

**ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTE**

**UPR9022 CNRS / Sino-French Hoffman Institute at Medicine University of Guangzhou**

# **THÈSE en cotutelle**

présentée par:

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soutenue le : **30 Juin 2022**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Immunologie

**Study of two Toll pathway effector genes  
involved in resilience and resistance to  
microbial infections in *Drosophila  
melanogaster***

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## Abbreviations

Abbreviations	Full name
<i>A. fumigatus</i>	<i>Aspergillus fumigatus</i>
AMP	Antimicrobial Peptide
ATP	Antitoxin Peptide
BaraA	BaramicinA
BBB	Blood Brain Barrier
BLUD	Bacterial Load Upon Death
<i>C. albicans</i>	<i>Candida albicans</i>
CFU	Colony Forming Unit
<i>C. glabrata</i>	<i>Candida glabrata</i>
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAP-type PGN	meso-diaminopymelic acid type Peptidoglycan
Dtx	Destruxins
DIF	Drosophila-induced Immune Molecule
DIM	Drosophila-induced Immune Molecule
DMSO	Dimethylsulfoxide
<i>Ecc15</i>	<i>Erwinia carotovora carotovora 15</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EntV	Enterocin O16
ETI	Effector-trigger immunity
FLUD	Fungal Load Upon Death
GAL4	Galactose4 gene
GNBP	Gram-negative Binding Protein
GNBP-like 3 (G13)	Gram-negative Binding Protein like 3
IMD	immune deficiency
KD	Knock Down
KO	Knock Out
KI	Knock In
LB	Luria-Bertani
Lys-type PGN	Lysine-type Peptidoglycan
<i>M. luteus (M. l)</i>	<i>Micrococcus luteus</i>
<i>M. robertsii</i>	<i>Metarhizium robertsii</i>

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MyD88	Myeloid differentiation primary-response gene 88
OMV	Outer Membrane Vesicles
PAMP	Pathogen Associated Molecular Pattern
PDA	Potato Dextrose Agar
PFTs	Pore-forming toxins
PGRP	Peptidoglycan Recognition Proteins
PO	Phenoloxidase
PPO	Prophenoloxidase
PrtA	Serralyisin A
PRRs	Pattern Recognition Receptors
RNAi	Ribonucleic Acid interference
ROS	Reactive Oxygen Species
TAG	Triacylglycerids
UAS	Upstream Activating Sequence
VitC	Vitamin C
WT	Wild Type
YPDA	Yeast extract- Peptone-Glucose Broth Agar

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## Abstract

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- $\kappa$ B sont activées et aboutissent directement ou indirectement à l'expression de cytokines et d'autres effecteurs de la réponse immunitaire innée, laquelle permet aussi de déclencher et d'orienter la réponse immunitaire adaptative chez les vertébrés<sup>1</sup>. Ainsi, les interférons de type I sont nécessaires à l'activation de centaines de gènes dont la fonction de la plupart reste évasive. Chez les invertébrés, lesquels sont 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. L'activation de voies NF- $\kappa$ 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<sup>2</sup>. Son système immunitaire est relativement bien étudié. Ainsi, trois types de réponses sont déclenchés suite à une blessure septique<sup>3</sup>. La première, la mélanisation, est relayée par le déclenchement de cascades de protéases qui aboutissent à l'activation d'une ou plusieurs phénol-oxydases, lesquelles 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 laquelle implique deux voies régulatrices de type NF- $\kappa$ B<sup>4</sup>. 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 n'est pas compris pourquoi une seule voie de signalisation a été sélectionnée au cours de l'évolution et permet d'assurer une protection contre des microorganismes aussi différents que des bactéries, procaryotes, et des champignons, eucaryotes, lesquels présentent peu de points communs identifiables de prime abord. De manière générale, chaque voie est efficace contre les pathogènes qui la déclenchent, à l'exception de certains pathogènes résistants aux principaux médiateurs de la réponse humorale, les peptides antimicrobiens (PAMs). D'autres pathogènes pourraient interférer avec la réponse NF- $\kappa$ B, voire la bloquer à l'instar de la gliotoxine sécrétée par *Aspergillus fumigatus* bloquant cette signalisation chez les mammifères<sup>5</sup>. Une des particularités de la voie Toll est qu'elle est déclenchée par des récepteurs circulants qui détectent soit les  $\beta$ -(1,3)-glucanes des parois fongiques soit le peptidoglycane de type Lys<sup>6,7</sup>. Ils initient 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<sup>8-10</sup>. 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*<sup>11,12</sup>. 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 pathogénique<sup>13,14</sup>. 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<sup>15</sup>. 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. 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 pathogènes : 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<sup>16</sup>. 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 approche les infections fongiques, et dans une bien 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*, lequel 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<sup>17</sup>. Cependant, peu d'études sur *A. fumigatus* dans ce modèle ont été conduites par la suite<sup>18</sup>. Il a pu toutefois être établi que la surexpression ectopique de la Drosomycine protège faiblement les mutants *Spätzle* contre cette infection<sup>12</sup>. De même, un mutant dans lequel les principaux gènes codant des peptides antimicrobiens sont délétés ne montre qu'une susceptibilité modeste à cette infection<sup>19</sup>.

