OMVs induce apoptosis in some

neurons (Fig. 5J-K), thereby significantly contributing to their pathogenicity.

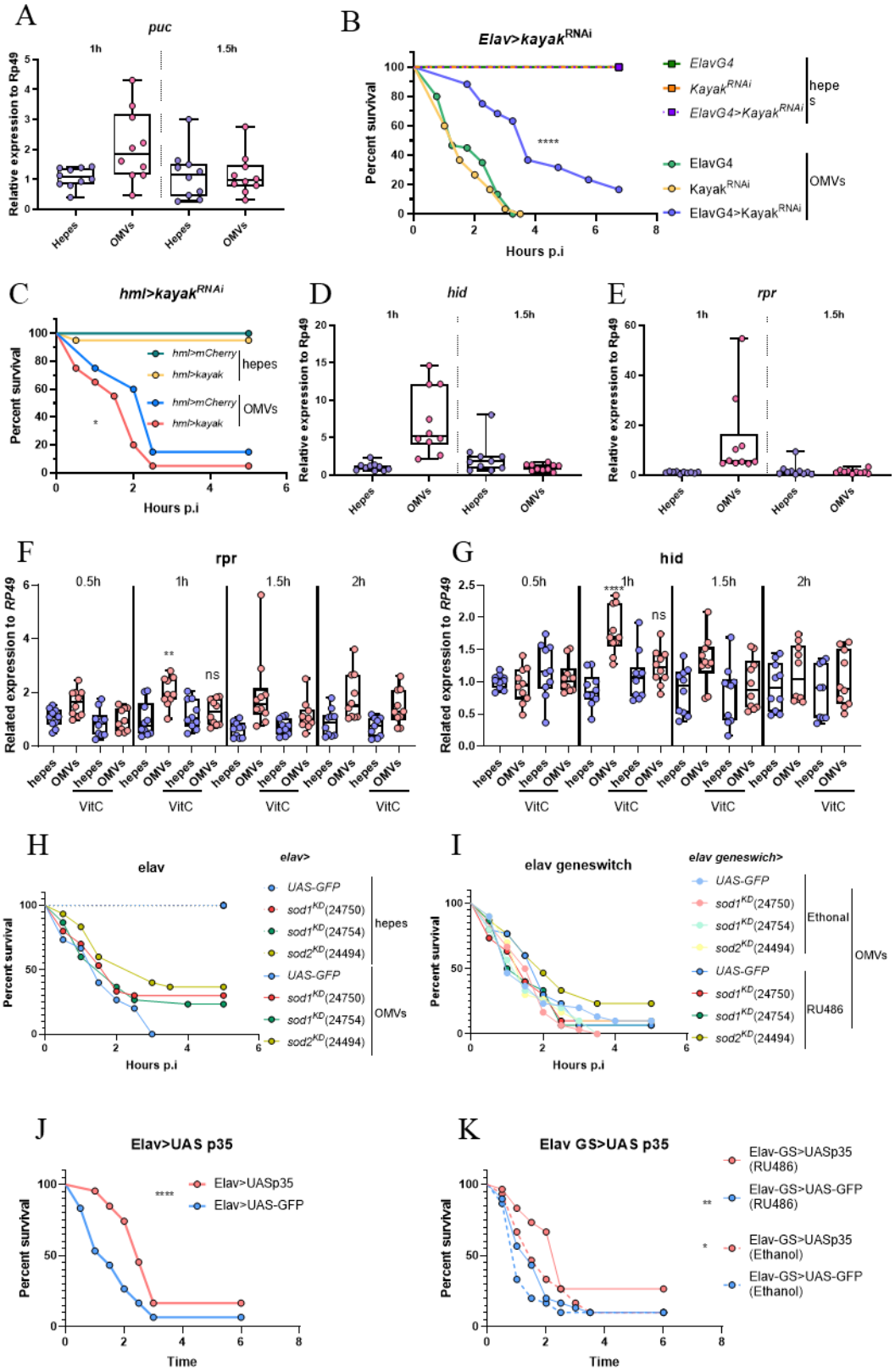

Figure 5. Voie JNK et OMVs. (A) Les niveaux d'expression transcriptionnelle de *pucker* (*puc)*, **(D)** *Head Involution Defective* (hid) et **(E)** *reaper* (*rpr*) dans la mouche entière lors de l'infection

par OMVs à 1 heure et 1,5 heure ont été contrôlés par RT-qPCR. **(B)** Knocking down *kayak* dans les neurones par Elav GAL4 et **(C) dans les** hémocytes par Hml GAL4. Test de survie comparant les mouches knock down aux mouches contrôles. **(F) Les** niveaux d'expression transcriptionnelle de reaper (rpr) et **(G)** hid lors de l'infection des OMVs avec et sans co-injection de VitC à différents moments ont été contrôlés par RT-qPCR. Les données regroupées de deux expériences indépendantes avec 5 répétitions à chaque fois ont été montrées. Le test de Mann-Whitney a été utilisé pour comparer les OMVs traitées au groupe Hepes. **(H)** Inhibition des gènes *Sods* dans les neurones par Elav GAL4 et **(I)** Elav gene switch GAL4. Test de survie comparant les mouches knock down aux mouches de contrôle. **(J)** Test de survie comparant les mouches surexprimant p35 dans les neurones avec Elav GAL4 et **(K)** Elav gene switch GAL4**.**

PrtA is a major virulence factor of OMVs

A previous proteomic analysis of OMV content revealed that the 56kD metalloprotease PrtA, also known as Serralysin, is packaged into OMVs. We thus prepared OMVs from a *PrtA* mutant strain and injected the preparation into flies. These *PrtA*-OMVs were avirulent when injected at our usual concentration but displayed a dose-dependent pathogenicity when injected at 10 to 100x higher concentrations (Fig. 6A-B). We next injected purified PrtA at a concentration equivalent to the one present in OMVs at the 0.1 ng/nL concentration and observed an equivalent demise of injected flies (Fig. 6C). We also showed that the injection of purified PrtA was able to rescue the decreased virulence of PrtA mutant OMVs (Fig. 6D).

We then assessed whether PrtA might act in a manner similar to that of injected wild-type OMVs. To this end, we injected purified PrtA into wild-type and mutant flies. Whereas *eater* and *kenny* mutants exhibited a phenotype similar to that observed upon the injection of OMVs, *i.e.*, respectively an enhanced resistance or sensitivity, it was striking that *hayan* mutant flies did not display any altered behavior in the “survival” experiment (Fig. 6E).

Finally, we addressed the relevance of OMVs to the high virulence of *S. marcescens* in a septic injury model. Since PrtA mediates most of the virulence of injected OMVs, we reasoned that if OMVs do contribute to the virulence of this bacterium *in vivo*, then a *PrtA* mutant would display a decreased virulence. As shown

in Fig. 6F, we did observe a mild but nevertheless significant lessened pathogenicity of the injected *PrtA* mutant bacteria as compared to wild-type bacteria.

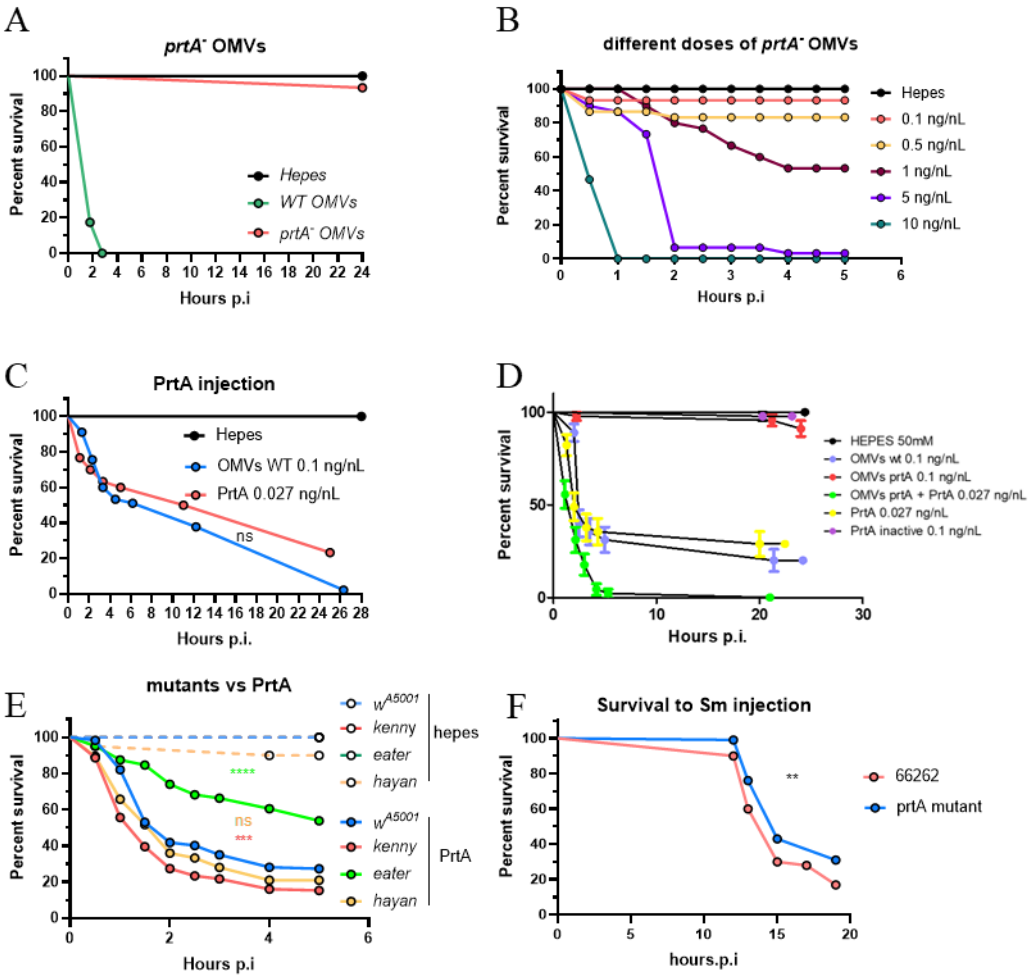

Figure 6. La toxicité de PrtA purifiée. (A) La même concentration d'OMV produite par *Serratia marcescens* WT et le mutant *PrtA*$^{-/-}$ a été injectée à des mouches de type sauvage. **(B)** La survie des mouches de type sauvage à différentes concentrations d'OMVs de *PrtA*$^{-/-}$. La concentration d'OMVs a été déterminée par le kit BCA. **(C)** La survie des mouches de type sauvage injectées avec des OMVs WT et de la PrtA purifiée. (**D)** Co-injection de PrtA purifiée avec des OMVs de mutant *PrtA*. **(E)** Test de survie comparant les mouches de type sauvage aux différents mutants après traitement à la PrtA purifiée. **(F)** La mouche de type sauvage a été injectée avec les bactéries *Serratia marcescens* et *PrtA*$^{-/-}$ mutant. Les données de pool de deux expériences indépendantes ont été montrées.

Discussion

S. marcescens is one of the most potent pathogens of *Drosophila* in a systemic infection model since the injection of a handful of bacteria causes the death of flies in less than a day [6]. In this work, we have established that purified *S. marcescens* OMVs lead to the demise of injected flies within hours, provided they are injected at a sufficiently high concentration, which might be reached toward the end of *in vivo S. marcescens* infection. An important observation was that flies were actually not killed but were initially paralyzed, suggesting an action of OMVs on the neuro-muscular system. We have shown that the different arms of the innate immune system play contrasted roles in the host defense against injected OMVs. Whereas the IMD pathway, phenol oxidases, and some ROS-generating enzymes such as NOS or Duox decrease the pathogenesis of the injected OMVs, other, notably the cellular immune response and Hayan, promote it. We further establish that the injection of OMVs leads to the production of harmful mitochondrial ROS that trigger apoptosis in neurons possibly through the activation of the JNK pathway.

The IMD pathway fosters a defense against injected OMVs, in keeping with its general role in fighting off Gram-negative bacterial infections [15, 16]. One may envision that membrane active AMPs such as Cecropins might lyse OMVs, thereby leading to the release of OMV content in the hemolymph. However, these peptides are unlikely to be active against injected purified PrtA, which kill IMD pathway mutant flies faster than wild-type flies. We have recently identified two effectors of the systemic immune response, the expressions of which are at least partially regulated by the IMD pathway, that are able to counteract the noxious activity of this metalloprotease, without apparently inhibiting its catalytic activity in *in vitro* assays (Cai *et al*., *in preparation*). Other IMD-regulated effectors such as *Drosophila* complement protein family members may further contribute to the host defense against OMVs (Cai *et al*., *in preparation*).

In contrast to the IMD pathway, the contribution of the Toll pathway to the

response against OMVs is much less clear. Its activation is detected late, although it cannot be excluded that in some cell types such as neurons it might be induced earlier. Mutant analysis suggests that OMVs may be sensed through the GNBP3 ß-(1-3)-glucan sensor. An activation of the other arm of Toll pathway that detects abnormal proteolytic activity such as that of the potential one of PrtA may however not be excluded [17-19]. In general, the results from "survival" experiments were variable, even when considering a single mutant such as *MyD88*. We however note that two of the IMD-dependent effectors referred to above are highly induced in a Toll pathway-dependent manner but yields a sensitivity phenotype instead of the mild protection observed for most Toll pathway mutants (Cai *et al*., *in preparation*).

The apparently paradoxical role of the melanization activation cascade is puzzling on first sight. However, a recent study has revealed an uncoupling between the Hayan-dependent synthesis and deposition of melanin at the wounding site and a killing activity mediated by the Sp7 protease in the host defense against low doses of injected *Staphylococcus aureus* bacteria [12]. One may thus envision that Sp7 activates PPO1 and PPO2 and generates an activity that counteracts the pathogenic effects of OMVs, possibly ROS, in keeping with those already potentially generated by NOS and Duox, or other metabolites.

The model we propose to account for our data is that injected OMVs trigger the production of ROS by mitochondria, which may be a consequence of mitochondrial dysfunction induced by internalized OMVs that has been reported in other biological systems [20, 21]. It is likely that mitochondrial dysfunction may directly or indirectly induce apoptosis via a ROS-dependent process. The JNK pathway is known to be triggered by ROS and to promote the expression of *rpr* and *hid* yet we observed that it was still induced in vitamin C-injected flies, for which *rpr* and *hid* induction was impaired. Thus, it may be that the JNK pathway and Rpr and Hid function in parallel in triggering apoptosis. Indeed, both pro-apoptotic proteins become associated with the mitochondria outer membrane where they may trigger apoptosis by inhibiting the apoptosis inhibitor DIAP-1 **[22]**. The induction of programmed cell death by OMVs

through an action in mitochondria has been documented in mammalian models, in which inflammation rather than overall survival is monitored [23]. It will be important to determine whether apoptosis in the brain occurs in a random widespread manner or more specifically targets neuronal circuits that regulate locomotion and posture. Of note, it is not clear at present whether flies die as a result of multiple actions of OMVs on different organs or whether the damage is limited to the brain of flies ultimately leading to death through starvation.

An interesting issue is whether OMVs can actually access the brain and have the ability to cross the blood-brain barrier (BBB). Our attempts to label *S. marcescens* OMVs by tagging some its endogenous constituents have failed and we have not been able to directly assess their presence using microscopy techniques. The OMVs from *Porphyromonas gingivalis* have been reported to weaken the BBB; thus, it is an open possibility that OMVs might directly act on neurons [24]. However, we have found that the cellular immune response also promotes the pathogenicity of OMVs like Hayan. In the latter case, it is interesting to note that the action of Hayan worked only on OMVs and not when purified PrtA was injected. These observations are compatible with a potential action of Hayan in releasing PrtA from OMVs. However, such a mechanism would not be at work as regards the cellular immune response since it promoted the pathogenicity of purified PrtA independently of OMVs. Thus, how hemocytes promote the pathogenicity remains enigmatic at present and it is an open possibility that OMVs and PrtA act directly on hemocytes, which are likely to internalize OMVs given their "professional" phagocytic abilities. They may then relay a signal to the nervous system, which is not Eiger, that promotes mitochondrial-derived ROS production and/or apoptosis in neurons.

Supplementary

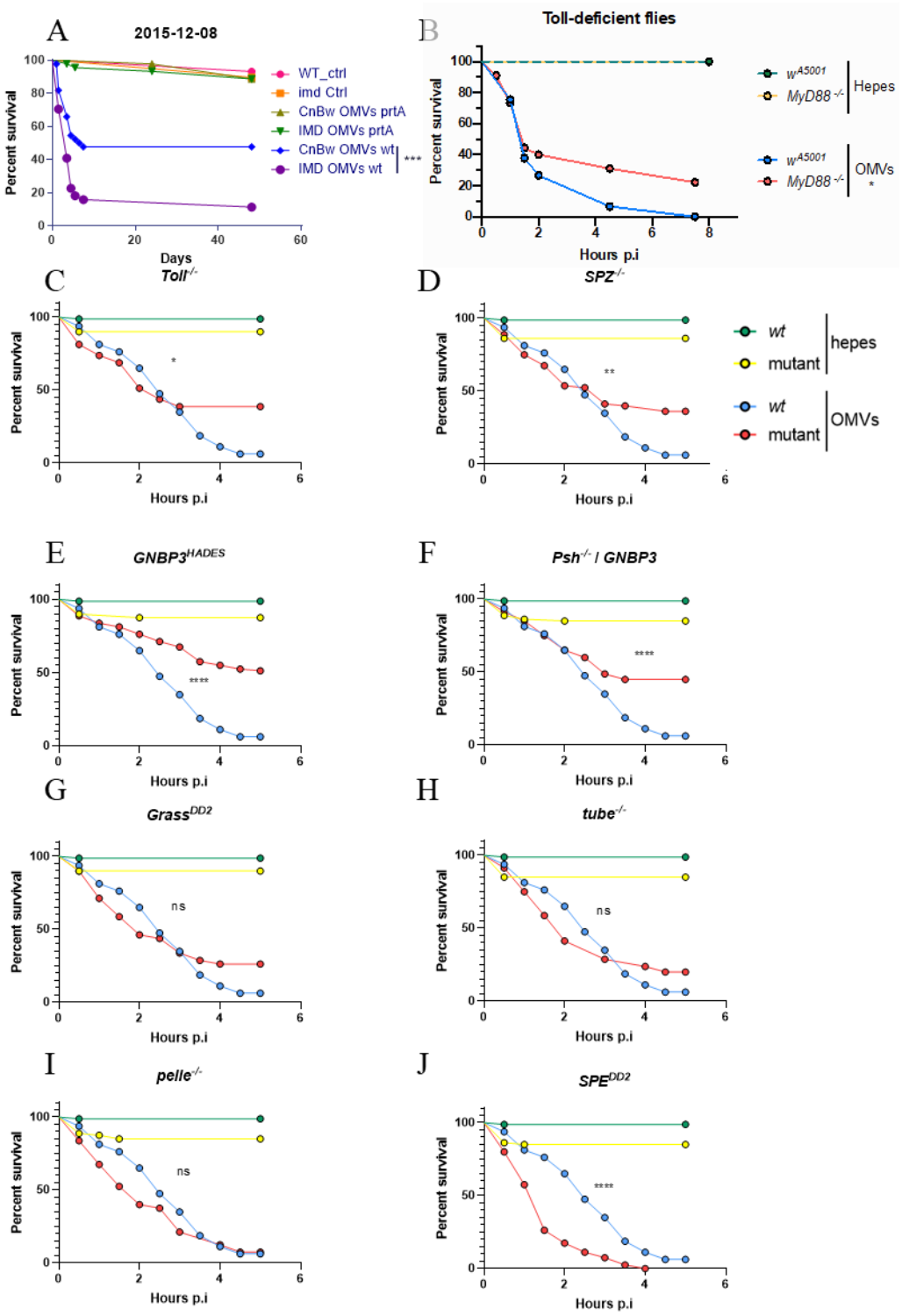

Figure S1. Taux de survie de différents mutants de la voie IMD **(A)** ou Toll **(B-J)** lors d'infections par OMVs.