Mon travail de recherche a porté sur la caractérisation des fonctions de deux gènes « effecteurs » de la voie Toll. En effet, peu d'effecteurs antimicrobiens de la voie Toll ont été identifiés comme des peptides actifs contre les champignons filamenteux (Drosomycine, Metchnikowine)<sup>11,20</sup>. Cependant, aucune activité contre les levures ou les bactéries à Gram-positif n'a été rapportée (la Défensine est partiellement coréglée par la voie Toll ; néanmoins, elle n'est plus exprimée dans les mutants de la voie IMD qui sont résistants face à ces infections : elle n'est donc pas nécessaire à la défense contre les

bactéries à Gram-positif)<sup>21</sup>. Des approches par puces à ADN avait révélé un grand nombre de gènes régulés par la voie Toll en réponse à des infections fongiques ou par des bactéries à Gram-positif<sup>22-24</sup>. Il y a plus d'une vingtaine d'années, une approche de spectrométrie de masse MALDI-TOF portant sur des échantillons d'hémolymphe extraite d'une seule mouche avait mis en évidence plusieurs pics correspondant à des peptides induits et sécrétés en réponse à des infections microbiennes, les DIMs (Drosophila Immune-Induced Molecules) alors qu'à l'époque les approches biochimiques classiques nécessitaient des extraits sur des dizaines de milliers de drosophiles infectées pour identifier des activités antimicrobiennes<sup>25,26</sup>. La plupart de ces DIMs correspondent à des produits placés sous le contrôle de la voie Toll alors que d'autres correspondent à des PAMs déjà caractérisés précédemment. Bien qu'identifiés moléculairement, la fonction de la majorité de ces peptides induits restait mystérieuse au début de mes travaux. Ils se répartissent en plusieurs familles dont l'une correspond à des gènes d'une même famille, les Bomanines. Les Bomanines sont définies par un court domaine d'une dizaine d'acides aminés qui se retrouvent dans douze protéines dont plusieurs DIMs. Dix de ces Bomanines sont regroupées en un amas au locus 55C. Ces molécules sont rangées en trois catégories : les Bomanines « courtes », BomS, qui comprennent un pré-domaine, le peptide signal et juste le domaine Bomanine. Une deuxième catégorie comprend deux gènes du locus 55C avec une queue C-terminale ajoutée, les BomT (T pour Tailed), et la troisième catégorie est formée par des molécules comprenant deux domaines Bomanines séparés par un segment intermédiaire, les BomBc (Bc pour Bicipital), représentée par deux membres au locus 55C. La propriété extraordinaire du locus 55C est que la délétion de ses dix gènes produit un phénotype de susceptibilité aux infections par des champignons, des levures et des bactéries à Gram-positif aussi fort que celui obtenu en bloquant la voie de signalisation Toll en amont, bien que celle-ci régule aussi l'expression de dizaines d'autres gènes<sup>13</sup>. Les résultats rapportés par le laboratoire de Steven Wasserman suggéraient donc que les Bomanines du locus 55C avaient potentiellement des fonctions antimicrobiennes contre des catégories spécifiques de pathogènes, soit individuellement, soit collectivement. La caractérisation partielle du locus 55C ainsi que des infections par *A. fumigatus* ont fait l'objet de deux thèses soutenues précédemment dans le cadre des accords de cotutelle entre l'Université de Strasbourg et la Guangzhou Medical University (thèses des Dr. Rui Xu et Yanyan Lou).

Je me suis quant à moi intéressée à une deuxième catégorie de DIMs correspondant à des peptides dérivés du clivage protéolytique d'un seul précurseur fortement induit par l'infection via la voie Toll, IMPPP maintenant nommé BaramicinA (BaraA) par nos soins ainsi que ceux de Mark Hanson qui a travaillé dans un autre laboratoire sur le même gène<sup>27</sup>.

Afin de comprendre la fonction de ce gène, j'ai initié la stratégie d'approche génétique mise en place pour l'étude des effecteurs de la voie Toll et dont la mise en œuvre dans le cas des Bomanines et de BaraA devait permettre de déterminer si cette stratégie pourrait être utilisée à grande échelle pour comprendre la fonction des gènes régulés par la voie Toll. La stratégie consiste d'une part à étudier des mutations perte de fonction obtenues soit par approche CRISPR-Cas9 (*knock-out* : *KO* ; *knock-in* : *KI*) soit par interférence à l'ARN (*knock-down* : *KD*) et d'autre part à surexprimer les gènes cibles de la voie Toll soit en contexte sauvage (surexpression simple) soit en contexte mutant de la voie Toll afin de déterminer si l'expression seule du gène étudié est susceptible de pallier un défaut d'activation de cette voie. J'ai donc généré et caractérisé plusieurs lignées mutantes indépendantes KO (produite par la plate-forme CRISPR-Cas9 du Sino-French Hoffmann Institute) et KI ainsi que deux lignées KD indépendantes. J'ai testé ces lignées dans des expériences de survie, mesuré la charge microbienne au cours de l'infection, déterminé si l'activation de la voie Toll était altérée dans ces lignées, et testé un effet éventuel sur les autres mécanismes de défense, phagocytose et mélanisation. Certains résultats n'étaient pas cohérents d'une lignée à l'autre vis-à-vis de certains de ces phénotypes. Cependant, en procédant à une isogénisation des lignées KO et KI dans un seul et même contexte génétique sauvage (contexte *w<sup>1118</sup>*) de la lignée A5001 de la compagnie Exelixis<sup>28</sup>), j'ai résolu ces problèmes et obtenus des phénotypes similaires et reproductibles. Après avoir testé un échantillon de souches microbiennes auxquelles les mutants des voies Toll ou IMD sont susceptibles, j'ai établi un profil de susceptibilité des mutants *BaraA* étonnamment spécifique. Ces mutants sont susceptibles aux infections par le champignon entomopathogène *M. robertsii*, uniquement dans un modèle d'injection de spores et non d'infection par la voie cuticulaire, ainsi qu'à l'injection de bactéries *Enterococcus faecalis* à l'exclusion des autres souches bactériennes à Gram-positif testées. Les expériences de surexpression transgénique de *BaraA* ou de séquences codant chacun des sous-peptides dérivés (le peptide C-terminal excepté), en contexte sauvage ou mutant

pour la voie Toll, n'ont pas permis de conférer une quelconque protection contre ces deux infections alors que la surexpression de *BaraA* en contexte *BaraA* mutant permettait de sauver leur phénotype de susceptibilité à *E. faecalis* et à *M. robertsii*. Dans les deux cas, la charge microbienne n'est augmentée à aucun moment de l'infection, ni lorsque les mouches ont récemment succombé à l'infection, ce qui exclut un rôle de ce gène dans un processus de résistance, bien que la voie Toll y joue un rôle cardinal. Ces résultats étaient donc difficiles à interpréter.