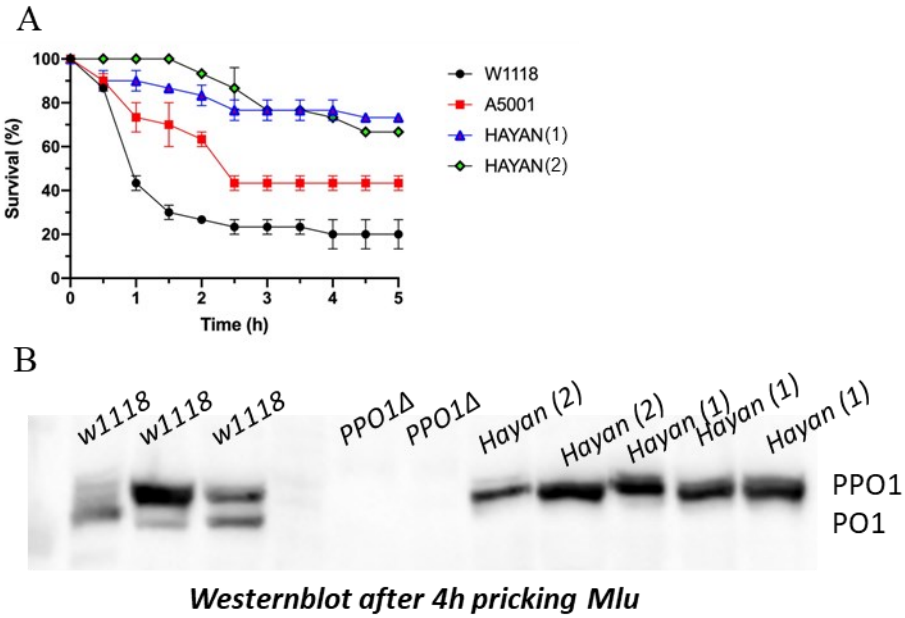

Figure S2. (A) Taux de survie de deux mutants Hayan indépendants après injection d'OMVs. **(B)** Analyse par Western bolt du clivage de PPO1 dans différents mutants à 4 heures après l'infection par M. luteus pour confirmer les mutant**s.**

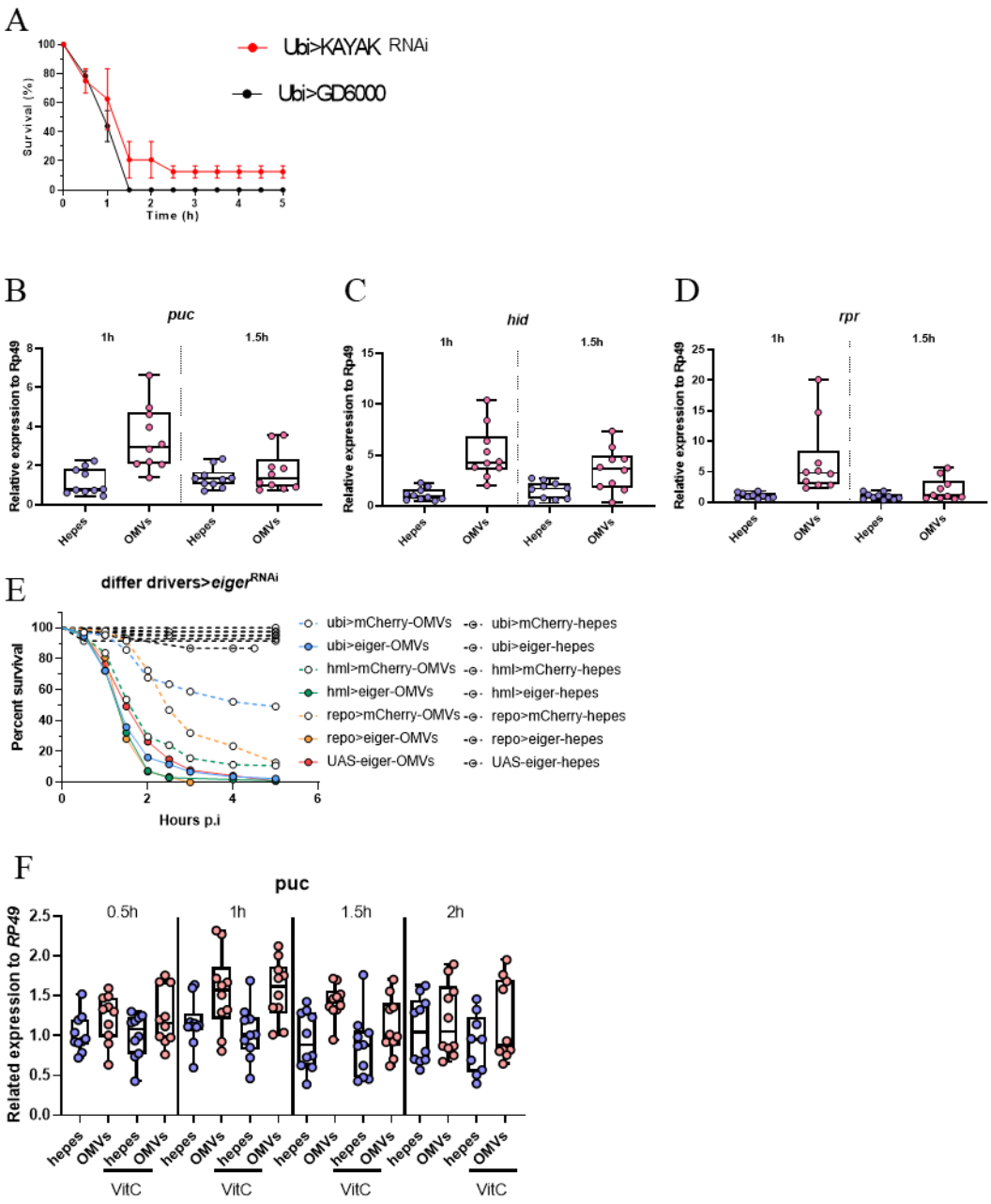

Figure S3. Apoptose et OMVs. (A) Inhibition de *kayak* dans le corps entier et infection par des OMVs. Test de survie comparant les mouches knock down aux mouches de contrôle. **(B) Les** niveaux d'expression transcriptionnelle de *pucker* (*puc)*, **(C)** *Head Involution Defective* (hid) et **(D)** *reaper* (*rpr)* dans le thorax et la tête lors de l'infection par les OMVs à 1 heure et 1,5 heure ont été contrôlés par RT-qPCR. **(E)** Eiger a été désactivé dans différents tissus avec différents pilotes GAL4 spécifiques aux tissus. Test de survie comparant les mouches knock down représentées en lignes pleines aux mouches knock down mCherry de contrôle représentées en lignes pointillées de la même couleur que les mouches knock down d'Eiger. **(F) Les** niveaux d'expression transcriptionnelle de *puc* lors de l'infection des OMVs avec et sans co-injection de VitC à différents moments ont été contrôlés par RT-qPCR. Les données regroupées de deux expériences indépendantes avec 5 répétitions à chaque fois ont été montrées.

Material and methods

Bacterial culture

S. marcescens RM66262 31 and the mutant *S. marcescens prtA* were cultured on Lysogeny Broth (LB)-agar plates containing ampicillin or chloramphenicol antibiotic. Bacteria were grown overnight on the agar plates at 37ºC. These plates are stored at 4ºC for one weeks. Only one colony was inoculated in liquid LB and the culture was kept at 30ºC with agitation for a maximum of 16 hours for OMV production.

Purification of outer membrane vesicles (OMVs)

A saturated culture of RM66262 or *prtA* mutant were diluted 1:500 in fresh LB and placed at 30ºC with agitation for a maximum of 16 hours. About 600 mL of cultures were centrifuged at 8,000 g for 15 min. The pellets were discarded, and the supernatants were filtered using 0.45 µm filter (Thermo scientific, cat# 126-0045) to remove all remaining cells. The filtered supernatants were further precipitated with ammonium sulfate (129g/250mL of supernatant) overnight at 4ºC. Then, the supernatants were centrifuged for 20 min at 10,000 g. The supernatants were discarded, and the pellets were re-suspended in 10 mL of 50 mM Hepes buffer (pH 7.5). The samples were dialyzed against Hepes (50 mM) overnight at 4ºC, then concentrated using Amicon® Ultra 15 mL (Millipore, UFC901024) by centrifugation at 5,000 g. Further, 4 mg from the samples were loaded on the top of a 30% to 60% sucrose/Hepes density gradient and centrifuged at 100,000 g at 4ºC for 16 hours. Fractions of 1 mL were collected from each gradient and checked on Coomassie gel. Only the 6 first fractions were selected and dialyzed against Hepes 50mM overnight at 4ºC. Finally, the samples were centrifuged at 100 000 g for 3 hours at 4ºC, the supernatants were discarded and the pellets that contain OMVs were re-suspended in 400 µL of fresh 50 mM Hepes (pH 7.5). The OMVs were stored at -80ºC.

Fly strains and maintenance

Fly lines were raised on media at 25°C with 65% humidity. For 25 L of fly food medium, 1.2 kg cornmeal (Priméal), 1.2 kg glucose (Tereos Syral), 1.5 kg yeast (Bio Springer), 90 g nipagin (VWR Chemicals) were diluted into 350 mL ethanol (Sigma-Aldrich), 120 g agar-agar (Sobigel) and water qsp were used.

Fly strains used in the experiments were: control flies w^{A5001}, w^{1118}, yw, GD line, KK line or mCherry as indicated, eater$^{-/-}$ mutant flies [10], MyD88$^{-/-}$[25], hayan$^{-/-}$ [13], PPO1$^{-/-}$ & and PPO2$^{-/-}$ & PPO1-2 $^{-/-}$ [26]. The RNAi lines used were from the Vienna *Drosophila* Research Center (VDRC) or were Bloomington TRiP lines (BL): UAS-*GBP2*^{RNAi} (VDRC GD #16696), UAS-*kayak*^{RNAi} (VDRC KK #19512), UAS-*EGFR*^{RNAi} (VDRC GD#43267, VDRC KK#107130), UAS-*Mthl10*^{RNAi} (BL# 51765382) UAS-*Jafrac2* ^{RNAi} (TRiP lines, TH03349.N), UAS-*DUOX*^{RNAi} (BL# 38907), UAS-*Eiger*^{RNAi} (BL# 55276). Crosses of UAS-transgene were performed at 18ºC. The progeny was kept at 18ºC until haching and was transferred to 29ºC.

Injections of OMVs and treatment

The injection of OMVs into the thorax of the flies was carried out with a Nanoject II auto-nanoliter injector (Drummond). depending on the OMVs batch 69 nL of 0.07 to 0.1 ng/nL of OMVs was injected. The Vitamin C antioxidant treatment or mito TEMPO treatment (SML0737) were performed through a co-injection of OMVs (0.1 ng/nL) with Vitamin C (20 mM) or mito TEMPO (20uM). All OMV samples and antioxidants were diluted in 50 mM Hepes (pH 7.5). For Pre-treatment OMV were incubate with or without Vitamin C (20mM) or mito TEMPO (20uM) for 30mn on ice. OMVs were centrifuge 15mn 10,000g using a 10kD amicon filter for to get rid of the treatment prior injection.

Purification of PrtA

Strain slpE- culture was grown at 30 °C (major expression of PrtA) for 16 h in SLB (SLB is LB broth medium without salt) since the molecular weight and isoelectric point of this metalloprotease (SlpE) encoded by our Serratia is similar and could interfere

with the purification of native PrtA. The cells were centrifuged and the supernatant was filtered and concentrated with a centricon. The supernatant was applied to an anion exchange column (Mono Q) and elution was carried out by a linear NaCl gradient. The active fractions were collected, and concentration and proteolytic activity were measured (using azocasein as substrate), to ensure that the protease is catalytically active after purification.

Survival tests to OMV injection

Survival tests were performed using 15-20 flies per vial in biological duplicate or triplicate. The number of survived flies was counted every 30 min after the injection. Of note, only flies that completely stopped moving their legs were considered to be dead.

Quantification of gene expression

Four to five whole flies were crushed into 100 µL of Trizol. Samples were filled with 900µl of Trizol and mixed with 500ul of chloroform. Samples were centrifuged at 12 000 xg for 15 min at 4ºC. The liquid upper phase of the samples was collected into an Eppendorf tube containing 100 µL of isopropanol. The tubes were vortexed and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 g for 15 min at 4ºC. The pellet was washed in 500 µL of 70% ethanol and dried. RNAs were then re-suspended in DEPC water. A volume of 10 µL was used to generate cDNA by reverse transcription, using the Transcript II all in one first strand synthesis supermix for qPCR (one step gDNA removal) synthesis kit (transgen biotech #AT341-02). The quantitative Polymerase Chain Reaction (qPCR) was performed with the same kit on cDNA diluted 20 times. The program-used was the following: 30 sec at 98ºC; 34 cycles of 5 sec at 95ºC, 30 sec at 98ºC and finally 30 sec at 65ºC. The data were analyzed using the CFX384 software (Bio-Rad). The Ct (Cycle threshold) values of the genes were normalized with the Ct values of Rpl32 (housekeeping gene that codes for a ribosomal protein). Further, the normalized values of treated conditions were normalized with the

normalized values of untreated conditions (delta delta Ct). All primers used in this report are listed in table 2.

H_2O_2 measurement

To quantify ROS, we used single fly detection. Briefly, flies are homogenate in specific buffer provide by Sigma alrich kit MAK165.then, 10ul of sample is use to determine H2O2 as mention by the kit

Statistical tests

All graphs and statistical tests were analyzed using the GraphPad software Prism 6 or 8. The statistical test used for the survival tests was Logrank. The Mann-Whitney test was performed on all other experiments. The number of stars represents the P values $P \geq 0.05$ (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

Western blot

For western blots, hemolymph samples were collected from 50 flies in a protease inhibitor cocktails solution. Protein concentration of the samples was determined by Bradford assay. 30 µg protein was separated on an 8% gel by SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% bovine serum albumin in PBST for 1 h at room temperature, samples were incubated at 4 °C overnight with rabbit antibodies against *Drosophila* PPO1 at a 1:10,000 dilution (a kind gift from Prof. Erjun Ling). After washes, a goat anti-rabbit-horseradish peroxidase (HRP) secondary antibody at a 1:20,000 dilution was incubated for 1 h at room temperature. Enhanced chemiluminescence substrate (ECL, General Electric Healthcare) was used according to the manufacturer's instructions to reveal the blot.

Reference

[1] P. A. Grimont and F. Grimont, "The genus Serratia," (in eng), *Annu Rev Microbiol,* vol. 32, pp. 221-48, 1978, doi: 10.1146/annurev.mi.32.100178.001253.

[2] R. Marre, J. Hacker, and V. Braun, "The cell-bound hemolysin of Serratia marcescens contributes to uropathogenicity," (in eng), *Microb Pathog,* vol. 7, no. 2, pp. 153-6, Aug 1989, doi: 10.1016/0882-4010(89)90034-x.

[3] M. J. Albers, J. W. Mouton, and D. Tibboel, "Colonization and infection by Serratia species in a paediatric surgical intensive care unit," *J Hosp Infect,* vol. 48, no. 1, pp. 7-12, May 2001, doi: 10.1053/jhin.2001.0939.

[4] M. J. Kuehn and N. C. Kesty, "Bacterial outer membrane vesicles and the host-pathogen interaction," *Genes Dev,* vol. 19, no. 22, pp. 2645-55, Nov 15 2005, doi: 10.1101/gad.1299905.

[5] K. J. McMahon, M. E. Castelli, E. Garcia Vescovi, and M. F. Feldman, "Biogenesis of outer membrane vesicles in Serratia marcescens is thermoregulated and can be induced by activation of the Rcs phosphorelay system," *J Bacteriol,* vol. 194, no. 12, pp. 3241-9, Jun 2012, doi: 10.1128/JB.00016-12.

[6] N. T. Nehme *et al.*, "A model of bacterial intestinal infections in *Drosophila melanogaster*," (in eng), *PLoS Pathog,* vol. 3, no. 11, p. e173, Nov 2007, doi: 10.1371/journal.ppat.0030173.

[7] M. McIntosh, B. A. Stone, and V. A. Stanisich, "Curdlan and other bacterial (1-->3)-beta-D-glucans," (in eng), *Appl Microbiol Biotechnol,* vol. 68, no. 2, pp. 163-73, Aug 2005, doi: 10.1007/s00253-005-1959-5.

[8] B. Charroux and J. Royet, "Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response," (in eng), *Proc Natl Acad Sci U S A,* vol. 106, no. 24, pp. 9797-802, Jun 16 2009, doi: 10.1073/pnas.0903971106.

[9] A. Defaye, I. Evans, M. Crozatier, W. Wood, B. Lemaitre, and F. Leulier, "Genetic ablation of *Drosophila* phagocytes reveals their contribution to both development and resistance to bacterial infection," (in eng), *J Innate Immun,* vol. 1, no. 4, pp. 322-34, 2009, doi: 10.1159/000210264.

[10] C. Kocks *et al.*, "Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*," (in eng), *Cell,* vol. 123, no. 2, pp. 335-46, Oct 21 2005, doi: 10.1016/j.cell.2005.08.034.

[11] A. J. Bretscher *et al.*, "The Nimrod transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in *Drosophila melanogaster*," (in eng), *Biol Open,* vol. 4, no. 3, pp. 355-63, Feb 13 2015, doi: 10.1242/bio.201410595.

[12] J. P. Dudzic, M. A. Hanson, I. Iatsenko, S. Kondo, and B. Lemaitre, "More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in *Drosophila*," (in eng), *Cell Rep,* vol. 27, no. 4, pp. 1050-1061.e3, Apr 23 2019, doi: 10.1016/j.celrep.2019.03.101.

[13] H. J. Nam, I. H. Jang, H. You, K. A. Lee, and W. J. Lee, "Genetic evidence of a redox-dependent systemic wound response via Hayan protease-phenoloxidase system in *Drosophila*," (in eng), *Embo j,* vol. 31, no. 5, pp. 1253-65, Mar 7 2012, doi: 10.1038/emboj.2011.476.

[14] A. O. Oyewole and M. A. Birch-Machin, "Mitochondria-targeted antioxidants," (in eng), *Faseb j,* vol. 29, no. 12, pp. 4766-71, Dec 2015, doi: 10.1096/fj.15-275404.

[15] B. Lemaitre and J. Hoffmann, "The host defense of *Drosophila melanogaster*," *Annu Rev Immunol,* vol. 25, pp. 697-743, 2007, doi: 10.1146/annurev.immunol.25.022106.141615.

[16] D. Ferrandon, J. L. Imler, C. Hetru, and J. A. Hoffmann, "The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections," *Nat Rev Immunol,* vol. 7, no. 11, pp. 862-74, Nov 2007, doi: 10.1038/nri2194.

[17] M. Gottar *et al.*, "Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors," *Cell,* vol. 127, no. 7, pp. 1425-37, Dec 29 2006, doi: 10.1016/j.cell.2006.10.046.

[18] L. El Chamy, V. Leclerc, I. Caldelari, and J. M. Reichhart, "Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways 'upstream' of Toll," *Nat Immunol,* vol. 9, no. 10, pp. 1165-70, Oct 2008, doi: 10.1038/ni.1643.

[19] N. Issa *et al.*, "The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway," (in eng), *Mol Cell,* vol. 69, no. 4, pp. 539-550.e6, Feb 15 2018, doi: 10.1016/j.molcel.2018.01.029.

[20] P. Deo *et al.*, "Outer membrane vesicles from Neisseria gonorrhoeae target PorB to mitochondria and induce apoptosis," (in eng), *PLoS Pathog,* vol. 14, no. 3, p. e1006945, Mar 2018, doi: 10.1371/journal.ppat.1006945.

[21] M. Bielaszewska *et al.*, "Enterohemorrhagic Escherichia coli hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis," (in eng), *PLoS Pathog,* vol. 9, no. 12, p. e1003797, 2013, doi: 10.1371/journal.ppat.1003797.

[22] A. Clavier, A. Rincheval-Arnold, J. Colin, B. Mignotte, and I. Guénal, "Apoptosis in *Drosophila*: which role for mitochondria?," (in eng), *Apoptosis,* vol. 21, no. 3, pp. 239-51, Mar 2016, doi: 10.1007/s10495-015-1209-y.

[23] P. Deo *et al.*, "Mitochondrial dysfunction caused by outer membrane vesicles from Gram-negative bacteria activates intrinsic apoptosis and inflammation," (in eng), *Nat Microbiol,* vol. 5, no. 11, pp. 1418-1427, Nov 2020, doi: 10.1038/s41564-020-0773-2.

[24] A. B. Pritchard, Z. Fabian, C. L. Lawrence, G. Morton, S. Crean, and J. E. Alder, "An Investigation into the Effects of Outer Membrane Vesicles and Lipopolysaccharide of Porphyromonas gingivalis on Blood-Brain Barrier Integrity, Permeability, and Disruption of Scaffolding Proteins in a Human in vitro Model," (in eng), *J Alzheimers Dis,* vol. 86, no. 1, pp. 343-364, 2022, doi: 10.3233/jad-215054.

[25] S. Tauszig-Delamasure, H. Bilak, M. Capovilla, J. A. Hoffmann, and J. L. Imler, "*Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections," *Nat Immunol,* vol. 3, no. 1, pp. 91-7, Jan 2002, doi: 10.1038/ni747.

[26] O. Binggeli, C. Neyen, M. Poidevin, and B. Lemaitre, "Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*," (in eng), *PLoS Pathog,* vol. 10, no. 5, p. e1004067, May 2014, doi: 10.1371/journal.ppat.1004067.

Annex II

PNAS RESEARCH ARTICLE | IMMUNOLOGY AND INFLAMMATION

A Toll pathway effector protects *Drosophila* specifically from distinct toxins secreted by a fungus or a bacterium

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The *Drosophila* systemic immune response against many Gram-positive bacteria and fungi is mediated by the Toll pathway. How Toll-regulated effectors actually fulfill this role remains poorly understood as the known Toll-regulated antimicrobial peptide (AMP) genes are active only against filamentous fungi and not against Gram-positive bacteria or yeasts. Besides AMPs, two families of peptides secreted in response to infectious stimuli that activate the Toll pathway have been identified, namely Bomanins and peptides derived from a polyprotein precursor known as Baramicin A (BaraA). Unexpectedly, the deletion of a cluster of 10 Bomanins phenocopies the Toll mutant phenotype of susceptibility to infections. Here, we demonstrate that *BaraA* is required specifically in the host defense against *Enterococcus faecalis* and against the entomopathogenic fungus *Metarhizium robertsii*, albeit the fungal burden is not altered in *BaraA* mutants. BaraA protects the fly from the action of distinct toxins secreted by these Gram-positive and fungal pathogens, respectively, Enterocin V and Destruxin A. The injection of Destruxin A leads to the rapid paralysis of flies, whether wild type (WT) or mutant. However, a larger fraction of wild-type than *BaraA* flies recovers from paralysis within 5 to 10 h. BaraAs' function in protecting the host from the deleterious action of Destruxin is required in glial cells, highlighting a resilience role for the Toll pathway in the nervous system against microbial virulence factors. Thus, in complement to the current paradigm, innate immunity can cope effectively with the effects of toxins secreted by pathogens through the secretion of dedicated peptides, independently of xenobiotics detoxification pathways.

Baramicin A | microbial toxins | Destruxin A | enterocin O16 | resilience/disease tolerance

Significance

Major immune response pathways control the expression of hundreds of genes that represent potential effectors of the immune response. The *Drosophila* Toll pathway is required in the host defenses against several Gram-positive bacterial infections as well as against fungal infections. The current paradigm is that peptides secreted in the hemolymph during the systemic immune response are either bona fide antimicrobial peptides or likely ones. The finding of a dual role for one Toll pathway effector in the resilience to both *Enterococcus faecalis* and *Metarhizium robertsii* infections underscores an original concept in insect innate immunity. Evolution can select effectors tailored to protect the host from the action of microbial toxins of prokaryotic or eukaryotic origin, independently of antibodies or detoxification pathways.

The study of host defense against infections has essentially focused on the immune response and the mechanisms used by the organism to directly attack, kill, or neutralize invading pathogens. This dimension of host defense is known as resistance and in insects is mediated primarily by antimicrobial peptides (AMPs) and also by the cellular immune response and melanization (1–4). However, there is a second complementary dimension known as disease tolerance or resilience whereby the organism is able to withstand and, in some cases, repair damages inflicted by the virulence factors of pathogens or the host's own immune response (5–7). Some instances of resilience have been reported in *Drosophila*, e.g., the removal of oxidized lipids by Malpighian tubules through the lipid-binding protein Materazzi, the requirement for *CrebA* in regulating secretion during the immune response or the enterocyte cytoplasmic purge against pore-forming toxins (8–10). One way to discriminate between resistance and resilience is to monitor the microbial burden of infected hosts. It will be increased during infection of immunodeficient as compared to immunocompetent hosts. In contrast, it will not change much in organisms with defective resilience, which will tend to succumb to a lower load of pathogens, as monitored by measuring the pathogen load upon death (PLUD) (11, 12).

In *Drosophila*, the Toll pathway is one of the two NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathways that regulate the systemic immune response to microbial infections and through the MyD88 adapter complex is required in the host defense against many Gram-positive and fungal infections. It regulates the expression of more than 250 genes (9, 13–16). A few AMPs active against filamentous fungi have been identified (Drosomycin, Metchnikowin, and Daisho) (17–19). However, effectors solely regulated by the Toll pathway able to attack pathogenic yeasts or Gram-positive bacteria in vitro have not been described so far. Mass spectrometry analysis performed on the hemolymph of single immune-challenged flies has led to the identification of more than 30 peaks corresponding to Drosophila immune-induced molecules (DIMs) (20, 21). Some of them correspond to known AMPs, whereas others belong to a family of 12 proteins that contain a domain known as the Bomanin domain (21, 22). Ten such *Bomanin* genes are located at the 55C locus, including DIMs 1 to 3, now referred to BomS1 to S3.

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The authors declare no competing interest.

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The deletion of this locus strikingly phenocopies the Toll mutant phenotype, being sensitive to filamentous fungi, pathogenic yeasts, and Gram-positive bacteria such as *Enterococcus faecalis* (22). Some short Bomanins that essentially contain only the Bomanin domain may be active against *Candida glabrata* in vivo (23).

Several DIMs (5, 6, 8, 10, 12, 13, 22, 24) are actually derived from a polyprotein precursor known as IMPPP and until recently their function has not been understood (20, 21). A recent study renamed this protein as Baramicin A (BaraA) and proposed that some of the derived peptides function as antifungal AMPs (24). Here, we report our analysis of *BaraA* mutants. While we confirm a sensitivity to entomopathogenic fungi, our data clearly establish a susceptibility also to *E. faecalis*, but not to other pathogens we have tested. Interestingly, the fungal burden does not appear to be altered in the mutants, from the beginning to the end of the infections. Our data indicate that a major function of *BaraA* is in the resilience against distinct toxins, Destruxin A (DtxA), a pore-forming toxin, and Enterocin V (EntV), a bacteriocin, respectively, secreted by *Metarhizium robertsii* and *E. faecalis*. BaraA helps the host recover from DtxA-induced paralysis and appears to be required in glial cells but not in neurons.

Results

The *BaraA* locus encodes a polyprotein precursor that is likely processed by a furin-like enzymatic activity, which leads to the release in the hemolymph of multiple DIM peptides. These peptides share extensive sequence similarity, except for the N-terminal DIM24 protein that defines an evolutionarily conserved independent domain (25) (Fig. 1 *A* and *B*). For convenience, we shall refer to specific BaraA-derived peptides by their DIM names. Of note, *BaraA* lies next to the *CG18278* gene and the two genes are found as a perfect duplication in some wild and laboratory lines (25) (*SI Appendix*, Fig. S1 *A* and *B*).

BaraA gene expression is induced by a challenge with the Gram-positive bacteria *Micrococcus luteus* (used as a reference) and *E. faecalis* and by injected *M. robertsii* spores in a *MyD88*-dependent manner (Fig. 1*C*), in keeping with previous data at the transcriptional and peptide levels (20, 24). In contrast, the *CG18278* gene does not appear to be induced by any of these challenges (*SI Appendix*, Fig. S2*A*) and also not upon natural infection (*SI Appendix*, Fig. S2*B*).

***BaraA* Contributes to the Host Defense against *E. faecalis*.** In this work, we have generated a CRISPR–Cas9-mediated knock-out (KO) line, a *mCherry* knock-in (KI) line, and also used an RNAi line for knockdown (KD) experiments (*SI Appendix*, Fig. S1 *A*, *C* and *D*). In these lines, the induction of *BaraA* expression by an immune challenge is hardly detected, both at the transcriptional (*SI Appendix*, Fig. S1*E*) and protein levels (*SI Appendix*, Fig. S3 and Table S1). We have also generated two *CG18278* KO lines and tested them along a KD line with an *E. faecalis* challenge: No consistent susceptibility phenotype was detected (*SI Appendix*, Fig. S2 *C–F*). In contrast, we observed a significant susceptibility of *BaraA* mutant lines isogenized in the wild-type (WT) w^{A5001} background after the injection of this Gram-positive bacterial strain (Fig. 2*A*). Contrary to the *MyD88* line, measurements of the bacterial burden did not reveal any difference between the KO/KI lines and the isogenic w^{A5001} control line except for the 24 h time point, also within 30 min after death (bacterial load upon death: BLUD (11); Fig. 2 *B–D*). Note that at the 24 h and 48 h time points, the distribution of bacterial loads is already bimodal in the *BaraA* mutant, reflecting that the fate of infected flies is likely settled earlier in the mutant than in the wild-type flies (11). These observations at 24 to 48 h opened the possibility that BaraA may be partially involved in resistance to *E. faecalis*. We therefore monitored the melanization arm of the immune response by visualizing the proteolytic cleavage that activates pro-phenoloxidase (PPO) into a catalytically active enzyme after a microbial challenge; we observed a lessened maturation of PPO1 into PO1 in the *BaraA* KO mutant in three out of four experiments (*SI Appendix*, Fig. S4 *A* and *A*). As regards the cellular immune response, we noted that the m*Cherry* gene that replaces the *BaraA–CG18278* locus was more strongly expressed in adult hemocytes after an *E. faecalis* challenge (*SI Appendix*, Fig. S4 *B* and *B*'). As expected, the *BaraA* KD line led to an enhanced sensitivity to *E. faecalis* and to *M. robertsii* when the short RNA hairpin was ubiquitously expressed (*SI Appendix*, Fig. S4 *C* and *D*). When we silenced *BaraA* by RNAi in hemocytes, we did observe that

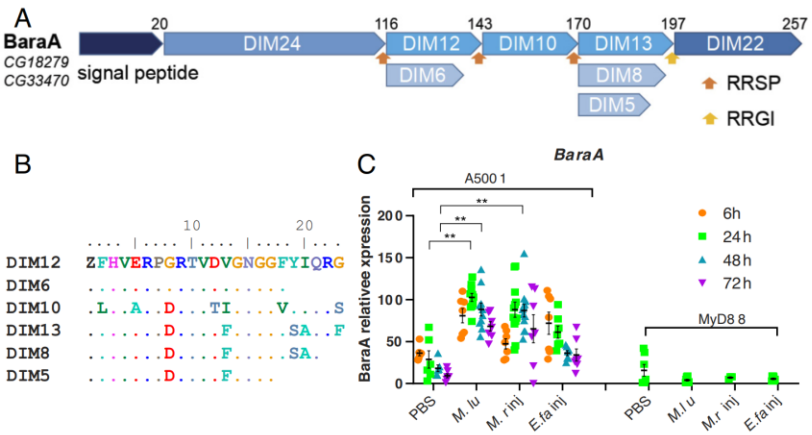

Fig. 1. Structure of the BaraA precursor protein and induction of *BaraA* expression by an immune challenge. (*A*) Schematic structure of the BaraA polyprotein. The name of the peptides derived from the processing of the precursor upon furin cleavage is shown as Drosophila-induced immune molecules (DIM), their original name. The type of internal furin-like cleavage sites is indicated by orange and yellow arrows (RRSP, RRGI). (*B*) Alignment of the short DIM peptides derived from BaraA, referred to by their DIM numbers. (*C*) Expression of the *BaraA* gene monitored by qRT-PCR at various time points after the injection of the indicated microbes; *M. luM. luteus*; *M. rM. robertsii*; *E. fa*: *E. faecalis*. The measured expression of *BaraA* 24 h after a *M. luteus* challenge is taken as reference for all other data points and given a 100% value. The means ± SEM are shown in black. Pooled data from three independent experiments, $^{**}P < 0.01$.

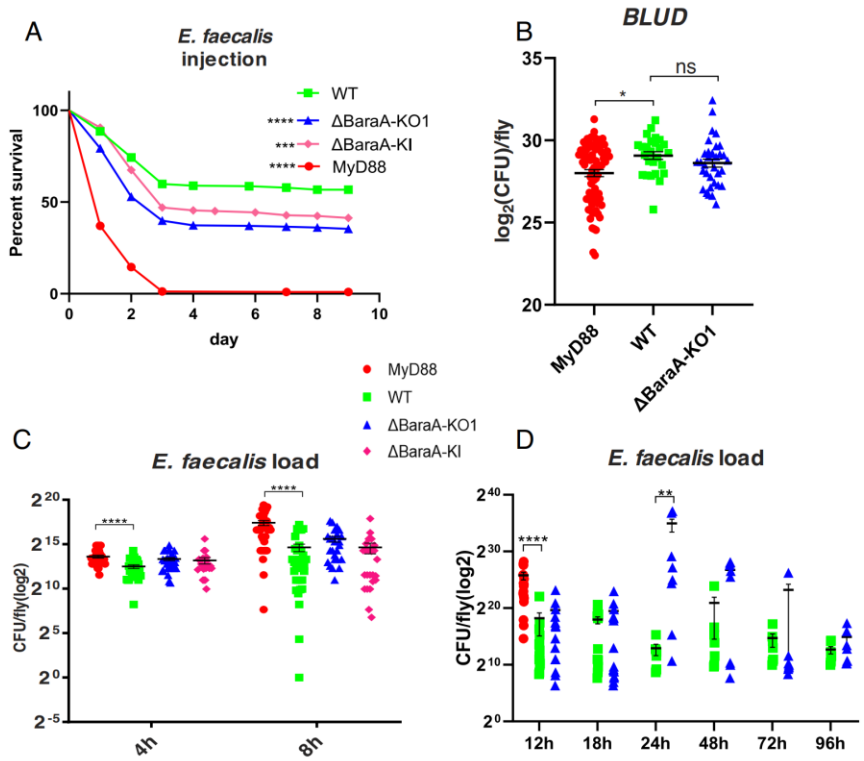

Fig. 2. Susceptibility of *BaraA* mutant flies to *E. faecalis* infection. (*A*) Survival curves of the isogenic *BaraA* KO and KI flies infected with *E. faecalis* NCTC 775. The WT corresponds to a wild-type w^{A5001} line isogenized in parallel to the KO and KI lines, which behaves like the w^{A5001} line used for isogenization. Pooled data from six independent experiments, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (*B*) Bacterial load upon death (BLUD) of *E. faecalis* OG1RF in WT and *BaraA* KO and KI lines. Pooled data from three independent experiments. (*C* and *D*) Bacterial load of *E. faecalis* NCTC 775 (*C*) and OG1RF (*D*) in WT and *BaraA* KO and KI lines from early time points to 6 d after infection. No significant differences were detected between WT and *BaraA* mutants at each time point, except for the 24 h one, $^{**}P < 0.01$. *MyD88* was significantly different from WT, $^{****}P < 0.0001$. The caption applies to panels *B*–*D*. Pooled data from three independent experiments. Data are expressed as means ± SEM.

these flies were significantly more susceptible to injected *E. faecalis* bacteria (*SI Appendix*, Fig. S4*E*).

We conclude that the *BaraA* mutant lines display an intermediate sensitivity to *E. faecalis* infection. It is however not fully clear whether the altered bacterial burden measured at 24 h results from a lessened activation of melanization, or a hemocyte-mediated response, albeit an impaired antimicrobial activity can also not be excluded.

The *BaraA* Mutant Is Susceptible to *M. robertsii* Infection Only in the Septic Injury Model. The *BaraA* KO and KI lines as well as the KD line consistently exhibited a moderately enhanced sensitivity to the injection of 50 *M. robertsii* conidia (Fig. 3*A* and *SI Appendix*, Fig. S4*C*). We did not detect an increased microbial titer in these mutants compared to wild-type controls during the infection, in contrast to *MyD88*; the fungal loads upon death (FLUD) were also similar (Fig. 3 *B* and *C*). Interestingly, no susceptibility to *M. robertsii* in the natural infection model was observed, even though the *BaraA* gene is induced by this challenge (*SI Appendix*, Fig. S5 *A* and *A'*). We have also tested a panel of other bacterial and fungal strains and did not observe any sensitivity to those infections (*SI Appendix*, Fig. S5 *B*–*F*).

In conclusion, we have found that *BaraA* appears to be required rather specifically in the host defense against a bacterial opportunistic pathogen, *E. faecalis*, and an entomopathogenic fungus, *M. robertsii*. Interestingly, the fungal burden did not appear to be altered in the *BaraA* mutants, which suggests that *BaraA* is not required in the resistance against *M. robertsii*.

The Transgenic Overexpression of BaraA Rescues the Sensitivity of *MyD88* Flies to *E. faecalis* and to *M. robertsii* to a Limited Degree. A complementary strategy to the loss-of-function analysis reported above consists in overexpressing the *BaraA* gene in a wild-type context, thus determining whether it might constitute a limiting factor in host defense against infections. The overexpression of *BaraA* at the adult stage using transgenic lines failed to enhance the protection of wild-type hosts against *M. robertsii* and to *E. faecalis* (*SI Appendix*, Fig. S6 *A* and *B*) yet rescued the *BaraA* sensitivity phenotype to these pathogens (*SI Appendix*, Fig. S6 *C* and *D*).

We next tested the overexpression of *BaraA* in a sensitized *MyD88* background. The *BaraA* transgene partially rescued the sensitivity of *MyD88* flies to *M. robertsii* and *E. faecalis* (*SI Appendix*, Fig. S6 *A* and *B*), suggesting that BaraA can function in the absence of Toll-induced Bomanins. We also checked by Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectrometry that the transgenic polyprotein was correctly processed, in the *MyD88* background, so that the endogenous signal would not mask that of the transgene-derived protein. As shown in *SI Appendix*, Fig. S6*E* and Table S2, *MyD88* is not required for the processing of the precursor into the DIM10, DIM12, DIM13, or DIM24 proteins by a putative furin. We also infer that the Toll

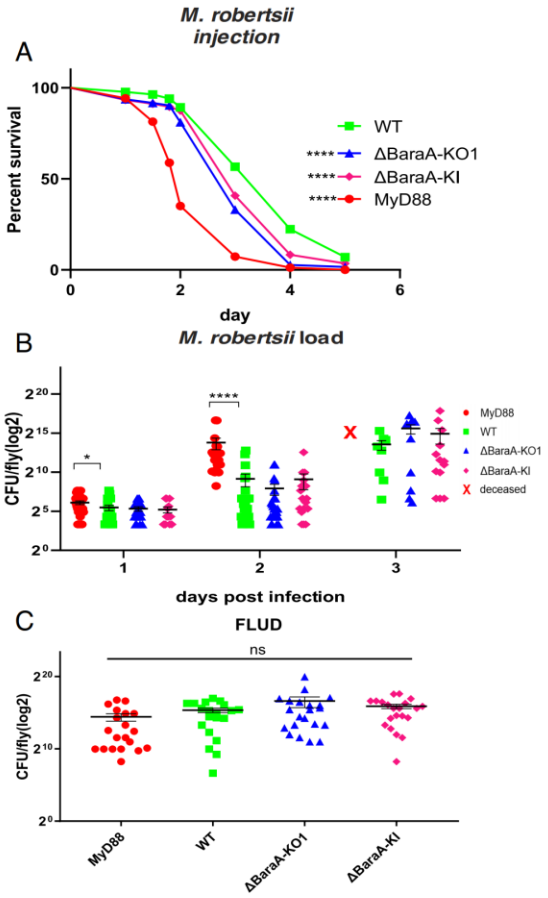

Fig. 3. Susceptibility of *BaraA* mutants to *M. robertsii* infection. (*A*) Survival of isogenic *BaraA* mutants injected with 50 *M. robertsii* conidia. Pooled data from 10 independent experiments. Statistical significance between WT and the KO or KI mutants: ****$P < 0.0001$. (*B*) Kinetics over 3 d of the fungal load of the *BaraA* KO and KI mutants injected with 50 *M. robertsii* conidia. No significant difference was detected between WT and *BaraA* mutants at each time point. (*C*) Fungal load upon death (FLUD) of single isogenic *BaraA* KO and KI flies injected with 50 *M. robertsii* conidia. No significant differences between WT and the isogenic mutant flies were detected. Three independent experiments have been performed and pooled (*B* and *C*). Data are expressed as means ± SEM.

pathway-dependent Bombardier activity needed to stabilize the expression of short Bomanins is not required for the stability of DIM10, DIM12, and DIM13 (26).

We conclude that the transgenic overexpression of BaraA is not sufficient to confer additional protection against *E. faecalis* or *M. robertsii* in the context of a wild type but can partially compensate for the Toll-deficient host defense against these two pathogens.

BaraA Does Not Modulate the Induction of the Toll Pathway. Besides a potential role of effectors, proteins that are induced by immune signaling pathways may play a role in their feedback regulation. We therefore monitored Toll pathway activation using the steady-state mRNA levels of AMP genes known to be regulated by the Toll pathway such as *Drosomycin*, *Metchnikowin*, and *IM1* (27–29). As shown in *SI Appendix*, Fig. S7, we did not observe any influence of the isogenized *BaraA* KO or KI null mutations over 48 h on their expressions.

***BaraA* Protects *Drosophila* from the Action of Secreted Microbial Toxins.** Our data thus far are not in keeping with a function for *BaraA* in resistance against *M. robertsii* and are compatible with this possibility as regards *E. faecalis*. The PLUD data are not indicative of a function in resilience.

The concept of pathogen load and PLUD relies on the assumption that the virulence of the pathogen correlates with the microbial burden. We have recently established that the function of the Toll pathway in the host defense against *Aspergillus fumigatus* is not to directly fight off this pathogen, as immunodeficient flies are killed by a limited number of pathogens that are trapped at the injection site. Rather, we have discovered that Toll function in the host defense against *A. fumigatus* is to limit or counteract some of its secreted mycotoxins (30). As mycotoxins, namely Destruxins (Dtx), have been described as important virulence factors from generalist *Metarhizium* entomopathogenic fungi (31, 32), we therefore injected DtxA into wild-type and *BaraA* flies. Interestingly, *BaraA* KO and KI mutants as well as *MyD88* flies reproducibly succumbed to a larger extent than wild-type flies to the injection of DtxA (Fig. 4*A*), a result confirmed in axenic flies (*SI Appendix*, Fig. S8*A*). We next determined that *BaraA* mutants are not more susceptible than wild-type flies to a challenge with a *Dtx* mutant *M. robertsii* strain (32) (Fig. 4*B*). Taken together, these results suggest that a major function of *BaraA* in the host defense against *M. robertsii* is to alleviate or counteract the effects of Destruxins secreted by the fungus in the septic injury model.

We then wondered whether *BaraA* might function in a similar manner in the host defense against *E. faecalis*. We therefore injected the *E. faecalis* culture supernatant into flies. Strikingly, whereas wild-type flies survived this challenge well, about 50% of *BaraA* KO flies and 20% of *BaraA* KI and *MyD88* flies succumbed to the injected supernatant (Fig. 4*C*). Filtration experiments allowed us to determine that the toxic component of the supernatant can be recovered in a 3 to 10 kD fraction (Fig. 4*D*). Even though the noxious activity in the *E. faecalis* supernatant was heat resistant, it was nevertheless susceptible to proteinase K treatment, suggesting a protein component (*SI Appendix*, Fig. S8 *B* and *C*). Interestingly, it has been reported that the bacteriocin enterocin O16 is an *E. faecalis* virulence factor in *Drosophila* (33). Enterocin O16 is also known as EntV, which is heat resistant and able to kill some lactobacilli strain as well as to inhibit the hyphal growth, the virulence, and the biofilm formation of *Candida albicans* (34–36).

We therefore asked whether the toxic activity in the supernatant is still present when using a bacterial *entV*$^-$ strain. We observed that the supernatant from the complemented *E. faecalis* strain *entV*$^+$/*entV*$^-$ behaved as that from the wild-type bacterial strain, that is, it killed *MyD88* and *BaraA* KO and KI mutants more than wild-type flies. Strikingly, the supernatant from an *entV*$^-$ *E. faecalis* mutant strain killed wild-type and *BaraA* mutants at a similar rate, whereas *MyD88* flies succumbed to the same extent to the mutant or complemented wild-type supernatants (Fig. 4*E*). Similarly, the direct infection-complemented *E. faecalis* strain *entV*$^+$/*entV* killed the immunodeficient *MyD88* and *BaraA* flies more than the wild-type control flies and thus behaved like the wild-type bacterial strain. In contrast, the *entV*$^-$ mutant *E. faecalis* strain did not kill *BaraA* KO flies to the same extent than the complemented bacterial strain (Fig. 4*F*). The EntV peptide derives from the open reading frame found in the *ef1097/entV* gene, which encodes a preproprotein. This precursor protein gets cleaved by the GelE protease into a 7.2 kDa active peptide (35, 37). As expected, a *gelE E. faecalis* mutant strain did not kill *BaraA* flies faster than wild-type flies (*SI Appendix*, Fig. S8*D*). We conclude that *BaraA* protects the flies from the action of the EntV bacteriocin.