Cependant, le travail du Dr. Yanyan Lou a permis d'établir que certaines Bomanines protègent les drosophiles contre l'action de mycotoxines émises par *A. fumigatus* agissant par des mécanismes pathologiques variés. Un des facteurs de virulence de *M. robertsii* est la voie de biosynthèse des Destruxines, lesquelles s'insèrent dans les membranes des cellules de l'hôte et agissent comme des ionophores permettant le libre passage de petits ions comme le potassium<sup>29</sup>. Ce sont donc des toxines apparentées à la classe des toxines formant des pores, les *pore-forming toxins* (PFTs). J'ai démontré que les mutants de la voie Toll et les mutants *BaraA* sont sensibles à l'injection de la DestruxineA alors que les mouches sauvages survivent à ce traitement. De manière symétrique, une souche de *M. robertsii* incapable de produire des Destruxines car le premier gène de la voie de synthèse est muté, ne tue plus les mutants *BaraA*, qui se comportent alors comme les mouches sauvages dans ces expériences de survie<sup>30</sup>. Un champignon entomopathogène de la même famille, *Beauveria bassiana*, tue aussi plus facilement les mutants *BaraA*. Cependant, j'ai déterminé que la toxine principale émise par ce champignon, la Beauvéricine, ne tue pas spécifiquement les mutants *BaraA* alors qu'elle tue les mutants de la voie Toll. Ces résultats indiquent donc que *BaraA* protège de manière spécifique contre l'action de mycotoxines. D'autres effecteurs de la voie Toll protègent donc la drosophile contre les effets de la Beauvéricine et restent à être identifiés. En ce qui concerne *E. faecalis*, nous avons d'abord testé et exclu l'hypothèse d'une protection contre la cytolysine émise par ce pathogène. J'ai alors collecté et concentré les surnageants de culture et démontré qu'ils étaient toxiques lorsqu'injectés dans les mutants de la voie Toll et les mutants *BaraA*. J'ai pu établir que l'activité nocive correspondait à une protéine résistante au traitement à la chaleur avec un poids moléculaire compris entre 3 et 10kDa. En analysant la littérature, j'ai trouvé un article rapportant qu'une bactériocine émise par *E. faecalis*, l'entérolineV, contribuait à la virulence de cette bactérie chez la drosophile<sup>31</sup>. Les bactériocines sont des



peptides émis par les bactéries dans le cadre de luttes microbiologiques. Ainsi, l'EntV tue certaines souches de bacilles<sup>32</sup>. Elle affecte aussi des cellules eucaryotes, en l'occurrence celle des hyphes émis par *Candida albicans* lorsqu'il forme un biofilm<sup>33</sup>. J'ai démontré que l'injection de surnageants concentrés de culture obtenus à partir d'une souche *EntV* mutante de *E. faecalis* n'affectait plus spécifiquement les mouches *BaraA*. De même, les mouches *BaraA* se comportaient comme les mouches sauvages lorsqu'elles sont infectées par la souche *EntV* mutante d'*E. faecalis*. Ces résultats établissent donc que la fonction de la BaramicineA est de neutraliser spécifiquement certaines toxines microbiennes, soit en les inactivant soit en permettant à l'hôte d'endurer ou de réparer les dégâts causés par ces toxines. Au rebours d'une étude concurrente<sup>27</sup>, nos résultats plaident donc pour une fonction de *BaraA* dans la résilience aux infections. Il s'agit d'un nouveau concept dans le champ d'étude de l'immunité innée, où les peptides sécrétés au cours de la réponse immunitaire ont été pensés comme ayant une activité dirigée directement contre les microorganismes et non contre les toxines. Nous étions arrivés indépendamment à ce même concept dans le cadre de l'étude des infections par *A. fumigatus*. Il est à noter que les propriétés protectrices de *BaraA* contre des pathogènes procaryotes et eucaryotes fournissent un point de convergence pour l'action de la voie Toll en sus de son activation par ces deux types de microorganismes.