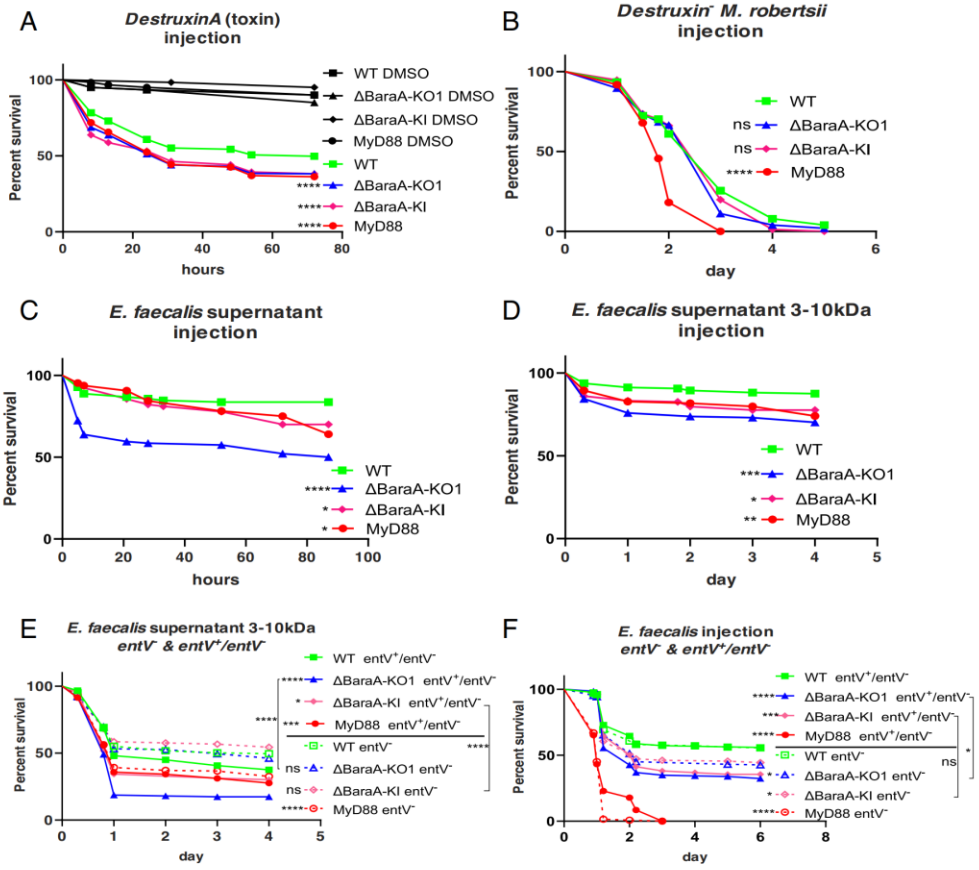

Fig. 4. *BaraA*-dependent protection of *Drosophila* flies from the noxious effects of microbial toxins. (*A*) Mutant flies were injected with 4.6 nl, 8 mM Destruxin A toxin. 80% DMSO was injected as vehicle control. Pooled data from eight independent experiments. Statistically significant differences between wild-type and *BaraA* mutants, ****$P < 0.0001$. (*B*) *BaraA* mutants were injected with 50 spores of *DestruxinS1*$^{-}$ *M. robertsii* mutant strain in which the biosynthesis of Destruxins is blocked. No significant difference was observed between wild-type and *BaraA* flies; pooled data from five independent experiments. (*C*) Wild-type, *MyD88*, and *BaraA* KO1 and KI mutant flies were injected with the concentrated supernatant from overnight *E. faecalis* OG1RF cultures. About 50% of *BaraA* KO1 mutant but not wild-type flies succumbed to this challenge, while 30% of the KI and MyD88 flies succumbed to this challenge. Pooled data from four independent experiments, *$P < 0.05$, ****$P < 0.0001$. (*D*) Same as (*C*), except that the supernatant was size filtered to retain molecules ranging from 3 to 10 kDa. Pooled data from four independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (*E*) The 3 to 10 kDa fraction supernatant from *E. faecalis* OG1RF was collected from *entV*$^{-}$ and *entV*$^{+}$*/entV*$^{-}$ strains. The supernatant from the *entV*$^{-}$ strain killed *BaraA* mutants at the same rate as wild-type flies, whereas *BaraA* KO1 mutants were killed by the complemented *entV*$^{+}$*/entV*$^{-}$ supernatant significantly faster than wild-type flies. Pooled data from eight independent experiments. (*F*) 0.5 OD, 4.6 nl of the mutant and rescued *E. faecalis* OG1RF strains were injected. Rescued strain *entV*$^{+}$*/entV*$^{-}$ killed *BaraA* KO1 faster than wild-type flies. Significant differences between wild-type and *BaraA* mutant flies were detected upon *entV*$^{-}$ infection. *BaraA* KO1 infected by *entV*$^{+}$*/entV*$^{-}$ strain was killed faster than *BaraA* KO1 infected by *entV*$^{-}$ strain, while no such significant difference was observed in the case of the *BaraA* KI line. Pooled data from four independent experiments. (*E* and *F*) For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies submitted to the same challenge for statistical analysis, *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

Taken together, our data suggest that a major function of *BaraA* in *Drosophila* host defense is to protect the fly from specific secreted microbial toxins, whether of prokaryotic or eukaryotic origin.

BaraA Helps *Drosophila* Recover from DtxA-Induced Paralysis and Is Required in Glial Cells. A striking phenotype observed upon the injection of DtxA is the immediate paralysis it induces as flies do not recover from anesthesia as untreated flies do. A careful scrutiny revealed that some 60% of wild-type flies progressively recovered their activity within 5 to 10 h of DtxA injection in contrast to less than 40% for *BaraA* KO flies (Fig. 5 *A* and *B* and Movies S1–S3). In contrast, the injection of the *E. faecalis* supernatant only temporarily slowed down the flies, a phenomenon difficult to quantify accurately.

This set of data suggested that the toxins may somehow interfere with the nervous system. We therefore silenced *BaraA* gene expression either in neurons or in glia and monitored the survival of flies to injected DtxA or *E. faecalis* supernatant. Silencing *BaraA* expression in glial cells but not in neurons enhanced the sensitivity of flies to these challenges (Fig. 5 *C*–*F*). In the case of DtxA, the effect was as strong as that observed upon using a ubiquitous driver (Fig. 5 *C* and *G*). In contrast, the degree of enhanced susceptibility to the injection of the *E. faecalis* supernatant was modest and unexpectedly none was detected upon the ubiquitous silencing of *BaraA* (Fig. 5*H*).

We conclude that BaraA function is required in glial cells where it may mediate the protection against the effects of DtxA on the nervous system.

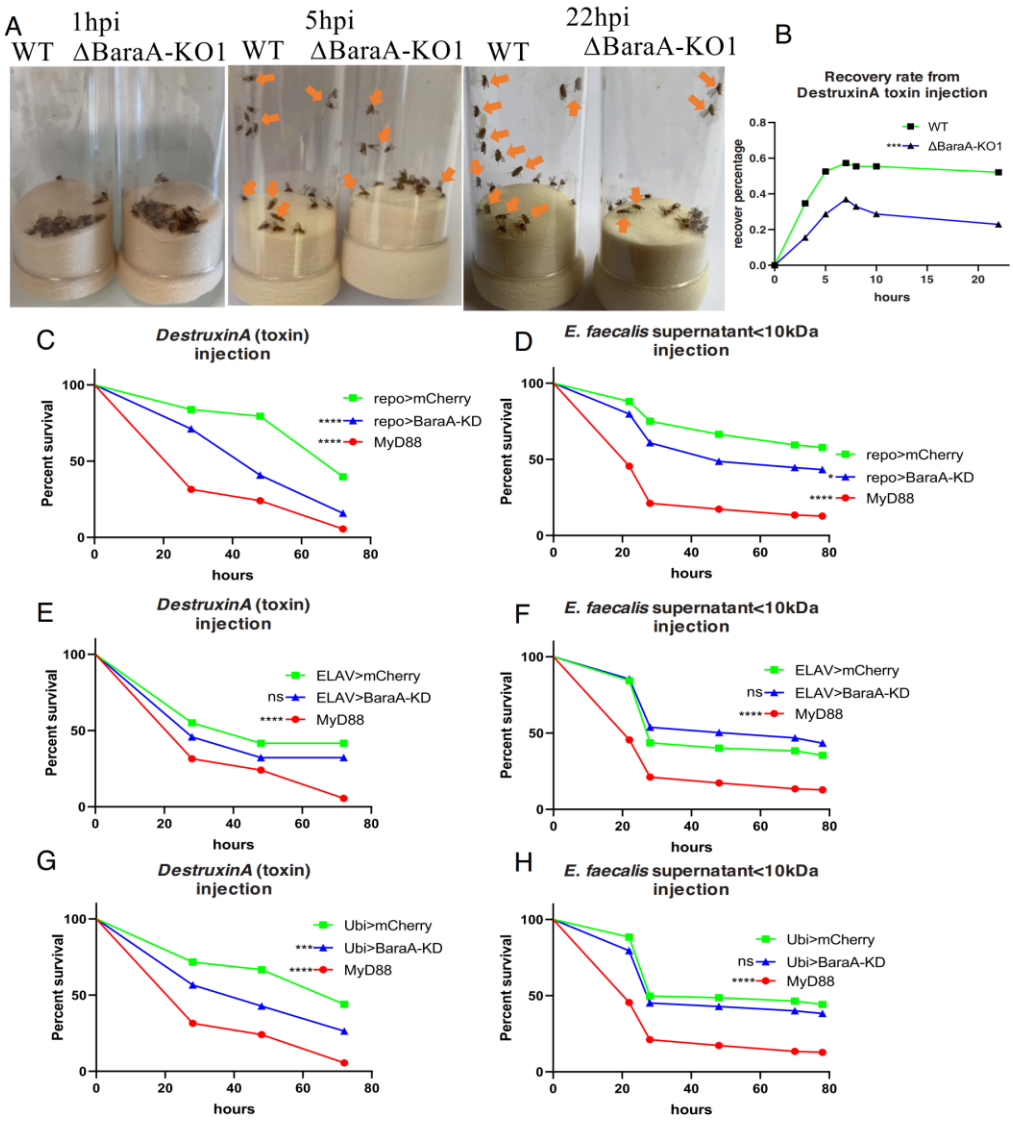

Fig. 5. BaraA counteracts the paralysis induced by exposure to Destruxin A and is required in glial cells. (*A*) Wild-type flies or *BaraA*-KO1 mutant flies were injected with 4.6 nl, 8 mM Destruxin A toxin. Pictures were taken at 1, 5, and 22 h postinfection (see also the corresponding Movies S1–S3). After 1 h postinfection, all flies were paralyzed. At 5 h postinfection, 11 wild-type flies woke up, whereas only six *BaraA*-KO1 mutant flies woke up from toxin injection. At 22 h postinfection, 12 wild-type flies woke up, whereas four *BaraA*-KO1 mutant flies woke up. (*B*) Quantification of (*A*). Pooled data from two independent experiments, ***$P < 0.0001$. (*C* and *D*) *BaraA*-KD flies were silenced in glial cells (*repo-Gal4*) and injected with 4.6 nl, 8 mM Destruxin A toxin (*C*) or 23 nl of *E. faecalis* supernatant <10 kDa (*D*). *BaraA*-KD flies displayed significant difference from wild-type control flies. Pooled data from four independent experiments, *$P < 0.05$, ****$P < 0.0001$. (*E* and *F*) *BaraA*-KD flies were silenced in neurons (*elav-Gal4*) and injected with 4.6 nl, 8 mM Destruxin A toxin (*E*) or 23 nl of *E. faecalis* supernatant <10 kDa (*F*). *BaraA*-KD flies showed no significant (ns) difference from wild-type control flies. Pooled data from four independent experiments. (*G* and *H*) *BaraA*-KD flies were silenced ubiquitously (*ubi-Gal4*) and were injected with 4.6 nl, 8 mM Destruxin A toxin (*G*) or 23 nl of *E. faecalis* supernatant <10 kDa (*H*). *Ubi-Gal4>BaraA* KD flies showed significant difference from wild-type control flies after Destruxin A injection (*G*). No significant difference between *BaraA* KD and wild-type control upon *E. faecalis* OG1RF supernatant injection. Pooled data from four independent experiments.

Discussion

Our analysis of the *BaraA* infection mutant phenotype revealed a sensitivity to specific pathogens and not to broad categories of microorganisms as is the case for Toll pathway mutants. Interestingly, we observed a susceptibility to *E. faecalis* and to *M. robertsii*, respectively, a Gram-positive bacterium and an entomopathogenic fungus. For both pathogens, specific secreted virulence factors killed a significant fraction of *BaraA* mutants, whereas the *BaraA* phenotype of enhanced sensitivity to infection was lost when the corresponding virulence factor genes were mutated in the pathogen. Taken together, these results indicate that a major function of *BaraA* in *Drosophila* host defense is to protect it from the action of specific secreted toxins. Indeed, whereas in a concurrent study we showed that Toll pathway mutant flies are sensitive to *A. fumigatus* restrictocin (30), *BaraA* mutants did not exhibit any enhanced sensitivity to

restrictocin, nor to Beauvericin, a toxin made by *Beauveria bassiana*, another entomopathogenic fungus that has been reported to kill *BaraA* mutants faster than wild-type flies (*SI Appendix*, Fig. S9) (24).

A recently published study proposed that BaraA is involved in resistance to infection to entomopathogenic fungi as an AMP since, besides being sensitive to *B. bassiana* and *Metarhizium rileyi*, *BaraA* mutants exhibit an increased *B. bassiana* load 48 h after infection (24). In addition, BaraA-derived IM10-like peptides synergize with a membrane-active antifungal compound to kill *C. albicans* in vitro (24). The fact that BaraA is a polyprotein that produces multiple DIM10-like peptides and that the *BaraA* locus is found to be duplicated in about 14% of wild-type *Drosophila* strains caught at one location is in keeping with this possibility, as these strains would be expected to produce twice as much BaraA-derived peptides (25). Because BaraA encodes a polyprotein precursor, we cannot formally exclude such an AMP function for one or several of these BaraA-derived peptides, possibly acting locally to achieve an effective antimicrobial concentration, for instance in the brain. Indeed, the Bomanin family presents a similar situation: Whereas we have shown a function for some specific 55C *Bomanins* in the resilience to *A. fumigatus* mycotoxins (30), it is known that at least some *Bomanin* genes are required for resistance to *E. faecalis* (22), a finding we have directly confirmed for at least one 55C *Bomanin* gene (38). We note that if DIM10-like peptides indeed act as AMPs, they would need to act rather specifically against *E. faecalis* and *M. robertsii* since we did not detect an enhanced sensitivity to the other pathogens tested in this study. Very specific antibacterial functions for some *Drosophila* AMPs have been documented (39–41); however, we are not aware of AMPs having dual specificities against both particular bacterial and fungal species. We observed an increased *E. faecalis* burden at one specific time point, which supports a function in resistance that may be mediated through antimicrobial activity as discussed above, or through a function in mediating melanization or the cellular immune response (Fig. 2 and *SI Appendix*, Fig. S4). Nevertheless, the findings that bacterial and fungal toxin mutants kill *BaraA* mutant as efficiently as wild-type mutant flies and that ubiquitous *BaraA* overexpression does not enhance the protection against *E. faecalis* or *M. robertsii* support the concept that an important function of BaraA is to neutralize or counteract the action of specific secreted microbial toxins in the case of *E. faecalis* or *M. robertsii* infections.

Interestingly, several studies have shown that besides their direct antimicrobial functions, mammalian α-defensins have the remarkable property to neutralize some microbial pore-forming toxins or enzymes that need to cross the host cell plasma membrane to act on their intracellular targets (42 and references therein). The proposed mechanism of action of these AMPs relies on a common property of these microbial virulence factors: a relative thermodynamic instability that is required for the necessary flexibility to insert into or cross the plasma membrane. α-defensins are constituted by amphipatic α-helices that through hydrophobic interactions with the targeted enzymes are able to destabilize them (42). The unfolded proteins can then be degraded. We know little about the biochemistry of BaraA-derived peptides and no antimicrobial activity at physiological concentrations has been yet found in vitro in the absence of a cofactor. While a similar mechanism may be at play with the EntV protein, it is less likely to function with a hexadepsipeptide that is circular (31) and thus likely difficult to destabilize and degrade because of its circular conformation, even though it is rather hydrophobic.

A study on the evolution of BaraA as well as two related paralogs generated by independent duplication events suggests that the core domain of these three proteins is the N-terminal DIM24 domain, which is associated with only two DIM10-like domains in BaraB and none in BaraC (25). The expression of *BaraB* and *BaraC* has been reported not to be induced by an immune challenge (25), a finding we have independently confirmed for *BaraB*. We did not find a susceptibility of *BaraB* KO mutant to *E. faecalis* infection (43), in keeping with a reported lack of detectable immune function (25). BaraB function is essential in neurons, whereas BaraC appears to be expressed in glial cells (25). Interestingly, a *BaraA* expression fluorescent reporter is detected in brain tissues (24). Thus, it is likely that the DIM24 domain may have a function distinct from the DIM10-like peptides that are thought to act more like AMPs, although definitive evidence is presently lacking. These observations taken together with our finding of a requirement for *BaraA* expression in glial cells to protect the host against the noxious effects of DtxA therefore open the possibility that the DIM24 peptide might mediate this function, a proposition that requires experimental validation.

The exact mode of action of BaraA-derived peptides in the resilience to microbial toxins remains to be characterized, in as much as they act against distinct types of toxins. Destruxins have been isolated 60 y ago and they appear to act as ionophores that deplete cellular ions such as H^+, Na^+, and K^+ through the formation of pores in the membranes in a reversible process (44), although many other functions have been proposed (31). ClassII bacteriocins also form pores on the membrane of targeted bacteria, but the specific molecular mechanism of action of EntV on eukaryotic cells remains unknown. It presents an activity against the formation of biofilms by the dimorphic yeast *C. albicans* or the monomorphic yeast *C. glabrata*. Furthermore, it prevents filamentation of the former in vitro and in vivo (34). Thus, it is unclear whether BaraA would act directly on both toxins, through the same or distinct BaraA-derived peptides, would counteract a common process triggered by Destruxins and EntV such as intracellular ion depletion, or would indirectly alter the physiology of cells exposed to the action of these toxins. It will be important to determine how the toxins act on the host and whether they target preferentially some tissues. For instance, it will be interesting to determine whether the function of BaraA in glial cells is linked to the blood–brain barrier. An emerging theme is that some of the Toll pathway effectors act in the brain and counteract the noxious effects of toxins that also act on the nervous system as exemplified here with the requirement for BaraA expression in glial cells. Interestingly, we have recently found that *BomS6* overexpression in the brain protects the flies from the effects of the injected *A. fumigatus* toxin verruculogen (30).

A specificity of the Toll pathway is that it is required in the host defense against both prokaryotic and eukaryotic pathogens. As compared to the Immune deficiency (IMD) pathway, one interesting feature is that the Toll pathway can be activated by proteases secreted by invading pathogens (45–47). It is interesting to note here that the function of BaraA against two distinct secreted virulence factors, likely pore-forming toxins, provides another point of convergence for the dual role of the Toll pathway, this time at the effector level. It is thus an open possibility that one of the selective pressures that shaped the function of the Toll pathway would be the need to cope with pathogens secreting virulence factors in the extracellular compartment.

Taken together with a concurrent study (30), our work underscores that the Toll pathway mediates resilience against the action of multiple toxin types such as pore-forming toxins, ribotoxins, or tremorgenic toxins, which are mediated by specific Bomanins or BaraA-derived proteins. It is likely that other uncharacterized effectors are able to counteract other toxins to which *Drosophila* flies are exposed to in the wild. In contrast to the current paradigm

according to which secreted peptides act as AMPs, our discoveries illustrate an original concept in insect innate immunity, the ability of the host to counteract secreted microbial virulence factors by dedicated effectors of the immune response.

Materials and Methods

Fly Strains. Fly lines were raised on media at 25° with 65% humidity. For 25 L fly food medium, 1.2 kg cornmeal (Priméal), 1.2 kg glucose (Tereos Syral), 1.5 kg yeast (Bio Springer), and 90 g nipagin (VWR Chemicals) were diluted into 350 mL ethanol (Sigma-Aldrich), 120 g agar-agar (Sobigel), and water qsp.

w^{A5001} (48) and occasionally *yw* flies were used as wild-type controls as needed. The positive controls for infection assays for Gram-positive/fungal infections and Gram-negative infections were, respectively, *MyD88* and *key* in the w^{A5001} background. Where stated, mutant flies were isogenized in the w^{A5001} background. For RNAi experiments, virgin females carrying the *Ubi-Gal4, ptub1-Gal80^{ts}* (*Ubi-Gal4, Gal80^{ts}*), *repo*-Gal4, and *elav*-Gal4 transposon were crossed to Trip lines males carrying an UAS-RNAi (Upstream Activation Sequence-RNA interference) transgene (TRiP) from the Tsinghua RNAi Center: THU0393 (*BaraA* KD), THU02336.N (*CG18278* KD). The control flies were the offspring of the cross of the driver to UAS-mCherry RNAi VALIUM20 (Bloomington Stock Center #BL35785). Crosses with the *Ubi-Gal4, Gal80^{ts}* driver were performed at 25 °C for 3 d, then the progeny was left to develop at the nonpermissive 18 °C temperature. The hatched flies were kept at 29 °C for 5 d prior to the experiment to allow Gal4-mediated transcription. All crosses involving flies without RNAi expression were performed at 25 °C. Unless stated otherwise, female flies were 5 to 7 d old at the beginning of each experiment.

To generate axenic flies, standard fly media was autoclaved. Antibiotics were added (ampicillin 50 µg/mL, kanamycin 50 µg/mL, tetracyclin 50 µg/mL, erythromycin 15 µg/mL) when it cooled down to 50 to 60°. The embryos were bleached and then cultured on the sterilized media. The sterility of axenic flies (20 d old) was checked on Lysogeny Broth (LB), Bushnell-Haas medium (BHB), Yeast Extract–Peptone–Dextrose (YPD), and deMan, Rogosa, Sharpe (MRS) plates.

Pathogen Infections. The bacterial strains used in this study include the Gram-negative bacterium *Pectobacterium carotovorum carotovorum* 15 (strain *Ecc15*, Optical Density $(OD)_{600} = 50$) and the Gram-positive strains *E. faecalis* National Collection of Type Cultures (NCTC) 775 (American Type Tissue Culture Collection (ATCC) 19433) or OG1RF (ATCC 47077) (OD = 0.1), *Micrococcus luteus* (OD = 200), and *Staphylococcus albus* (OD = 10), as well as *gelE*, *entV*$^-$, and complemented *entV*$^+$/*entV*$^-$ and, which are derivatives of the wild-type *E. faecalis* OG1RF strain (OD = 0.5) (kind gifts of Profs. Garsin and Lorenz, Houston, USA) (34). The fungal strains we used include filamentous fungi, *A. fumigatus* (5×10^7 spores/mL, 250 spores in 4.6 nL), *M. robertsii* (ARSEF2575, 1×10^7 spore/mL, natural infection 5×10^4/mL), and *DestruxinS1* mutant strain (1×10^7 spore/mL), a kind gift from Prof. Wang, Shanghai, China (32). Besides, we used yeast as well, *C. albicans* (pricked) and *C. glabrata* (1×10^9 yeasts/mL). The following media were used to grow the strains: Yeast extract–Peptone–Glucose Broth Agar (YPDA, *C. albicans* and *C. glabrata*) or LB–all others at 29 °C (*Ecc15*, *M. luteus*, *C. albicans*, *C. glabrata*) or 37 °C, *entV*$^-$, *entV*$^+$/*entV*$^-$ *E. faecalis*, Brain Heart Infusion (BHI) medium, 37 °C overnight, rifampicin 100 µg/mL. Spores of *M. robertsii* and *A. fumigatus* were grown on Potato Dextrose Agar (PDA) plates at 25 °C or 29 °C (*A. fumigatus*) for approximately 1 wk or 3 wk (*A. fumigatus*) until sporulation. We injected 4.6 nL of the suspension into each fly thorax using a Nanoject III (Drummond). Natural infections were initiated by shaking anesthetized flies in 5 mL 0.01% tween-20 solution containing *M. robertsii* conidia at a concentration of 5×10^4/mL. Infected flies were subsequently maintained at 29 °C (*C. albicans*, *C. glabrata*, *A. fumigatus, M. robertsii*) or at 25 °C (for all other pathogens, except for experiments with RNAi KD flies performed at 29 °C). The flies were anesthetized with light CO2 for about 3 min during the injection procedure and were observed 3 h after injection to confirm recovery from manipulations. Survival experiments were usually performed on three batches of 20 flies tested in parallel and independent experiments pooled for statistical analysis using the log-rank test.