Il importe de souligner que les fonctions de *BaraA* sont multiples et susceptibles d'être assurées par des peptides dérivés spécifiques. Ainsi, alors que la plupart des peptides issus de *BaraA* partagent un court domaine, le peptide N-terminal DIM24 présente une structure différente et il a été proposé que des gènes paralogues codant un domaine similaire seraient impliqués dans le système nerveux<sup>34</sup>. J'ai pu mettre en évidence que *BaraA* est nécessaire pour une activation complète de la réaction de mélanisation et par ailleurs qu'il agit comme une opsonine permettant une meilleure phagocytose par les hémocytes des conidies injectées de *M. robertsii*. Ces deux dernières défenses correspondent à des mécanismes de résistance et ne semblent pas jouer un rôle essentiel dans la mesure où la charge microbienne n'est pas altérée dans les mutants *BaraA* et où le phénotype de sensibilité à *M. robertsii* ou à *E. faecalis* sont perdus lors de l'utilisation de souches microbiennes incapables de produire des toxines.

Par la suite, il sera important de déterminer dans quels tissus la fonction de *BaraA* est requise, aussi en fonction du mode ubiquitaire ou spécifique de certains tissus des

Destruxines et Entéroisine V. Bien que les peptides soient sécrétés, une fonction dans le système nerveux que suggèrent certains résultats préliminaires nécessiterait une expression dans l'encéphale, dont la perméabilité avec l'hémolymphe est limitée par la barrière hémato-encéphalique et constitue donc un compartiment physiologique distinct.

J'ai aussi repris l'étude du gène *GNBP-like3* initiée par Jessica Quintin lors de sa thèse à l'Université de Strasbourg<sup>35</sup>. Ce travail n'avait pas été publié car il n'avait pas permis de mettre en évidence de manière indubitable un phénotype de sensibilité à une infection dans des mouches KD. La protéine GNBP-like3 a un domaine de liaison au  $\beta$ -(1-3)-glucane des parois fongiques, lequel constitue l'essentiel de la protéine. Au contraire, la protéine GNBP3 dispose non seulement d'un domaine similaire en N-ter, mais aussi d'un autre domaine glyco-hydro16 en C-terminal. Alors que GNBP3 est un senseur circulant des infections fongiques qui active par son domaine C-ter la voie Toll via le déclenchement d'une cascade de protéases extracellulaires qui mûrent Spätzle en une cytokine active, ligand de Toll, GNBP-like3 est la protéine la plus fortement induite par les infections fongiques dans des études protéomiques. Jessica Quintin avait documenté une induction aussi par d'autres types d'infections, bactéries à Gram-négatif ou positif. La structure tridimensionnelle de GNBP-like3 a été déterminée par notre collaborateur Alain Roussel (Marseille) et Vishukumar Aimanianda (Institut Pasteur) avait mesuré une affinité pour des multimères de  $\beta$ -(1-3)-glucanes de taille 12-16 motifs alors que la partie N-terminale de GNBP3 se lie plutôt à des polymères plus longs. J'ai caractérisé un mutant nul de *GNBP-like3* obtenu par la technique CRISPR-Cas9 (plateforme CRISPR-Cas9 du SFHI). J'ai initialement trouvé une susceptibilité de ces mutants aux infections par *C. glabrata* et *M. robertsii* (infection par voie naturelle à travers la cuticule). Cependant, ce dernier phénotype n'a plus été observé de manière reproductible une fois la lignée *GNBP-like3* isogénéisée en contexte *wA5001*. Le phénotype de sensibilité à *C. glabrata* est intermédiaire avec le fort phénotype des mutants de la voie Toll, que ce soit en termes de survie ou de charge fongique. Comme cette charge augmente dans les mutants *GNBP-like3*, la fonction de ce gène est donc dans la résistance aux infections par *C. glabrata*. Alors que l'injection de protéine GNBP-like3 recombinante améliore faiblement la survie des mutants de la voie Toll, elle protège complètement les mutants *GNBP-like3*. *In vitro*, la protéine recombinante à forte dose inhibe la croissance de *C.*

*glabrata* mais ne semble pas être candidacide, au rebours de la gomésine, PAM de l'araignée *Acanthoscuria gomesina* utilisé comme contrôle positif.