Quantification of the pathogen burden in infected flies. To characterize the dynamics of within-host microbial loads or BLUDs or FLUDs, live flies were taken at each time point postinjection for pathogen load or flies were infected with *E. faecalis* or *M. robertsii* and vials were monitored every 30 min for newly dead flies (PLUD). These flies were then individually homogenized with a bead in 100 µL Phosphate Buffered Saline (PBS) with 0.01% tween-20 (PBST: Phosphate Buffered Saline tween) or PBS. Homogenates were diluted serially (10-fold dilutions, checked by fivefold dilutions for some BLUD experiments) in PBST (or PBS) and spread on LB (*E. faecalis*) or PDA (*M. robertsii*) plates for incubation at 37 °C (*E. faecalis*) or 25 °C (*M. robertsii*) overnight. Colonies were counted manually. Data were obtained from at least three independent experiments and pooled.

Western Blots. For western blots, hemolymph samples were collected from 50 flies in a protease inhibitor solution (PBS+:phenylmethylsulfonyl fluoride (PMSF)). Protein concentration of the samples was determined by Bradford assay. 30 µg protein was separated on an 8% gel by SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% bovine serum albumin in PBST for 1 h at room temperature, samples were incubated at 4 °C overnight with rabbit antibodies against *Drosophila* PPO1 at a 1:10,000 dilution (a kind gift from Prof. Erjun Ling). After washes, a goat anti-rabbit-horseradish peroxidase (HRP) secondary antibody at a 1:20,000 dilution was incubated for 1 h at room temperature. Enhanced chemiluminescence substrate (ECL, General Electric Healthcare) was used according to the manufacturer's instructions to reveal the blot.

Gene Expression Quantitation. We followed the protocol as described (49) using primer pairs displayed in *SI Appendix*, Table S4.

Survival Tests. Survival tests were performed using 20 to 25 flies per vial in biological triplicates. Female adult flies used for survival tests were 5 to 7-d old. For survival tests using RNAi-silencing genes, flies were crossed at 25 °C for 3 d for laying eggs and then transferred to 18 °C; after hatching, flies were kept for at least 5 d at 29 °C prior to infections. Flies were counted every day. Each experiment shown is representative of at least two independent experiments.

Toxin Injection. Destruxin A (MedChemExpress) was resuspended in high-quality-grade dimethyl sulfoxide (DMSO) and was diluted in PBS to a 8-mM concentration. 4.6 nL of the solution or of control DMSO diluted in PBS at the same concentration was injected into flies using the Nanoject III microinjector (Drummond). Restrictocin (Sigma) was resuspended in PBS to the concentration of 1 mg/mL, 4.6 nL was injected. Beauvericin (Sigma) was resuspended in high-quality-grade DMSO. 20 mM, 9.2 nL was injected.

Collection and Preparation of *E. faecalis* Supernatants. Filter-sterilized supernatant phases were obtained from 10 mL overnight cultures grown in LB medium that were collected by centrifugation at 4,000 rpm for 10 min. The supernatants were sterilized by passing through a 0.2-µm-pore-size sterile syringe filter. The sterilized supernatants were centrifuged through a 15-mL Amicon Centricon filter (Millipore) to separately collect the molecules larger or lower than 10 kDa. 1.5 mL Eppendorf tubes were used to collect the supernatant lower than 10 kDa, which were vacuum freeze-dried for 24 h. The powder was resuspended with H_2O and thus concentrated 10 to 20-fold. The solution was filtered on 3 kDa Amicon Centricon filter columns (Millipore) by centrifugation at 10,000 rpm for 30 min. The nonfiltered fraction was then injected into flies with a volume optimized according to the batch (16 to 69 nL) and the same volume of buffer was used for the controls. All experiments were performed at least three times.

Statistics. Statistical analyses were performed using GraphPad software Prism 6 and R. Data were expressed as means ± SEM. RT-qPCR data were analyzed by ANOVA (one-way) with Dunnett's multiple comparisons test, with a significance threshold of $P = 0.05$. Log-rank tests were used to determine whether survival curves of female flies were significantly different from each other. Experiments measuring microbial loads (log2 values) were analyzed using linear models (lm) or linear mixed-effect models (lmer, package lme4) (50) in order to include the different factors of the experiment, such as the fly line or the treatment, and to include random factors, such as the experimental replicates. Significance of interactions between factors was tested by comparing models fitting the data with and without the interactions, using ANOVA. Models were simplified when interactions were not significant. Pairwise comparisons of the estimates from fitted models were analyzed using lmerTest, lsmean, and multcomp packages. Details are included in the legend of each figure. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Data, Materials, and Software Availability. Raw data have been deposited in Figshare (51).

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1. B. Lemaitre, J. Hoffmann, The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25**, 697–743 (2007).
2. B. P. Lazzaro, M. Zasloff, J. Rolff, Antimicrobial peptides: Application informed by evolution. *Science* **368**, eaau5480 (2020).
3. S. J. H. Lin, L. B. Cohen, S. A. Wasserman, Effector specificity and function in *Drosophila* innate immunity: Getting AMPed and dropping Boms. *PLoS Pathog.* **16**, e1008480 (2020).
4. M. A. Hanson, B. Lemaitre, New insights on *Drosophila* antimicrobial peptide function in host defense and beyond. *Curr. Opin. Immunol.* **62**, 22–30 (2020).
5. R. Medzhitov, D. S. Schneider, M. P. Soares, Disease tolerance as a defense strategy. *Science* **335**, 936–941 (2012).
6. D. Ferrandon, The complementary facets of epithelial host defenses in the genetic model organism *Drosophila melanogaster*: From resistance to resilience. *Curr. Opin. Immunol.* **25**, 59–70 (2013).
7. M. P. Soares, L. Teixeira, L. F. Moita, Disease tolerance and immunity in host protection against infection. *Nat. Rev. Immunol.* **17**, 83–96 (2017).
8. X. Li, S. Rommelaere, S. Kondo, B. Lemaitre, Renal purge of hemolymphatic lipids prevents the accumulation of ROS-induced inflammatory oxidized lipids and protects *Drosophila* from tissue damage. *Immunity* **52**, 374–387.e376 (2020).
9. K. Troha, J. H. Im, J. Revah, B. P. Lazzaro, N. Buchon, Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in *D. melanogaster*. *PLoS Pathog.* **14**, e1006847 (2018).
10. K. Z. Lee *et al.*, Enterocyte Purge and Rapid Recovery Is a Resilience Reaction of the Gut Epithelium to Pore-Forming Toxin Attack. *Cell Host. Microbe.* **20**, 716–730 (2016).
11. D. Duneau *et al.*, Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in *D. melanogaster*. *eLife* **6**, e28298 (2017).
12. P. Lafont, C. Lauzeral, D. Duneau, J.-B. Ferdy, A within-host infection model to explore tolerance and resistance. bioRxiv (2021), https://doi.org/10.1101/2021.10.19.464998 (Accessed 20 October 2021).
13. P. Irving *et al.*, A genome-wide analysis of immune responses in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 15119–15124 (2001).
14. E. De Gregorio, P. T. Spellman, G. M. Rubin, B. Lemaitre, Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12590–12595 (2001).
15. M. Boutros, H. Agaisse, N. Perrimon, Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev. Cell* **3**, 711–722 (2002).
16. E. De Gregorio, P. T. Spellman, P. Tzou, G. M. Rubin, B. Lemaitre, The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *Embo J.* **21**, 2568–2579 (2002).
17. P. Fehlbaum *et al.*, Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J. Biol. Chem.* **269**, 33159–33163 (1995).
18. E. A. Levashina *et al.*, Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila* with antibacterial and antifungal properties. *Eur. J. Biochem.* **233**, 694–700 (1995).
19. L. B. Cohen, S. A. Lindsay, Y. Xu, S. J. H. Lin, S. A. Wasserman, The Daisho peptides mediate *Drosophila* defense against a subset of filamentous fungi. *Front. Immunol.* **11**, 9 (2020).
20. S. Uttenweiler-Joseph *et al.*, Differential display of peptides induced during the immune response of *Drosophila*: A matrix-assisted laser desorption ionization time-of- flight mass spectrometry study. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 11342–11347 (1998).
21. F. Levy *et al.*, Peptidomic and proteomic analyses of the systemic immune response of *Drosophila*. *Biochimie* **86**, 607–616 (2004).
22. A. W. Clemmons, S. A. Lindsay, S. A. Wasserman, An effector peptide family required for *Drosophila* Toll-mediated immunity. *PLoS Pathog.* **11**, e1004876 (2015).
23. S. A. Lindsay, S. J. H. Lin, S. A. Wasserman, Short-form bomanins mediate humoral immunity in *Drosophila*. *J. Innate. Immun.* **10**, 306–314 (2018).
24. M. A. Hanson *et al.*, The *Drosophila Baramicin* polypeptide gene protects against fungal infection. *PLoS Pathog.* **17**, e1009846 (2021).
25. M. A. Hanson, L. Bruno, Repeated truncation of a modular antimicrobial peptide gene for neural context. *PLoS Genet.* **18**, (2022).
26. S. J. H. Lin, A. Fulzele, L. B. Cohen, E. J. Bennett, S. A. Wasserman, Bombardier enables delivery of short-form bomanins in the *Drosophila* Toll response. *Front. Immunol.* **10**, 3040 (2019).
27. B. Lemaitre, E. Nicolas, L. Michaut, J. M. Reichhart, J. A. Hoffmann, The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983 (1996).
28. D. Ferrandon, J. L. Imler, C. Hetru, J. A. Hoffmann, The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. *Nat. Rev. Immunol.* **7**, 862–874 (2007).
29. M. S. Busse, C. P. Arnold, P. Towb, J. Katrivesis, S. A. Wasserman, A kappaB sequence code for pathway-specific innate immune responses. *Embo J.* **26**, 3826–3835 (2007).
30. R. Xu *et al.*, The Toll pathway mediates *Drosophila* resilience to *Aspergillus* mycotoxins through specific Bomanins. *EMBO Rep.* e56036 (2022), 10.15252/embr.202256036.
31. M. S. Pedras, L. Irina Zaharia, D. E. Ward, The destruxins: Synthesis, biosynthesis, biotransformation, and biological activity. *Phytochemistry* **59**, 579–596 (2002).
32. B. Wang, Q. Kang, Y. Lu, L. Bai, C. Wang, Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 1287–1292 (2012).
33. N. Teixeira *et al.*, *Drosophila* host model reveals new *Enterococcus faecalis* quorum-sensing associated virulence factors. *PLoS One* **8**, e64740 (2013).
34. C. E. Graham, M. R. Cruz, D. A. Garsin, M. C. Lorenz, *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 4507 (2017).
35. H. Dundar *et al.*, The fsr Quorum-sensing system and cognate gelatinase orchestrate the expression and processing of proprotein EF_1097 into the mature antimicrobial peptide Enterocin O16. *J. Bacteriol.* **197**, 2112–2121 (2015).
36. M. R. Cruz *et al.*, Structural and functional analysis of EntV reveals a 12 amino acid fragment protective against fungal infections. *Nat. Commun.* **13**, 6047 (2022).
37. A. O. Brown *et al.*, Antifungal activity of the *Enterococcus faecalis* peptide EntV requires protease cleavage and disulfide bond formation. *mBio* **10**, e01334-19 (2019).
38. Y. Lou, *Study of the Function of Bomanin Genes at the 55C Locus in Drosophila melanogaster Host Defense against Microbial Infections* (Université de Strasbourg, 2021).
39. R. L. Unckless, V. M. Howick, B. P. Lazzaro, Convergent balancing selection on an antimicrobial peptide in *Drosophila*. *Curr. Biol.* **26**, 257–262 (2016).
40. M. A. Hanson *et al.*, Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *eLife* **8**, e44341 (2019).
41. M. A. Hanson, S. Kondo, B. Lemaitre, *Drosophila* immunity: The *Drosocin* gene encodes two host defence peptides with pathogen-specific roles. *Proc. Biol. Sci.* **289**, 20220773 (2022).
42. E. Kudryashova *et al.*, Human defensins facilitate local unfolding of thermodynamically unstable regions of bacterial protein toxins. *Immunity* **41**, 709–721 (2014).
43. J. Huang, *Study of Two Toll Pathway Effector Genes Involved in Resilience and Resistance to Microbial Infections in Drosophila* (Université de Strasbourg, Strasbourg, 2022), p. 151.
44. S. R. Shanbhag, A. T. Vazhappilly, A. Sane, N. M. D'Silva, S. Tripathi, Electrolyte transport pathways induced in the midgut epithelium of *Drosophila melanogaster* larvae by commensal gut microbiota and pathogens. *J. Physiol.* **595**, 523–539 (2017).
45. M. Gottar *et al.*, Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors. *Cell* **127**, 1425–1437 (2006).
46. L. El Chamy, V. Leclerc, I. Caldelari, J. M. Reichhart, Sensing of "danger signals" and pathogen-associated molecular patterns defines binary signaling pathways "upstream" of Toll. *Nat. Immunol.* **9**, 1165–1170 (2008).
47. N. Issa *et al.*, The circulating protease persephone is an immune sensor for microbial proteolytic activities upstream of the *Drosophila* Toll pathway. *Mol. Cell* **69**, 539–550.e536 (2018).
48. S. T. Thibault *et al.*, A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nat. Genet.* **36**, 283–287 (2004).
49. C. Huang *et al.*, Differential requirements for mediator complex subunits in *Drosophila melanogaster* host defense against fungal and bacterial pathogens. *Front. Immunol.* **11**, 478958 (2020).
50. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. *J. Stat. Software* **67**, 1–48 (2015).
51. J. Huang, A toll pathway effector protects Drosophila specifically from distinct toxins secreted by a fungus or a bacterium. Figshare. https://doi.org/10.6084/m9.figshare.21967805.v3. Deposited 31 January 2023.

Supporting Information Appendix for:

A Toll pathway effector protects Drosophila *specifically from distinct toxins secreted by a fungus or a bacterium*

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This PDF file includes:

Supporting text
Figures S1 to S9

Other supporting materials for this manuscript include the following:

Movies S1 to S3
Tables S1-S4

Supplementary Material and Methods

Generation of CRISPR/Cas9-mediated null mutants

The *BaraA* (*CG18279*) and *CG18278* null mutants were generated using CRISPR/Cas9 technology based on the expression of gRNA transgenes that were then crossed to a transgenic line expressing a *pnos-Cas9* transgene. The 20bp-long gRNAs for the target genes were devised using web-based CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/). The plasmids carrying DNA sequences for the production of single strand gRNAs were constructed using standard methods. Briefly, the oligonucleotides were synthesized, denatured, and annealed to get double strand DNA before ligation into the expression vector, in which the gRNA coding sequences were transcribed under the control of the U6:3 promoter.

Plasmids carrying different gRNA targets were grouped by three or six for microinjection to obtain the gRNA transgenic fly lines, which were checked by sequencing. The gRNAs expressing plasmids were designed to be inserted on the 3rd chromosome using y^{1} M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb (BL24749) flies. The gRNA flies were balanced before being crossed to flies carrying the *nosP-Cas9* transgene, to induce inheritable mutations. The primers used to generate the knock out mutants are shown in Table S3.

Knock-in strategy

PCRs were done with the Q5 Hot-start 2× master mix (New England BioLabs, NEB), and cloning was performed using the Gibson Assembly 2× Master Mix (NEB) following the manufacturer's instructions. The pCFD5 (U6:3-(t :: RNA^{Cas9})) plasmid vector was used. A cloning protocol to generate the pCFD5 plasmids encoding one to six tRNA-flanked sgRNAs was followed as described (1). The primers used to generate the pCFD5 vector containing the gRNAs are shown in Table S1. We used a pSK vector as donor plasmid with the homology arms flanking the mCherry:

a fragment 1552bp upstream of *BaraA* had been amplified as a left arm; a fragment 1952bp downstream of *CG30059* as a right arm. Left arm + mCherry + right arm have been assembled (Gilson Assembly) and the resulting fragment ligated to Pst1-Spe1 double-digested pSK and checked by sequencing. The plasmid mixture containing the two plasmids at a ration pCFD5:pSK=3:1, was injected into recipient y^1 M{Act5C-Cas9.P.RFP-}ZH-2A w^{1118} DNAlig4^{169} embryos.

Overexpression strategy

Normal PCRs in first and second round were performed to amplify the ORF of BaraA constructing in pDONR221 with attP site (2). The primers used are shown in Table S3. BP recombination reaction was performed using with DH5α competent cells (Invitrogen); next, sequence-confirmed ORF entry clones were transferred to the destination vector pGW-HA.attB using a Gateway LR reaction (Gibson assembly). After validation by sequencing, the plasmids were injected in a pool into y^1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb embryos and missing constructs were reinjected alone.

Molecular mass fingerprints by MALDI MS

Each individual hemolymph sample was analyzed with the Bruker AutoFlex™ III based on Bruker Daltonics' smartbeam laser technology. The molecular mass fingerprints (MFP) were acquired using a sandwich sample preparation on a MALDI MTP 384 polished ground steel plate (Bruker Daltonics Inc., Germany). Briefly, the hemolymph samples were 10-fold diluted in acidified water (0.1% trifluoroacetic acid - 0.1% TFA, Sigma Aldrich, France), 0.6µL was deposited on a thin layer of an air-dried saturated solution (0.6µL) of the matrix

alpha-cyano-4-hydroxycinnamic Acid (4-HCCA, Sigma Aldrich, France) in pure acetone. Then 0.4 µL of a saturated solution of 4-HCCA prepared in 50% acetonitrile acidified with 0.1% TFA was mixed with the *Drosophila* hemolymph. Following co-crystallization of the hemolymph spots with the second matrix droplet and evaporation under mild vacuum, MALDI MS spectra were recorded in a linear positive mode and in an automatic data acquisition using FlexControl 4.0 software (Bruker Daltonics Inc.). The following instrument settings were used: the pseudo-molecular ions desorbed from the hemolymph were accelerated under 1.3kV, dynamic range of detection of 600 to 18,000 Da, between 50-60% of laser power, a global attenuator offset of 60% with 200Hz laser frequency, and 2,000 accumulated laser shots per hemolymph spectrum. The linear detector gain was setup at 1,906V with a suppression mass gate up to *m/z* 600 to prevent detector saturation by clusters of the 4-HCCA matrix. An external calibration of the mass spectrometer was performed using a standard mixture of peptides and proteins (Peptide Standard Calibration II and Protein Standard Calibration I, Bruker Daltonik) covering the dynamic range of analysis. All of the recorded spectra were processed with a baseline subtraction and spectral smoothing using FlexAnalysis 4.0 software (Bruker Daltonics Inc.).

Supplementary References

1. F. Port, S. L. Bullock, Augmenting CRISPR applications in Drosophila with tRNA-flanked sgRNAs. *Nat Methods* **13**, 852-854 (2016).
2. J. Bischof, E. M. Sheils, M. Bjorklund, K. Basler, Generation of a transgenic ORFeome library in Drosophila. *Nat Protoc* **9**, 1607-1620 (2014).

Legends to Supplementary movies

Supplementary movie 1

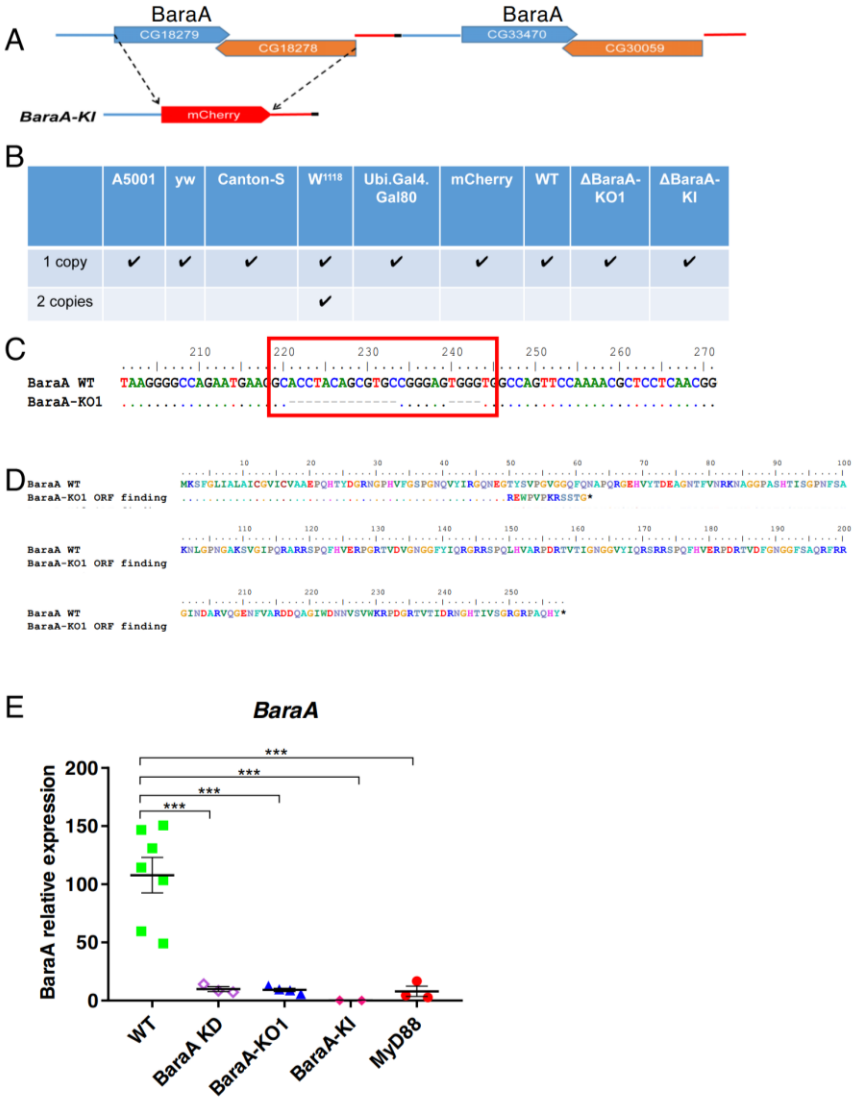
Wild-type (left) and *BaraA* KO1 (right) flies were inspected one hour after the injection of Destruxin A.

Supplementary movie 2

Wild-type (top) and *BaraA* KO1 (bottom) flies were inspected five hours after the injection of Destruxin A.

Supplementary movie 3

Wild-type (left) and *BaraA* KO1 (right) flies were inspected 22 hours after the injection of Destruxin A.



	A5001	yw	Canton-S	W1118	Ubi.Gal4. Gal80	mCherry	WT	ΔBaraA-KO1	ΔBaraA-KI
1 copy	✔	✔	✔	✔	✔	✔	✔	✔	✔
2 copies				✔					

Figure S1. Mutants affecting the BaraA locus.

(A) Scheme of the tandem duplication of the *BaraA/CG18278(CG30059)* locus according to the Drosophila genome sequence; *CG18279* and *CG33470* (*BaraA*) on the one hand and *CG18278* and *CG30059* are perfectly duplicated, including 1172bp 5' to *CG18279* or *CG33470* start codon (shown as a blue line) and 774bp 5' to *CG18278* or *CG30059* start codon (shown as a red line). The black line represents the short unique region at the overlap of the duplicated loci. In the KI fly line, the two genes (*CG18279*, *CG18278*) were replaced by *mCherry* coding sequence after the START codon from *CG18279*. **(B)** Table recapitulating the tested strains and the presence of the duplication. The KI line was originally generated in a *yw* background with only one copy of the locus. **(C)** CRISPR Cas9 knock out mutants of *BaraA*: KO1 has a complex deletion pattern removing 17bp in total. **(D)** The small deletions found in the KO1 line leads to a frame shift mutation that generates an early stop codon (*). **(E)** *BaraA* expression level measured by RTqPCR in wild-type, knock down (KI), knock out (KO), knock in (KI), and *MyD88* flies, 24h after a *M. luteus* challenge. Data are expressed as means ± SEM. Pooled data from two independent experiments, **** $p<0.0001$

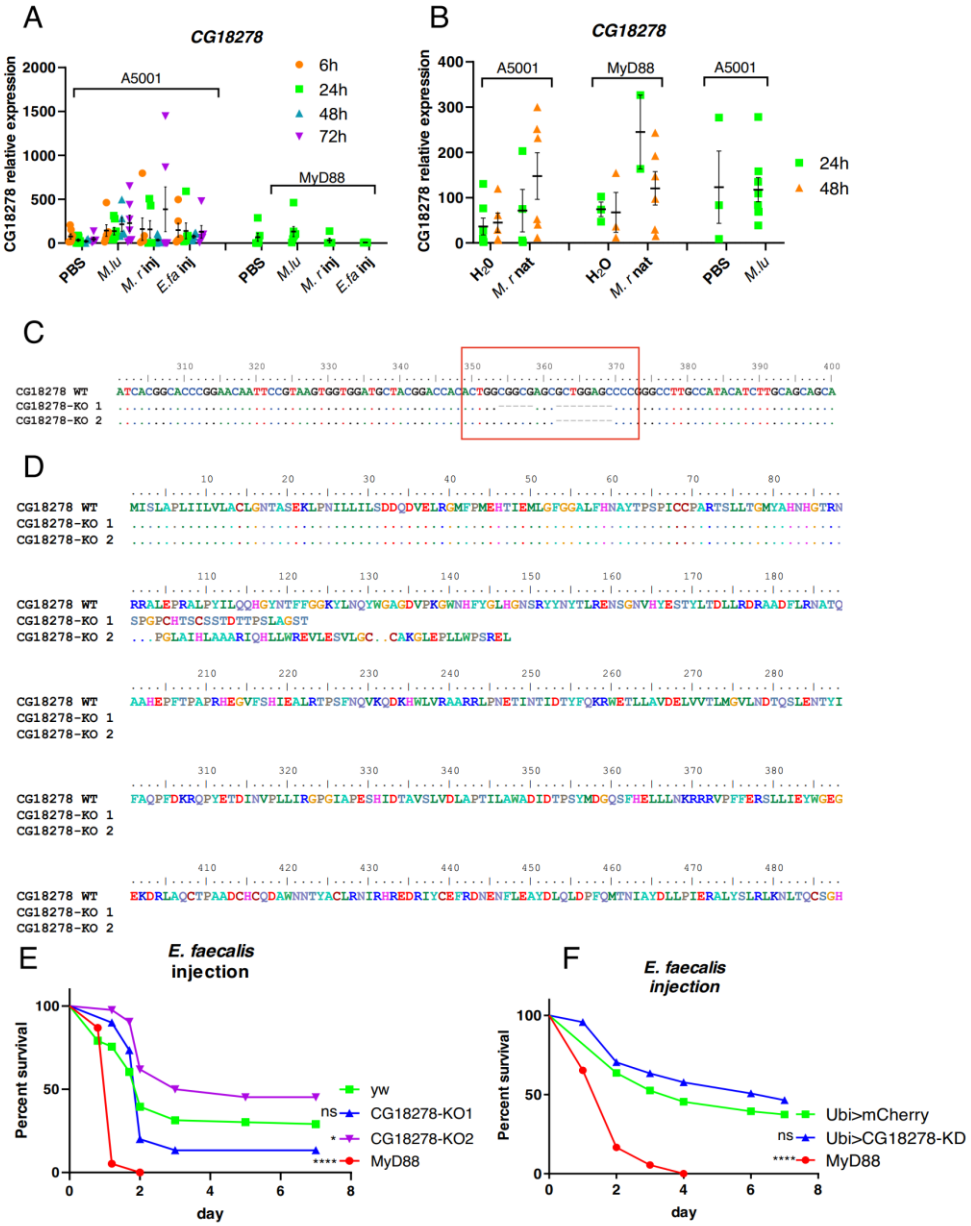


Figure S2. The *BaraA* neighboring gene *CG18278* is not involved in host defenses against *E. faecalis*.

(A) Expression of the *CG18278* gene monitored by RTqPCR in wild-type w^{A5001} and *MyD88* mutant flies at various time points after the injection of the indicated microbes; *M. lu: M. luteus*; *M. r: M. robertsii*; *E. fa: E. faecalis*. Data are expressed as means ± SEM. **(B)** Expression of the *CG18278* gene monitored by RTqPCR at 24 and 48 hours after a "natural" *M. robertsii* infection achieved by plunging the flies in a solution of conidia. Data were normalized to w^{A5001} with *M. luteus* challenged after 24 hours. Ct values from the RTqPCR for *CG18278* were in the 35-38 range while the Ct value for the Rpl32 were in the 18-20 range, which indicates that *CG18278* has low basal expression in fly. Data are expressed as means ± SEM. **(C, D)** Two lines of the CRISPR Cas9 mutant have been generated: *CG18278*-KO1 has a set of two small deletions removing altogether 13bp deletion whereas 8bp are deleted in the *CG18278* KO2 line. These deletions lead to frame shift mutations and early stop codons (D). **(E)** Nonisogenized *CG18278* KO1 mutant behaved like the *yw* reference line when infected by *E. faecalis* NCTC 775 while *CG18278* KO2 mutant displayed a slight protection compared to *yw*. Two independent experiments have been pooled, * $p<0.05$, **** $p<0.0001$. **(F)** Flies in which *CG18278* is attenuated by RNAi KD driven by *Ubi*-Gal4 displayed a sensitivity to *E. faecalis* NCTC 775 similar to that of the wild type. Two independent experiments have been pooled.

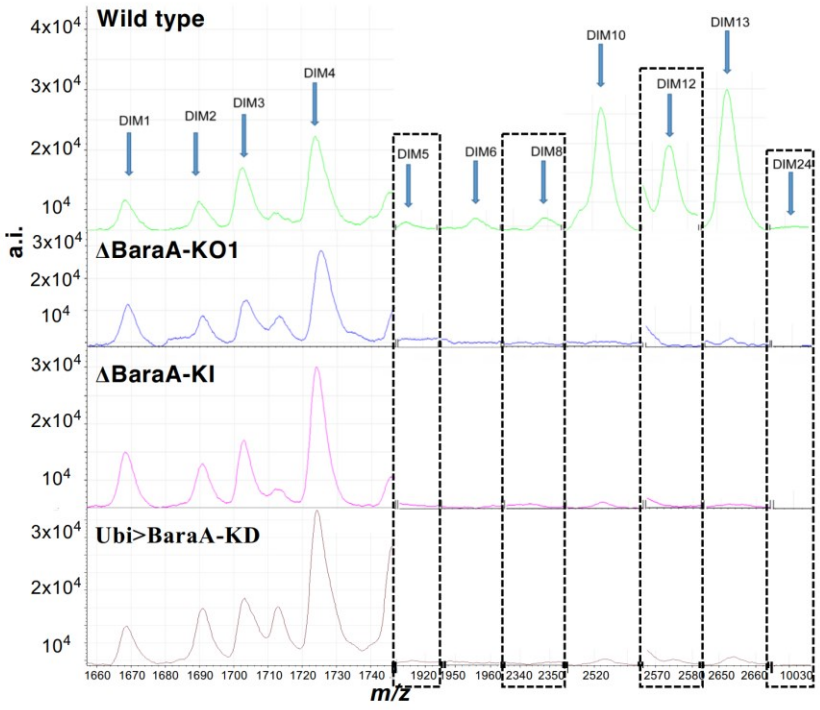


Figure S3. Detection of *BaraA*-derived DIM peptides in wild-type and *BaraA* KD, KO, and KI mutants by mass-spectrometry analysis.

The hemolymph was collected from single flies; four single flies were analyzed per genotype and yielded similar spectra by MALDI-TOF mass spectrometry. *BaraA* derived peptides were induced in wild type fly by *E. faecalis* NCTC 775. See also Table S1 for quantification of the peaks. a.i. : absolute quantitation.

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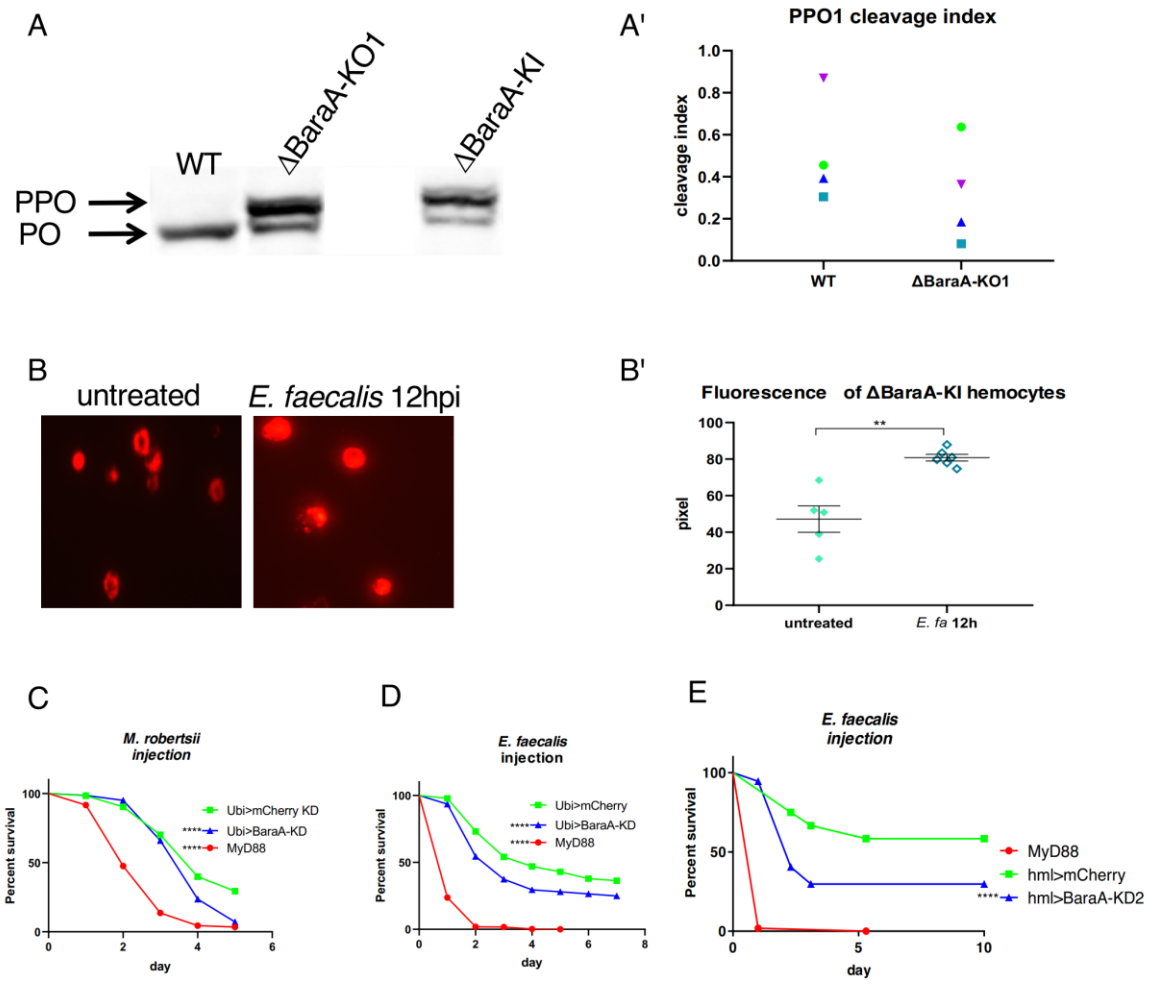

Fig. S4 *BaraA* may contribute to resistance against *E. faecalis* infection.
(A) The cleavage of prophenoloxidase was analyzed 4h after *M. luteus* septic injury by Western blotting using an anti-PPO1 antibody. **(A')** Quantification of the cleavage ratio of PPO1 in control and *BaraA KO1* flies in four independent experiments. Different colors correspond to different experiments; the purple triangles correspond to the experiment shown in Panel A in which PPO1 cleavage was nearly complete. The cleavage of PPO1 was stronger in wild-type than in *BaraA* KO1 in three out of four experiments. **(B)** Hemolymph was collected from adult *BaraA-mCherry* knock-in flies injected with *E. faecalis* for 12 hours or from untreated flies and the hemocytes were observed by fluorescence microscopy at 40X magnification. **(B')** Quantification of the fluorescence of hemocytes of *ΔBaraA*-KI displayed in (B). **(C)** Survival of *Ubi Gal4>UAS-BaraA* RNAi KD after an immune challenge with *M. robertsii*. Four out of seven experiments showed significant difference between BaraA-KD and control. Pooled data from seven independent experiments. ****P < 0.0001. **(D, E)** Survival of *Ubi Gal4>UAS-BaraA* RNAi KD (D) and *hmlGal4>UAS-BaraA* RNAi KD2 (E) after an immune challenge with *E. faecalis*. Statistical significance between wild type and KD flies (five out of five experiments in (D), three out of four experiments in (E)). LogRank test on pooled data, * p<0.05, ** p < 0.01, ****P < 0.0001.

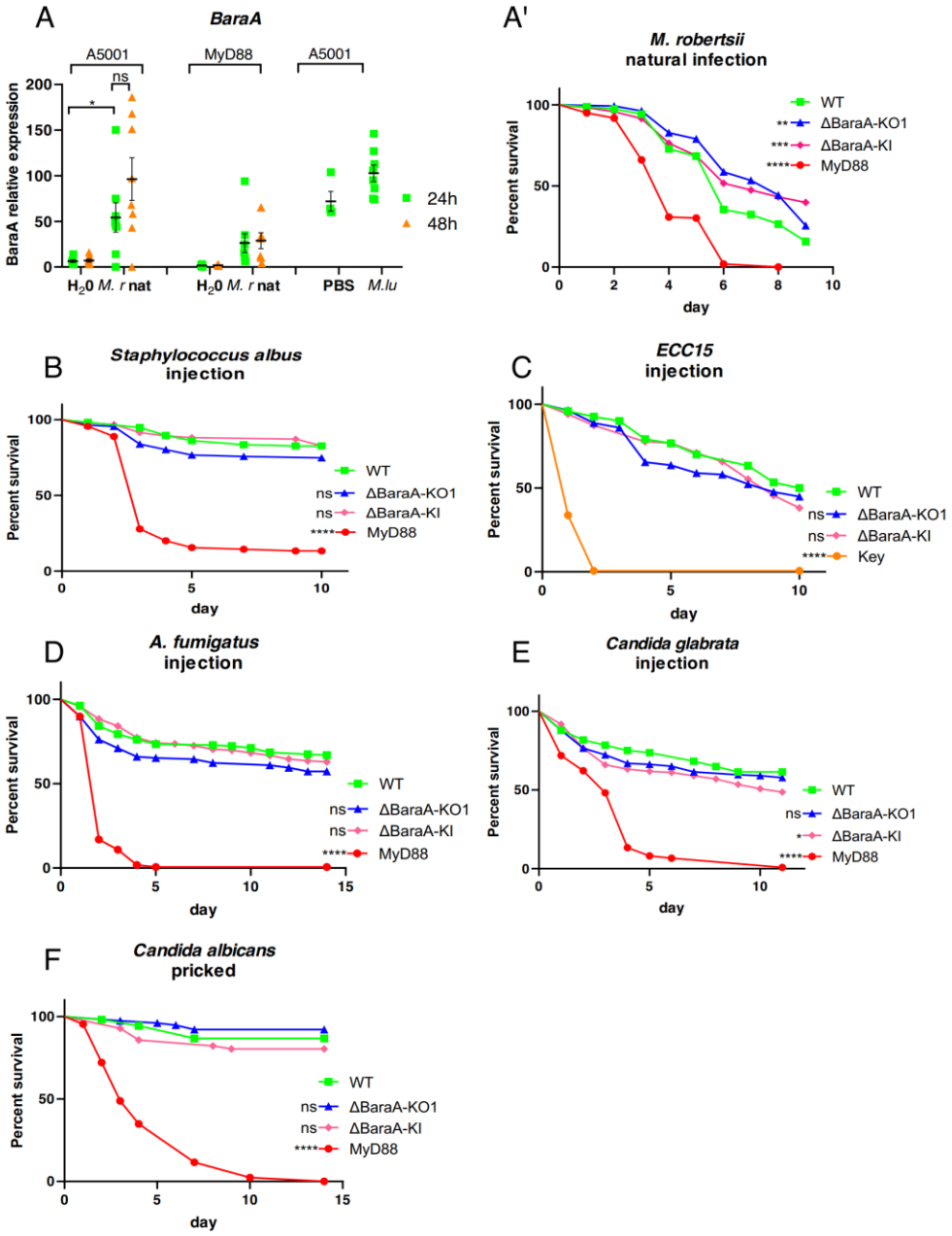

Figure S5: *BaraA* mutants survive as well as wild-type (WT) flies to different types of infection.

(A-A') Expression of *BaraA* steady-state transcripts as measured by RTqPCR (A) and survival after a natural *M. robertsii* infection challenge (A'). **(B-F)** Survival experiments after the indicated infectious challenge in the septic injury model are presented. The appropriate controls for the different microbes have been used: Gram-positive bacteria, fungi: *MyD88*, mutant of the Toll pathway; Gram-negative bacteria: *key*, mutant of the Immune deficiency pathway. None of the *BaraA* mutants displayed a reproducible susceptibility or resistance to infection (KO, KI). We used the log-rank test to determine the significance between wild-type and mutant survival curves. (A', F) Pooled data from at least two independent experiments. * $p<0.05$, **** $p<0.0001$. Data are expressed as means ± SEM.

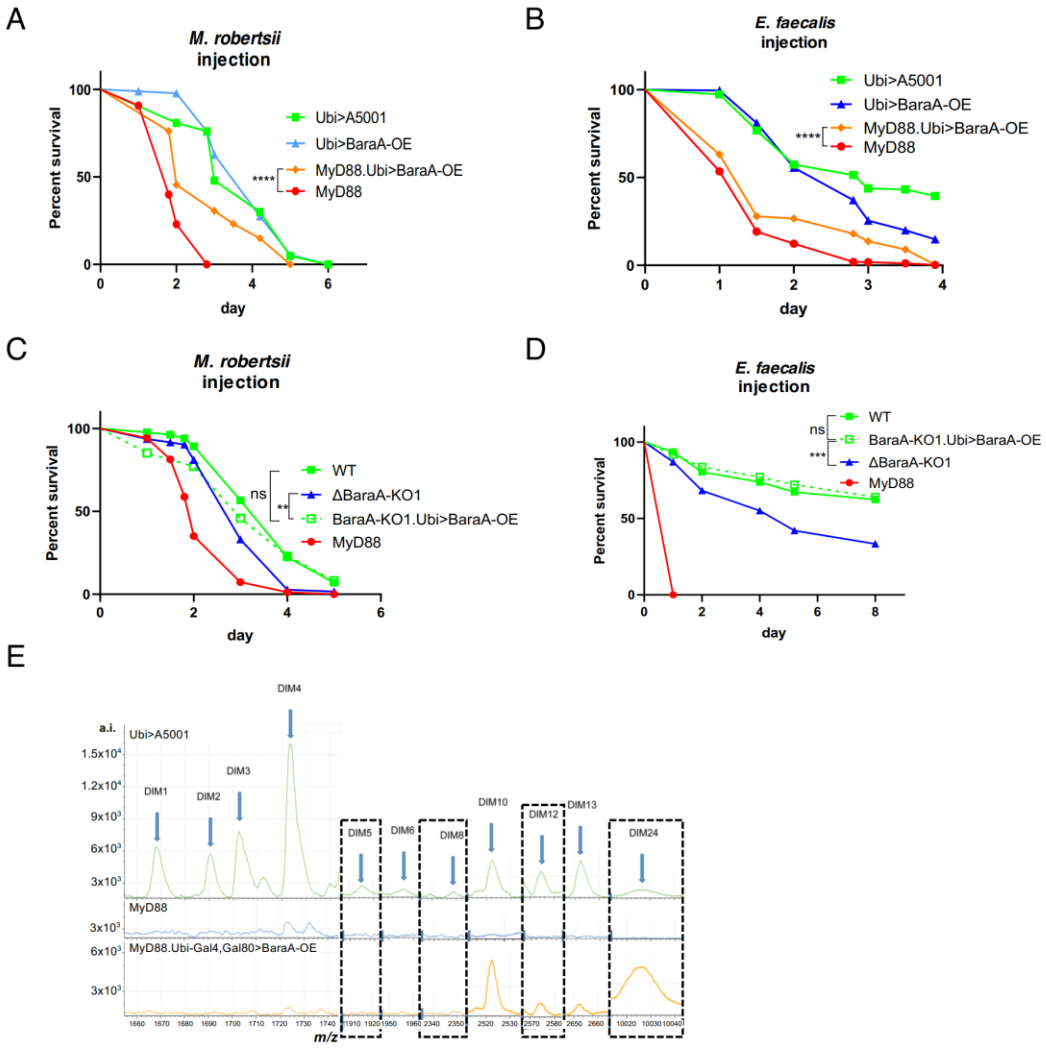

Figure S6: *BaraA* overexpression in *MyD88* but not in WT background confers an enhanced protection against *E. faecalis* and *M. robertsii* infection *in vivo*.