Les travaux de l'équipe de Steve Wasserman avaient documenté un rôle potentiel des BomS du locus 55C dans la résistance aux infections par *C. glabrata*. J'ai testé l'hypothèse selon laquelle GGBP-like3 formerait un complexe d'attaque avec BomS3 dans lequel GGBP-like3 permettrait de cibler la levure au point faible de son armure, la cicatrice de bourgeonnement, seul endroit où le  $\beta$ -(1-3)-glucane est à nu dans la paroi. Les données obtenues jusqu'à présent ne permettent pas d'étayer cette hypothèse.

Mon travail de thèse apporte donc une contribution originale à notre compréhension des mécanismes mis en jeu par les défenses innées de l'hôte lors d'infections et souligne l'importance de la résilience aux toxines microbiennes par BaraA alors que GGBP-like 3 joue un rôle dans la résistance à *C. glabrata*. Le point commun de ces deux études est qu'il met en évidence un fort degré de spécificité d'action de ces effecteurs de la voie Toll.

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## Introduction to the thesis

During evolution in metazoan, the host has evolved diversified resistance mechanisms that afford it a degree of protection against pathogens. The immune responses are divided into two arms: innate and adaptive immunity.

Acquired immunity is found mostly in vertebrates. The adaptive immune response refers here to the whole process of proliferation and differentiation of antigen-specific T/B lymphocytes into effector cells, relying on antigen-specific recognition, resulting in a series of biological effects. The adaptive immune response can produce immune memory after the initial infection with a pathogen and develop stronger resistance the next time it is infected with the pathogen. This feature is the theoretical basis for vaccination. However, the process of the adaptive immune response takes three to five days to produce a sufficient number of clones that differentiate into effector cells. In contrast, innate immunity includes antimicrobial peptides, phagocytes, and the alternative complement pathways, which are activated immediately after infection and rapidly restrict replication of the infected pathogen [1].

Comparative analysis of innate and adaptive immune recognition [2] and the analysis of associated molecular components [3] suggest that in the evolution of vertebrates, the development of innate immunity appeared before the adaptive immune response. Pattern recognition receptors (PRRs), signaling transduction pathways and downstream effectors are conserved to some degree in plants, insects and vertebrates [4]. Indeed, as the first line of defense against pathogenic microorganisms, all multicellular organisms including vertebrates are considered to have innate immunity [5]. In addition, studies of model organisms that lack adaptive immunity like most animal species, have shown that signaling pathways involving innate immune response are significantly conserved in a variety of organisms, including humans and *Drosophila*. There are conserved class of transmembrane Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) that act directly or indirectly as PRRs in the living world [4, 6-9]. The pathogen-associated molecular patterns (PAMPs) should be highly conserved and relatively invariant characteristic of microorganisms [2], PAMPs include bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, mannans,

DNA, double-stranded RNA, and glucans. The first receptor of the Toll family was described in the study of *Drosophila* as a component of a signaling pathway that controls the dorsal-ventral embryonic polarity during *Drosophila* development [10, 11]. Remarkably, the sequence of the Toll-interleukin 1 (IL-1) receptor (TIR) domain (the cytoplasmic domain of Toll) was turned out to be homologous to the cytoplasmic domain of mammalian IL-1 receptor [12]. Analysis of gene promoters encoding antimicrobial peptides in humans and *Drosophila* showed that they regulated the signaling transduction pathways, activating the NF- $\kappa$ B transcription factors, which also played a role in the Toll pathway [13-16].