(A-B) BaraA overexpression in *MyD88* but not in WT background enhanced the protection against *M. robertsii* (A) and *E. faecalis* OG1RF (B) infection compared respectively to *MyD88* and WT flies. Pooled data from three independent experiments, **** $p<0.0001$. **(C-D)** Rescue of the nonisogenic *BaraA* KO1 mutant with a *BaraA* transgene expressed under the control of a p*Ubi*-Gal4^{ts} driver (cross performed at 18°C and induced at the adult stage at 29°C) after *M. robertsii* (C) or *E. faecalis* OG1RF injection (D). Three independent experiments with each pathogen have been performed and pooled. *$p < 0.05$. **(E)** Mass-spectra of hemolymph from single flies was collected 24h after a *M. luteus* challenge for wild-type control flies, *MyD88* or flies overexpressing *BaraA* in a *MyD88* mutant background. In *MyD88* flies, only DIM4 (Daisho) was slightly expressed in contrast to the wild-type control in which DIM12, DIM10, DIM13 and DIM24 were detected, as well as other DIMs. Only the relevant parts of the spectra are shown.

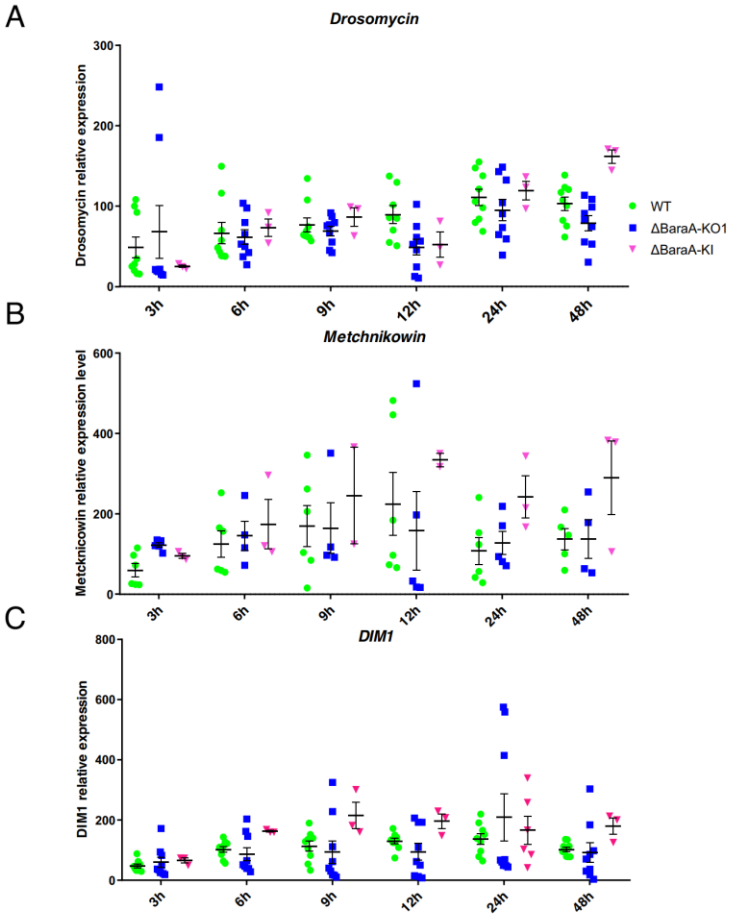

Figure S7. Toll-mediated activation of some of its target effector genes is not altered in *BaraA* KO or KI flies.

Steady-state transcript levels of *D. melanogaster* Toll pathway-regulated genes were measured by quantitative RT-PCR at different time points after a *E. faecalis* challenge: *Drosomycin* (A), *Metchnikowin* (B), and *DIM1=BomS1* (C). These experiments are representative of three independent experiments. Gene expression was normalized against *rpl32* gene expression and the results are normalized to the expression at 48h measured with WT. No significant difference between WT and isogenic *BaraA* mutants was detected. The Kruskall-Wallis multiple comparisons test has been used. Data are expressed as means ± SEM.

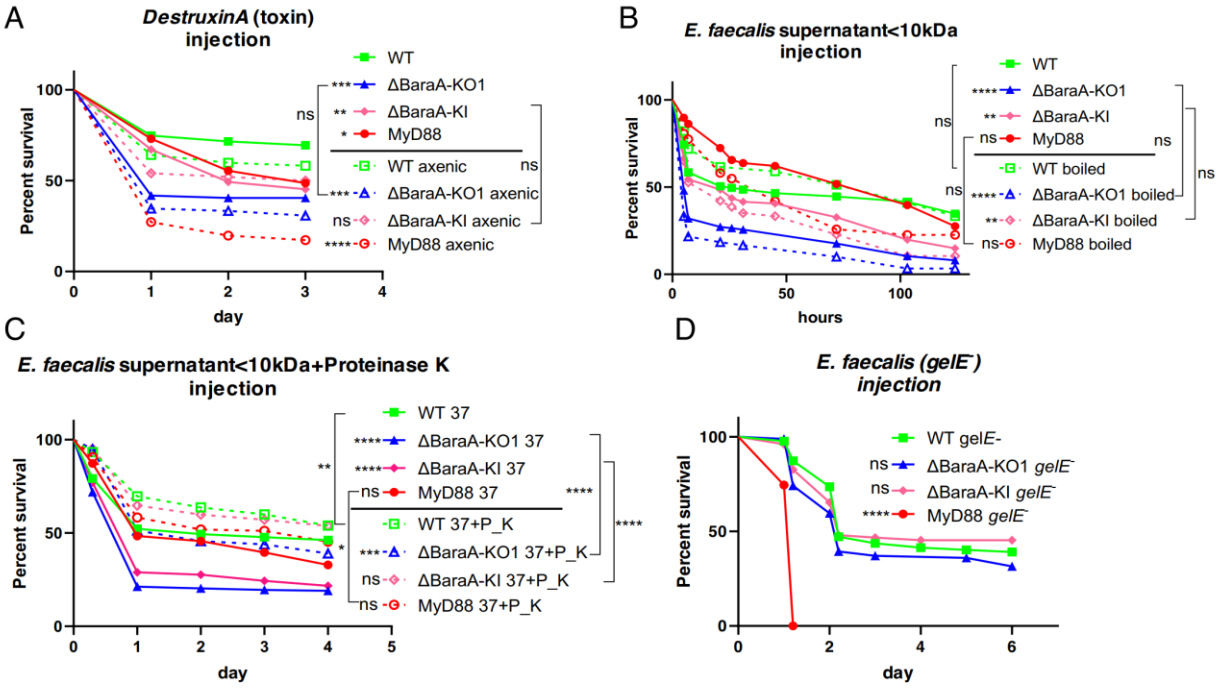

Figure S8. Further characterization of toxins secreted by *M. robertsii* or *E. faecalis*
(A) 4.6nL of 8mM DestruxinA were injected into axenic (dashed lines) or conventionally-raised flies. Axenic *BaraA* mutants showed no significant (ns) difference from control conventionally-raised *BaraA* mutant flies. Pooled data from two independent experiments, * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001: comparison of mutant flies to WT flies for each condition. **(B)** Supernatant from *E. faecalis* OG1RF was boiled at 95°C for 5 min. Flies were injected with 23nl of the boiled (dashed lines) or untreated supernatant. For both conditions, injected mutant flies were significantly more susceptible than control WT flies. For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies submitted to the same challenge for statistical analysis. Pooled data from two independent experiments, ** p< 0.01, **** p<0.0001. **(C)** Supernatant was incubated with 100ug/ml Proteinase K or with PBS (the same volume as Proteinase K) at 37°C for 18 hours. 23nl of supernatant was injected. *BaraA*-KO1 with Proteinase K treated supernatant (dashed lines) died slower than the flies injected with untreated supernatant. The same result was observed for *BaraA*-KI. Pooled data from seven independent experiments, * p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001. **(D)** 4.6nL of 0.5 OD of the *GelE- E. faecalis* mutant strain was injected. No statistically significant difference was observed. Pooled data from five independent experiments.

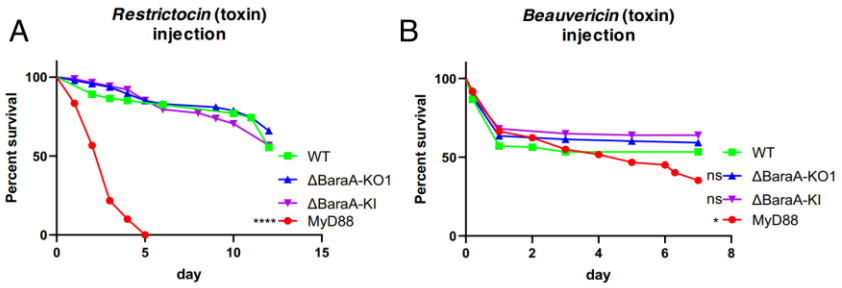

Fig. S9 BaraA is specifically counteracting toxins.
(A) Survival of *BaraA* mutants following Restrictocin injection. No statistically significant difference between wild type flies and *BaraA* mutant flies was observed. Pooled data from two independent experiments. **(B)** Upon Beauvericin injection (toxin from *Beauveria bassiana*), *MyD88* and not *BaraA* mutants displayed sensitivity to this challenge. Pooled data from two independent experiments, * $p<0.05$.

Table S1: MS results of hemolymph collected from *BaraA* mutant flies 24h after *M. luteus* challenge

	Average molecular masse (*m/z*)		Wild type		ΔBaraA-KO1		ΔBaraA-KI	
	Theroretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
DIM1(BomS1)	1667	1668	67258	16767	62061	12083	103407	21980
DIM2(BomS2)	1690	1690	40706	11767	61520	11580	82204	16824
DIM3(BomS3)	1704	1703	80236	20008	72387	15006	121265	25515
DIM4(Dso1)	1723	1724	134713	26255	260151	44055	289787	52637
DIM5	1914	1915	47815	15539	1863 (0)	2276 (0)	3125 (0)	5929 (0)
DIM6	1955	1957	121705	30592	-	-	-	-
DIM8	2348	2349	46950	18659	2182 (0)	1672 (0)	-	-
DIM10	2521	2523	257018	51458	2738	1797	9619	6569
DIM12	2573	2575	165811	36743	-	-	-	0
DIM13	2651	2653	355175	68504	10071 (0)	2287 (0)	-	6020
DIM24	10031	10034	141464	4599	-	-	-	-
Drosomycin (Drs)	4890	4892	2065642	168320	155898	13213	639405	50870

Quantitation of Fig. S3
(0) correspond to signals almost undistinguishable from the background

Table S2: MS results of hemolymph collected on *BaraA*-overexpressing *MyD88* flies 24h after *M. luteus* challenge

	Average molecular masse (*m/z*)		Ubi>A5001		MyD88		BaraA-OE (MyD88)	
	Theroretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
DIM1(BomS1)	1667	1668	39814	5291	2262	283	-	105
DIM2(BomS2)	1690	1691	36729	4492	-	111	-	136
DIM3(BomS3)	1704	1703	70522	10659	1113	283	-	108
DIM4(Dso1)	1723	1724	120283	15820	6007	1337	2387	510
DIM5	1914	1915	6829	1223	-	-	-	-
DIM6	1955	1957	10952	1874	-	-	-	-
DIM8	2348	2349	13152	2049	-	-	531	175
DIM10	2521	2523	154384	20693	685	164	26381	4382
DIM12	2573	2575	104939	14020	-	-	4563	926
DIM13	2651	2653	169357	24429	-	-	4211	828
DIM24	10031	10033	29166	1054	-	-	134314	4227
Drosomycin (Drs)	4890	4892	184661	14427	9057	977	7168	844

Quantitation of Fig. S6E

Table S3: Primers used for cloning

BaraA Fw	AAAAAGCAGGCTTCAACATGAAATCGTTTGGATTGATTGC
BaraA Rv	AGAAAGCTGGGTCTTAAACTTTTTGGAGGCATATGA
BaraA Rv-HA	AGAAAGCTGGGTCAACTTTTTGGAGGCATATGA
2nd-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT
2nd-R	GGGGACCACTTTGTACAAGAAAGCTGGGT
BaraA gRNA-F(KI)	GCGGCCCGGGTTCGATTCCCGGCCGATGCAATCTGTGGCGT TATCTGCGTGTTTTAGAGCTAGAAATAGCAAG
BaraA gRNA-R(KI)	ATTTTAACTTGCTATTTCTAGCTCTAAAACCTGCCTTTTACA ACACTGCATGCACCAGCCGGGAATCGAACCC
BaraA gRNA	ACCCACTCCCGGCACGCTGT
CG18278 gRNA	ACCACACTGGCGGCGAGCGC

Table S4: Primers used for RTqPCR

RpL32 Fw	GCTAAGCTGTCGCACAAATG
RpL32 Rv	GTTCGATCCGTAACCGATGT
Drosomycin Fw	TACTTGTTCGCCCTCTTCG
Drosomycin Rv	GAGCGTCCCTCCTCCTTGC
IM1 Fw	CAATGCTGTTCCACTGTCGC
IM1 Rv	CGTGGACATTGCACACCCTG
Metchnikowin Fw	CGTCACCAGGGACCCATTT
Metchnikowin Rv	CCGGTCTTGGTTGGTTAGGA
BaraA Fw	GGTGAGCATGTGTACACCGA
BaraA Rv	GGCGGAAAAATTGGGACCAC
CG18278 Fw	GCCCATGAACCCTTCACTCC
CG18278 Rv	CACCAACCAGTGCTTATCCTGC

Summary

Les recherches des 25-30 dernières années ont souligné le rôle fondamental de l'immunité innée dans les défenses de l'hôte contre les infections microbiennes. Suite à la détection de la présence d'infection, assurée généralement par des récepteurs capables de se lier à des structures moléculaires portées par des microbes, des voies de signalisation intracellulaires telles que les voies NF- B sont activées et aboutissent directement ou indirectement à l'expression de cytokines et d'autres effecteurs de la réponse immunitaire innée. L'activation de ces voies permet aussi de déclencher et d'orienter la réponse immunitaire adaptative chez les vertébrés[1]. Ainsi, les interférons de type I sont nécessaires à l'activation de centaines de gènes dont la fonction de la plupart reste élusive. Chez les invertébrés, dépourvus de réponse immunitaire adaptative au sens de celle des mammifères, un paradigme similaire prévaut : les infections sont détectées par des récepteurs capables de se lier à des motifs moléculaires de la paroi microbienne ou par des récepteurs capables de percevoir l'activité enzymatique de facteurs de virulence, essentiellement des protéases. En aval de ces récepteurs, l'activation de voies NF- B aboutit à l'expression de centaines de gènes, dont les plus connus sont ceux codant des peptides antimicrobiens lesquels agiraient directement sur les pathogènes bactériens ou fongiques. Cependant, ces voies contrôlent l'expression de nombreux autres gènes dont la fonction commence juste à être élucidée.

La mouche du vinaigre *Drosophila melanogaster* constitue un modèle d'étude très puissant, en particulier en raison de sa génétique sophistiquée développée depuis plus d'un siècle[2]. Son système immunitaire est relativement bien étudié. Ainsi, trois types de réponses sont déclenchés suite à une blessure septique[3]. La première, la mélanisation, est relayée par le déclenchement de cascades de protéases qui aboutissent à l'activation d'une ou plusieurs phénol-oxydases qui sont requises pour le dépôt de mélanine au site de blessure et pourraient générer des espèces oxygénées réactives et

radicaux libres susceptibles d'agir sur les microbes introduits au niveau de la blessure. Une deuxième réponse est cellulaire et implique la phagocytose des microorganismes par les hémocytes de la drosophile. La troisième est la réponse humorale systémique qui implique deux voies régulatrices de type NF- B[4]. Alors que la voie Immune deficiency (IMD) est déclenchée par des bactéries à Gram-négatif et des bacilles dont la paroi comprend du peptidoglycane de type di-amino-pimélique, la voie Toll quant à elle est préférentiellement induite par des infections fongiques et des infections bactériennes d'espèces dont le peptidoglycane est de type Lysine. Dans ce dernier cas, il est étonnant d'observer qu'une seule voie de signalisation ait été sélectionnée au cours de l'évolution, permettant d'assurer une protection contre des microorganismes aussi différents que des bactéries, procaryotes, et des champignons, eucaryotes, qui présentent peu de points communs identifiables de prime abord. De manière générale, chaque voie est efficace contre les microorganismes qui la déclenchent, à l'exception de certains microorganismes résistants aux principaux médiateurs de la réponse humorale, les peptides antimicrobiens (PAMs). D'autres microorganismes pourraient interférer avec la réponse NF- B, voire la bloquer, à l'instar de la gliotoxine sécrétée par *Aspergillus fumigatus* bloquant cette signalisation chez les mammifères[5]. Une des particularités de la voie Toll est qu'elle est déclenchée par des récepteurs circulants dans l'hémolymphe avec donc l'étape de détection de l'infection prenant place au niveau extracellulaire[6,7]. Cette perception d'agents infectieux initie alors des cascades protéolytiques qui aboutissent à activer par clivage le ligand Spätzle (homologue des neurotrophines humaines) du récepteur Toll. Une deuxième cascade de protéases est quant à elle déclenchée par les activités protéolytiques de facteurs de virulence sécrétés par des pathogènes fongiques ou bactériens[8-10]. Ainsi, des bactéries à Gram-négatif sécrétant des protéases comme facteurs de virulence extracellulaires induisent les deux voies, surtout si elles ne sont pas annihilées par les défenses de l'hôte, comme c'est le cas pour les bactéries pathogènes *Serratia marcescens* ou *Pseudomonas aeruginosa*. Les voies de transduction intracellulaires IMD et Toll aboutissent chacune à l'expression d'un éventail spécifique de gènes codant des peptides antimicrobiens. Ainsi,

la Drosomycine dont l'expression est activée par la voie Toll agit sur certains champignons filamenteux et aboutit à leur lyse, ce qui a pu être confirmé *in vivo*[11,12]. Par ailleurs, d'autres peptides dont dix gènes « *Bomanines* » regroupés au locus 55C du génome seraient actifs contre une variété de pathogènes, y compris *Candida glabrata*, une levure pathogène[13,14]. Celle-ci ne prolifère pas et ne tue pas les drosophiles sauvages. Au contraire, elle se multiplie dans les mouches déficientes pour l'activation de la voie Toll[15]. En aboutissant au contrôle de la prolifération de certains pathogènes, voire leur lyse, la voie Toll apparaît donc comme une voie de résistance de la défense de l'hôte contre les infections fongiques et infections bactériennes à Gram-positif. La **résistance** est une des deux dimensions de la défense de l'hôte contre les infections et aboutit généralement à la neutralisation ou à l'annihilation des microorganismes : elle correspond à la réponse immunitaire. Cependant, une deuxième dimension de la défense de l'hôte contre les infections existe et a été nettement moins étudiée : la **résilience** (aussi connue sous le nom de tolérance à la maladie) correspond à la capacité de l'hôte à endurer et à réparer les dommages occasionnés par l'infection, soit suite à l'action des facteurs de virulence du pathogène, soit infligés par la propre réponse immunitaire de l'hôte[16]. Cette deuxième dimension de la réponse immunitaire n'a presque pas été étudiée dans le cas des infections fongiques.

L'équipe animée par le Pr. Dominique Ferrandon au sein du Sino-French Hoffmann Institute de la Guangzhou Medical University s'intéresse aux infections fongiques, et dans une moindre mesure celles par les bactéries à Gram-positif, chez la drosophile de manière globale, d'une part à l'aide de mutagénèses relativement peu biaisées car le paramètre suivi est la survie à l'infection fongique, et d'autre part en étudiant la voie Toll et le rôle des gènes régulés par cette voie dans la défense de l'hôte contre les infections fongiques ou bactériennes. En ce qui concerne les infections fongiques, un premier pathogène est le champignon entomopathogénique *Metarhizium robertsii*, lequel tue les drosophiles soit dans un modèle d'infection par injection soit en traversant la cuticule après dépôt des spores sur la carapace des mouches. Un deuxième modèle d'étude est le champignon opportuniste *A. fumigatus*, qui doit être injecté et est

incapable de tuer les lignées de drosophile sauvages. Celui-ci avait été utilisé comme illustration du rôle antifongique de la voie Toll dans la publication princeps de notre laboratoire CNRS dirigé par Jules Hoffmann à Strasbourg[17]. Cependant, peu d'études sur *A. fumigatus* dans ce modèle ont été conduites par la suite[18]. Il a pu toutefois être établi que la surexpression ectopique de la Drosomycine protège faiblement les mutants *Spätzle* contre cette infection[12]. De même, un mutant dans lequel les principaux gènes codant des peptides antimicrobiens sont délétés ne montre qu'une susceptibilité modeste à cette infection[19]. En fait, il a été démontré que les mouches mutantes de la voie Toll succombent à *A. fumigatus* non en raison de la prolifération et de la dissémination du champignon dans l'hôte immuno-déficient mais suite à une susceptibilité aux mycotoxines sécrétées par le champignon. Ainsi, trois Bomanines (BomS3, BomS6 et BomBc1) protègent l'hôte de l'action d'une ribotoxine protéique, la restrictocine, tandis qu'une seule, BomS6, permet à l'hôte de contrecarrer les effets trémorgéniques du verruculogène, une neurotoxine du métabolisme secondaire d'*A. fumigatus*[20]. **La voie Toll permet donc aussi de protéger l'hôte de l'activité de toxines microbiennes sécrétées**, une conclusion renforcée par l'étude d'un autre effecteur de la voie Toll, la polyprotéine BaramicineA (BaraA), précurseuse de multiples peptides, qui protège à la fois de l'action de la Destruxine A (DtxA), un hexadepsipeptide cyclique neurotoxique sécrété par *M. robertsii* et de l'Enterocine V (EntV) produite par la bactérie à Gram-positif *Enterococcus faecalis* (Huang *et al.*, *PNAS*, sous presse).

Par ailleurs, l'équipe du Pr. Ferrandon à Strasbourg s'intéresse aussi aux infections intestinales par *S. marcescens* et à l'étude des relations hôtes-pathogène avec ce pathogène[21-25]. Comme la génétique de cette bactérie est difficile à mettre en œuvre, un deuxième système d'étude a été développé avec *P. aeruginosa* pour laquelle beaucoup plus d'outils et de connaissances sont disponibles[26-28].