Common characteristics of vertebrate, invertebrate, and plant innate immunity include defined receptors of microbial-associated molecules, conserved mitogen-associated protein kinase signaling cascades, and antimicrobial peptides (AMPs) production. The innate immunity of both plants and animals relies on germline-encoded pattern recognition receptors (PRRs) to sense PAMPs [2], such as cell wall components from bacteria or fungi, leading to the activation of innate immune signaling. Afterwards, NF- $\kappa$ B-like transcription factors translocate into the nuclear and yields the transcription of immune effector genes, antimicrobial peptides (in both insects and vertebrates) and cytokines (in vertebrates, such as interferons (IFNs)) [8]. Cytokines produced in innate immune response are also one of the important factors in activating the adaptive immune response [17].

The innate immune response plays an important role in regulating various aspects of immunity; therefore, dysfunction of the innate immune components can lead to diseases, such as inflammatory diseases and cancer. Thus, it is important to study innate immunity, which shows potential therapies of these diseases [18].

*Drosophila melanogaster* lacks the adaptive immunity, and has been studied for more than a century. Powerful genetic tools can be performed with this model organism. Of note, the *Drosophila* genome is 60% homologous to humans, and *Drosophila* shares evolutionarily conserved NF- $\kappa$ B transcription factors to mammals [19, 20]. These advantages make *Drosophila* an ideal model to study innate immunity. Hence, we use *Drosophila* as a model to study the innate immunity.

# General introduction

## 1. *Drosophila* model

### 1.1 *Drosophila* as a model organism

Biologists use the scientific research on selected species as model organisms to reveal biological principles. For example, Morgan chose fruit flies as experimental material to study the principles of heredity in living organisms. Due to evolutionary reasons, many of the fundamental ways of life activity are conserved across the various species of organisms. In addition to genetic research, some species were recognized as excellent model organisms, such as nematodes and fruit flies. Based to the advantages that these organisms were easier to observe and manipulate experimentally, model organism research strategies have been widely used in developmental biology.

*Drosophila melanogaster* has been extensively used in various fields for more than 100 years, and it is the most thoroughly studied model organism. *D. melanogaster* continues to be widely used for biological research in genetics, physiology, microbial pathogenesis, and life history evolution. Radiation, chemicals, transposable elements, provoke mutations, have been used for *Escherichia coli*, yeast, and also *Drosophila*, allowing us to analyze gene functions by studying their mutant phenotypes. For example, Jules Hoffman won the Nobel Prize for the discovery of the Toll receptor in 2011. In 2017, the Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash, Michael W. Young for their works with *Drosophila* in understanding the "molecular mechanisms controlling the circadian rhythm" [21-24].

*Drosophila* is a great model organism. One of the major advantages is their particularly rapid life cycle. Under optimal conditions, the *Drosophila* lifespan is about 60-80 days from the egg to death. The short development time takes on average 10 days from a fertilized egg to the adult at 25°C (Fig. 1), producing hundreds of progenies. The female can produce up to 100 eggs per day and perhaps 2000 eggs in its lifetime. The life cycle includes four developmental stages: fertilized egg, larva, pupa, adult. The embryogenesis takes 24 hours and yields first instar larvae. At 25°C, the resulting larvae grow for about 4 days, molting twice. These processes include three larval stages, termed first, second