Mon travail de recherche a porté initialement sur la caractérisation des gènes d'ARN longs annotés comme non-codant (lncARNs). En effet, l'analyse de données de séquençage d'ARN totaux lors d'une cinétique d'infection par *M. robertsii* a mis en

évidence que plusieurs gènes lncARNs étaient exprimés de manière différentielle, certains étant plutôt réprimés comme *CR45601*, un gène positionné sur l'autre brin d'ADN du gène codant une protéine majeure du vitellus, la Yolk Protein1 (YP1). Mes travaux n'ont pas permis d'établir un lien entre ce lncARN, la protéine YP1 et la défense contre les infections microbiennes. D'autres lncARNs au rebours de *CR45601* étaient très fortement induits, tel le gène *CR44404*, lequel arrivait en tête de liste des gènes de cette catégorie, ce qui a motivé son étude approfondie. J'ai donc développé les outils transgéniques et mutants nuls CRISPR-Cas9 pour son étude génétique. Entretemps, une étude d'un laboratoire concurrent a rapporté que la surexpression de ce gène, nommé *IBIN* par ces chercheurs, en contexte d'une voie Toll pré-activée conférait une survie légèrement améliorée à une infection par *E. faecalis* sans toutefois vérifier que le titre microbien était diminué, ce qui ne permettait pas de déterminer un rôle de résistance ou de résilience pour ce lncRNA dans cette infection. Des phénotypes d'un nombre accru de plasmatocytes et d'un taux de glucose dans l'hémolymphe légèrement augmenté avait été aussi décrits par cette approche d'expression ectopique[29].

Une analyse bioinformatique détaillée de l'évolution de ce gène dans les diverses espèces de drosophile séquencées[30,31] nous a permis de confirmer que ce lncARN contient un cadre ouvert de lecture conservé au cours de l'évolution et qu'en sus il comprend la séquence d'un peptide signal, ce qui nous a amené à émettre l'hypothèse que ce gène code un peptide sécrété lors de la réponse immunitaire. Nous avons alors cherché ce peptide dans des données d'analyse de spectrométrie de masse LC/MS portant sur de l'hémolymphe de drosophiles infectées par *E. faecalis*. Nous n'avons pas réussi à identifier ce peptide mais en avons identifié un autre de séquence proche, lui aussi fortement inductible par certains stimuli immunitaires. Nous nous sommes alors aperçus que ce peptide dérive d'un autre gène initialement annoté comme lncARN, *CR45045*. Il y a aussi un cadre de lecture conservé au cours de l'évolution ainsi qu'un peptide signal. Les deux gènes ont donc une forte similitude de séquences dans la partie codante. Nous avons réalisé que ce gène lncARN était déjà étudié dans le laboratoire de Strasbourg, essentiellement en culture de cellules, par Adrian Acker, alors doctorant

dans l'équipe du Pr. Nicolas Matt. Nous avons donc décidé de joindre nos forces sur ce projet, en particulier pour la génération d'un double mutant plus susceptible de révéler des phénotypes en cas de redondance des deux gènes. Par ailleurs, la banque de données Flybase a réannoté ces gènes comme étant codant, d'où leurs nouveaux noms *CG44404/IBIN* et *CG45045*.

Une des premières étapes a été de déterminer les voies génétiques qui régulent l'induction très forte de ces deux gènes. Alors que l'induction de *CG44404* est relayée par la voie Toll et/ou la voie IMD, *CG45045* est uniquement induit par l'intermédiaire de la voie IMD. Il reste toutefois faiblement inductible, via la voie IMD, en réponse à une blessure, par exemple lors de l'injection de spores de *M. robertsii* ou de PBS comme contrôle. Lors d'infection « naturelle » par ce champignon qui traverse la cuticule en creusant des trous microscopiques dans l'exosquelette de l'insecte, il n'y a pas d'activation de la voie IMD, laquelle est observée seulement lors de blessures macroscopiques. L'utilisation de transgènes rapporteurs exprimant des protéines fluorescentes sous le contrôle des promoteurs de ces deux gènes a permis d'établir que le tissu principal où cette induction prend place est le corps gras, principal tissu de biosynthèse de l'insecte où sont aussi produits les PAMs. Cet organe représente un composite entre foie et tissu adipeux des vertébrés.

J'ai généré plusieurs types de rapporteurs transgènes, certain étant en fusion avec la séquence du peptide et d'autres remplaçant la partie codante de *CG44404* par celles de la *GFP*. Ces expériences m'ont permis de démontrer que l'expression de *CG44404* aboutit bien à la production d'un peptide sécrété, lequel a été détecté dans l'hémolymphe par Western blot en utilisant un anticorps contre la GFP.

Après avoir généré moi-même un mutant nul à l'aide de la technique CRISPR-Cas9 pour *CG44404* tandis qu'Adrian Acker faisait de même en ce qui concerne *CG45045*, j'ai généré des lignées isogénéisées simple et double mutantes pour ces gènes. J'ai pu alors analyser leurs phénotypes de survies par plusieurs pathogènes représentatifs des différentes catégories d'infection microbienne. La conclusion de multiples expériences est que les simples et double mutants sont susceptibles à

l'injection de spores de *M. robertsii* et non à une infection naturelle par la cuticule. Par ailleurs, une susceptibilité accrue aux infections par *P. aeruginosa* a été observée pour le double-mutant mais pas les simples mutants (*S. marcescens* n'a pas révélé de phénotype car il tue les drosophiles trop vite, en moins de 24 heures). Aucune modification du titre microbien au cours de ces deux infections fongique et bactérienne n'a pu être mise en évidence.

J'ai aussi vérifié si les principales défenses de l'hôte restaient fonctionnelles chez les simples et double mutants. Alors que la capacité de phagocytose des bactéries ainsi que l'activation des gènes de la voie de mélanisation restaient normales, j'ai cependant noté que l'induction de certains, mais pas de tous les gènes codant des AMPs régulés essentiellement par la voie IMD était affectée en réponse à un stimulus *Escherichia coli*, une bactérie induisant préférentiellement la voie IMD. Ce phénotype affectant seulement certains des gènes régulés par la voie IMD reste mal compris. Une altération significative de cette voie aurait dû se traduire par une susceptibilité plus élevée, non observée, aux autres bactéries à Gram-négatif que j'ai testées.

L'absence de prolifération augmentée des pathogènes est compatible avec un rôle dans des phénomènes de résilience, à l'instar de la situation pour *BaraA*. J'ai donc testé la susceptibilité des mutants simples et double de *CG44404* et *CG45045* à la DtxA. Comme pour *BaraA*, une susceptibilité accrue à l'exposition à la DtxA a été observée dans tous les cas. Les phénotypes de susceptibilité augmentée à l'infection par *M. robertsii* disparaissait lorsqu'une souche mutée incapable de produire des Destruxines était utilisée. Étant donné le phénotype de paralysie partiellement réversible induit par l'exposition à la DtxA, j'ai réalisé des expériences complémentaires sur la perméabilité de la barrière hémato-encéphalique : celle-ci n'est pas augmentée par l'injection de DtxA et n'est pas non plus altérée chez les simple et double-mutants *CG44404* & *CG45045*.

L'équipe étudie depuis longtemps les relations hôte-pathogène entre *S. marcescens* et la drosophile. Dans le cadre d'une collaboration avec une équipe de microbiologistes argentins animée par la Pr. Eleonora Garcia-Vescovi, la virulence de vésicules issues de

la paroi bactérienne libérées par *S. marcescens* et connues sous le nom anglais de « Outer-Membrane Vesicles » "(OMVs) a pu être établie. Ces OMVs agissent sur le système nerveux de l'insecte et induisent une paralysie irréversible en cinq-six heures. Les OMVs induisent une réponse immunitaire et les mutants de la voie IMD sont plus sensibles à leurs actions. Au contraire, la réponse cellulaire semble favoriser l'action des OMVs. La virulence des OMVs peut être atténuée par un traitement avec des molécules anti-oxydantes comme la vitamine C. Le modèle actuel est que les OMVs induisent un stress oxydatif dans les neurones par l'intermédiaire de leurs mitochondries, stress qui semble induire l'apoptose dans le système nerveux vraisemblablement par l'activation de la voie JNK. Un des facteurs de virulence essentiel porté par les OMVs est la protéase PrtA. Les OMVs de ces mutants *PrtA* ont besoin d'être concentrés dix fois plus pour avoir une activité. L'injection directe de PrtA purifiée induits des phénotypes très similaires à ceux induits par l'injection d'OMVs.

J'ai donc testé la survie du mutant double *CG44404-CG45045* à une exposition aux OMVs issus de *S. marcescens* ou de *P. aeruginosa* et observé leur sensibilité accrue, ainsi qu'à la PrtA purifiée. Une des fonctions de ces deux peptides est donc de protéger l'hôte contre l'action des OMVs et d'une métalloprotéase émise par des pathogènes bactériens. Je n'ai pas pu mettre en évidence une inhibition de l'activité protéolytique de PrtA *in vitro* par l'addition de peptides CG44404 et CG45045 synthétiques. Par ailleurs, la co-injection de vitamine C a permis d'améliorer autant la survie des drosophiles sauvages que celle des mutants, ce qui suggère que ces deux peptides n'influencent pas la production de ROS.

Afin de comprendre le mécanisme de protection conféré par les deux peptides, j'ai conduit des expériences d'immuno-précipitation d'une protéine transgénique de fusion CG44404-GFP présente dans des extraits de mouches entières ou dans de l'hémolymphe collectée, en collaboration avec la plate-forme de spectrométrie de masse de l'Institut de Biologie Moléculaire et Cellulaire. Ces expériences ont révélé l'existence d'une liaison directe ou indirecte avec un des facteurs du complément de la drosophile, la protéine contenant un motif thioester « thioester-containing protein 2 »

TEP2 de la drosophile. De manière inattendue, j'ai trouvé que les mutants *Tep2* montraient une susceptibilité diminuée à l'action de PrtA purifiée alors que le phénotype inverse était obtenu avec des mutants *Tep4* (TEP4 est resté non identifié dans les expériences d'immuno-précipitations). Cependant, les mutants *Tep2* et *Tep4* se sont comportés comme les drosophiles sauvages après l'injection de DtxA. Ces résultats ouvrent donc la possibilité que TEP2 soit une des facteurs émis par les hémocytes qui favoriserait l'action des OMVs. Il faudra déterminer si l'action des peptides CG44404 et CG45045 permet de neutraliser l'activité de TEP2, par exemple en séquestrant ce facteur du complément dans un complexe comprenant ces peptides. Comment TEP2 promeut l'activité de PrtA sera aussi un sujet d'étude important.

Le phénotype de sensibilité à la DtxA a poussé la Dr. Jianqiong Huang à tester si le double mutant *CG44404-CG45045* présentait un phénotype de sensibilité accrue à la toxine EntV d'*E. faecalis*, ce qui est le cas. Réciproquement, elle a établi que les mutants *BaraA* sont aussi plus sensibles à l'injection de PrtA purifiée. Le mutant *BaraA* présente donc un profil de phénotypes de sensibilité aux toxines/facteurs de virulence sécrétés très proche de celui du double mutant *CG44404-CG45045*, une exception étant l'absence de susceptibilité de ce dernier à l'infection par *E. faecalis*. J'ai testé une interaction éventuelle des peptides synthétiques CG44404 et CG45045 entre eux ou avec deux peptides (synthétiques) générés à partir du précurseur BaraA (DIM12&DIM13) par des expériences d'ultracentrifugation et n'ai observé aucune interaction entre ces peptides.

En conclusion, le travail sur BaraA, auquel j'ai participé, et mon travail de thèse ont contribué à établir la notion d'effecteurs de l'hôte dédiés à la neutralisation ou à la réparation des effets de toxines et facteurs de virulence microbiens sécrétés. Une découverte est qu'un ensemble de peptides d'origine différentes permettent à l'hôte de contrecarrer dans une certaine mesure les effets de toxines et facteurs de virulence d'activités biochimiques très diverses. Ce sera un défi formidable au vu de sa complexité de comprendre le mode d'action protecteur de ces divers effecteurs de la réponse immunitaire. Le rôle potentiel de Tep2 dans la promotion de l'effet toxique de

PrtA mais pas de DtxA laisse entrevoir un certain degré de spécificité d'action des effecteurs selon la toxine ou le facteur de virulence considéré.

Bibliographie sommaire

1 Ishii, K. J., Koyama, S., Nakagawa, A., Coban, C. & Akira, S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* **3**, 352-363, doi:S1931-3128(08)00151-0 [pii] 10.1016/j.chom.2008.05.003 (2008).

2 Bellen, H. J., Tong, C. & Tsuda, H. 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. *Nat Rev Neurosci* **11**, 514-522, doi:nrn2839 [pii] 10.1038/nrn2839 (2010).

3 Lemaitre, B. & Hoffmann, J. The Host Defense of *Drosophila melanogaster*. *Annu Rev Immunol* **25**, 697-743 (2007).

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

5 Dolan, S. K., O'Keeffe, G., Jones, G. W. & Doyle, S. Resistance is not futile: gliotoxin biosynthesis, functionality and utility. *Trends Microbiol* **23**, 419-428, doi:10.1016/j.tim.2015.02.005 (2015).

6 Michel, T., Reichhart, J., Hoffmann, J. A. & Royet, J. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756-759 (2001).

7 Gobert, V. *et al.* Dual Activation of the *Drosophila* Toll Pathway by Two Pattern Recognition Receptors. *Science* **302**, 2126-2130 (2003).

8 Gottar, M. *et al.* Dual Detection of Fungal Infections in *Drosophila* via Recognition of Glucans and Sensing of Virulence Factors. *Cell* **127**, 1425-1437 (2006).

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

10 Issa, N. *et al.* The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway. *Mol Cell* **69**, 539-550 e536, doi:10.1016/j.molcel.2018.01.029 (2018).

11 Fehlbaum, P. *et al.* Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J. Biol. Chem.* **269**, 33159-33163 (1995).

12 Tzou, P., Reichhart, J. M. & Lemaitre, B. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. *Proc Natl Acad Sci U S A* **99**, 2152-2157 (2002).

13 Clemmons, A. W., Lindsay, S. A. & Wasserman, S. A. An effector Peptide family required for *Drosophila* Toll-mediated immunity. *PLoS Pathog* **11**, e1004876, doi:10.1371/journal.ppat.1004876 (2015).

14 Lindsay, S. A., Lin, S. J. H. & Wasserman, S. A. Short-Form Bomanins Mediate Humoral Immunity in *Drosophila*. *J Innate Immun* **10**, 306-314, doi:10.1159/000489831 (2018).

15 Quintin, J., Asmar, J., Matskevich, A. A., Lafarge, M. C. & Ferrandon, D. The Drosophila

Toll Pathway Controls but Does Not Clear Candida glabrata Infections. *J Immunol* **190**, 2818-2827, doi:10.4049/jimmunol.1201861 (2013).

16 Ferrandon, D. The complementary facets of epithelial host defenses in the genetic model organism *Drosophila melanogaster*: from resistance to resilience. *Curr Opin Immunol* **25**, 59-70, doi:10.1016/j.coi.2012.11.008 (2013).

17 Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973-983 (1996).

18 Lionakis, M. S. *et al.* Toll-deficient Drosophila flies as a fast, high-throughput model for the study of antifungal drug efficacy against invasive aspergillosis and Aspergillus virulence. *J Infect Dis* **191**, 1188-1195 (2005).

19 Hanson, M. A. *et al.* Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *eLife* **8**, e44341, doi:10.7554/eLife.44341 (2019).

20 Xu, R. *et al.* The Toll pathway mediates *Drosophila* resilience to *Aspergillus* mycotoxins through specific Bomanins. *EMBO Rep*, e56036, doi:10.15252/embr.202256036 (2022).

21 Kurz, C. L. *et al.* Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. *Embo J* **22**, 1451-1460 (2003).

22 Nehme, N. T. *et al.* A Model of Bacterial Intestinal Infections in Drosophila melanogaster. *PLoS Pathog* **3**, e173 (2007).

23 Lestradet, M., Lee, K. Z. & Ferrandon, D. Drosophila as a model for intestinal infections. *Methods Mol Biol* **1197**, 11-40, doi:10.1007/978-1-4939-1261-2_2 (2014).

24 Lee, K. Z. *et al.* Enterocyte Purge and Rapid Recovery Is a Resilience Reaction of the Gut Epithelium to Pore-Forming Toxin Attack. *Cell Host Microbe* **20**, 716-730, doi:10.1016/j.chom.2016.10.010 (2016).

25 Sina Rahme, B. *et al.* The fliR gene contributes to the virulence of S. marcescens in a Drosophila intestinal infection model. *Scientific reports* **12**, 3068, doi:10.1038/s41598-022-06780-w (2022).

26 Limmer, S. *et al.* Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. *Proc Natl Acad Sci U S A* **108**, 17378-17383, doi:1114907108 [pii] 10.1073/pnas.1114907108 (2011).

27 Haller, S. *et al.* Quorum-sensing regulator RhlR but not its autoinducer RhlI enables Pseudomonas to evade opsonization. *EMBO Rep* **19**, e44880, doi:10.15252/embr.201744880 (2018).

28 Haller, S., Limmer, S. & Ferrandon, D. Assessing Pseudomonas virulence with a nonmammalian host: Drosophila melanogaster. *Methods Mol Biol* **1149**, 723-740, doi:10.1007/978-1-4939-0473-0_56 (2014).

29 Valanne, S., Salminen, T. S., Jarvela-Stolting, M., Vesala, L. & Ramet, M. Immune-inducible non-coding RNA molecule lincRNA-IBIN connects immunity and metabolism in Drosophila melanogaster. *PLoS Pathog* **15**, e1007504, doi:10.1371/journal.ppat.1007504 (2019).

30 Kim, B. Y. *et al.* Highly contiguous assemblies of 101 drosophilid genomes. *eLife* **10**, e66405, doi:10.7554/eLife.66405 (2021).

31 Stark, A. *et al.* Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. *Nature* **450**, 219-232 (2007).

Chuping CAI

From long non-coding RNA genes to the expression of secreted effector peptides involved in *Drosophila melanogaster* defenses against toxins and virulence factors secreted by pathogenic microbes: the case of *CG44404* and *CG45045*

Résumé

Mon doctorat visait à étudier le rôle des ARN longs non-codant (lncRNAs) dans l'immunité de la drosophile. Grâce à des données obtenues précédemment, nous avons découvert que l'expression de 23 lncRNAs étaient significativement augmentée ou diminuée lors de l'infection par l'entomopathogène *Metarhizium robertsii.* Dans les chapitres I et II, j'ai abordé l'étude d'un gène de chaque catégorie dont l'expression variait le plus.

Dans le chapitre I, j'ai étudié le gène non codant, *CR45601*, dont l'expression est atténuée significativement lors de l'infection. *CR45601* chevauche complètement le deuxième exon d'un gène codant, la *yolk protein 1 (Yp1)* dans une orientation antisens. En utilisant des mutants affectant soit *Yp1* seul, soit *Yp1* et *CR45601* simultanément, nous n'avons pas observé de phénotypes reproductibles lors des infections testées.

Dans le chapitre II, nous avons découvert que *CG44404* et *CG45045*, qui étaient précédemment annotés comme des gènes non codants, codent en fait de courts peptides sécrétés. Nous avons révélé qu'au lieu d'agir directement contre les pathogènes eux-mêmes, les deux peptides agissent dans la défense de l'hôte contre des facteurs de virulence distincts sécrétés par des pathogènes, tels que la Destruxine A de *M. robertsii*, la Protéase A de *Serratia marcescens* et l'Entérocine V d'*Enterococcus faecalis*. Nos données suggèrent que les deux peptides agissent en conjonction avec un effecteur de la voie Toll, BaraA. Dans le cas de la défense contre la protéase A, une fonction de CG44404 serait de séquestrer le facteur du complément TEP2.

À l'annexe I, j'ai participé à la finalisation d'une étude sur les vésicules de la membrane externe (OMV) de *S. marcescens*. Nous avons révélé que les OMVs déclenchent la production de ROS par les mitochondries dans les neurones et entraînent leur apoptose. En outre, nous avons découvert que la réponse cellulaire contribue à la pathogenèse des OMVs.

Dans l'annexe II, j'ai cosigné une étude publiée révélant que BaraA protège la mouche de toxines distinctes telles que la DestruxineA et l'Enterocin V sécrétées respectivement par *M. robertsii* et *E. faecalis*.

Mots clés: CR45601, yolk protein 1, CG44404, CG45045, DestruxinA, ProtéaseA, EnterocinV, BaramicinA, protéine contenant des thioesters, vésicule de la membrane externe.

Résumé en anglais

My doctoral work aimed to investigate the role of long noncoding RNAs (lncRNAs) in Drosophila immunity. Based on previous studies performed in the laboratory, we have found that the expression of 23 lncRNAs were significantly elevated or decreased upon the infection of entomopathogen *Metarhizium robertsii*. I worked on the most significantly and consistently suppressed and induced genes, respectively in Chapter I and Chapter II.

In Chapter I, I studied the lncRNA gene *CR45601*, the expression of which is suppressed significantly upon infection. *CR45601* completely overlaps the second exon of a coding gene *yolk protein 1(Yp1)* in the antisense orientation. By using the mutant depriving either *Yp1* alone or both *Yp1* and *CR45601*, we did not observe the mutant showing any reproducible phenotype in the infections we have tested.

In Chapter II, we found that *CG44404* and *CG45045,* which were previously annotated as lncRNAs, actually encode short secreted peptides. We have revealed that instead of directly attacking the pathogens, both peptides act in the host defense against distinct virulence factors secreted by pathogens, such as DestruxinA from *M. robertsii*, Protease A from *Serratia marcescens* and Enterocin V from *Enterococcus faecalis*. Our data suggest that both peptides function in concert with the Toll pathway effector BaraA. In the case of defense against Protease A, one function of CG44404/CG45045 may be to sequester the TEP2 complement factor.

In Annex I, I was involved in finishing a study about the outer membrane vesicles (OMVs) from *S. marcescens*. We have discovered that OMVs trigger the production of ROS by mitochondria in neurons, resulting in the apoptosis in neurons. Besides, we have found that the cellular response from the host contributes to the pathogenesis of OMVs.

In Annex II, I co-authored of a published study revealing that BaraA protects fly from distinct toxins such as Destruxin A and Enterocin V secreted by *M. robertsii* and *E. faecalis* respectively.

Key words: CR45601, yolk protein 1, CG44404, CG45045, DestruxinA, ProteaseA, EnterocinV, BaramicinA, thioester containing protein, outer membrane vesicle