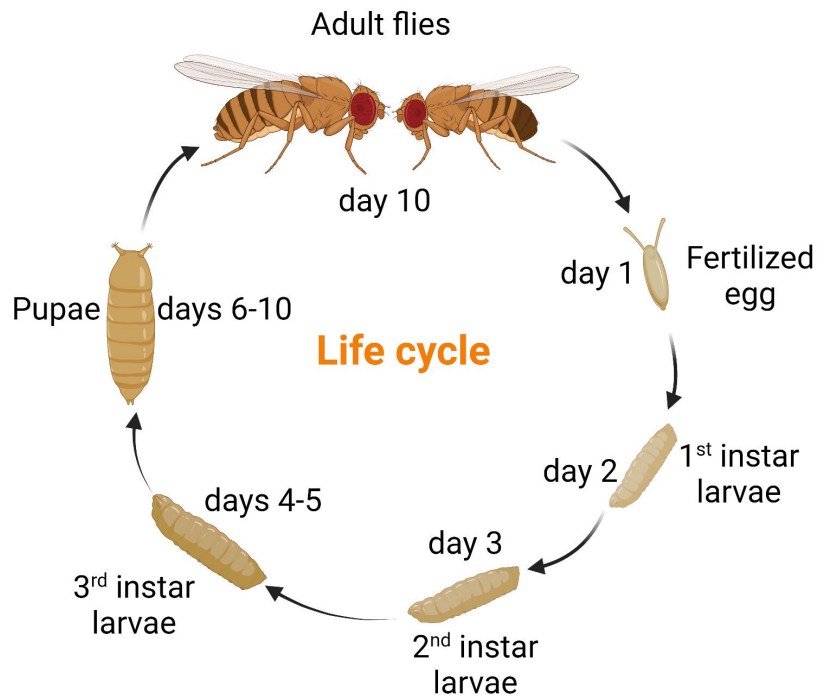
and third instar. Then the larvae are encased in the pupae and undergo a 4-day long metamorphosis (at 25°C), after which the adults emerge and become sexually mature in 8-12 hours. Temperature can have an impact on the speed of the life cycle, with flies at 18°C takes about 19 days from egg to adult. Thus, the short life cycle allows researchers to collect a large number of flies in a short time, allowing for multiple experiments to be performed.

In summary, a short lifespan, rapid generation time, small size, the ability to be safely and readily anesthetized, low experimental cost make *Drosophila* an interesting model to study.

## **1.2 *Drosophila* genetic model**

### **1.2.1 *Drosophila* is a powerful genetic tool for research**

*Drosophila* is a classical model for genetic study and has been used to identify genes involved in the developmental and cellular processes. The genome of *Drosophila* contains four pairs of chromosomes, three autosomes, and one pair of sex chromosomes. The *Drosophila* sequenced genome of 139.5 million base pairs has been annotated in NCBI (National Center for Biotechnology Information) Genome Database. About 75% of known human disease genes have recognizable matches in the *Drosophila* genome [25]. The genetics of *Drosophila* is relatively simple compared to that of mammals and is therefore easy to study. Its complete genome was sequenced and first published in 2000 [26]. There are approximately 14,000 classical genes in the genome of *Drosophila*. The subsequent expansion of new post-genomic technologies, including proteomics, microarrays, RNA-seq, and RNAi (RNA interference) have greatly broadened the possibilities for immune system analysis in this model organism. There is only limited genetic redundancy in *Drosophila*. Genetic redundancy means that there are multiple genes responsible for the same biological function. For example, in mice there may be three copies of a gene that control a particular phenotype. When one of these is mutated, the other genes can compensate so that no developmental or physiological changes are observed in the phenotype. Mutation experiments in mice can therefore be more difficult. In contrast, a gene in *Drosophila* may have only one copy, and when the gene is mutated,



**Figure 1. Life cycle of *Drosophila*.**

The life cycle of *Drosophila* is about 10 days at 25°C and divided into four developmental stages: embryo, larva, pupa, and adult.

it causes a phenotypic change that allows us to understand the specific function of the target gene.

Genetic markers are commonly used in *Drosophila* research, for instance within balancer chromosomes or *P*-element inserts, most phenotypes are easily identified either with the naked eye or under a microscope. Moreover, male flies do not have meiotic recombination, facilitating genetic studies. A recessive lethal "balancer chromosomes" carrying a visible genetic markers can be used to keep a stock of lethal alleles in a heterozygous state without recombination due to multiple inversions in the balancer.

The heart, lung, kidney, reproductive tract and gut from *Drosophila* share functional similarity to mammals. *Drosophila* shows evolutionary conservation between insects and vertebrates. For instant, signaling transduction mechanisms are functionally conserved in mammals, and demonstrates that *Drosophila* is an effective model for deciphering general innate immune mechanisms in animals. *Drosophila* is being used as a genetic model for several human diseases, including the neurodegenerative disorders Parkinson's, Huntington's and Alzheimer's disease [27]. Additionally, *Drosophila* is widely used as a model to study virulence factors of pathogenic microorganisms, and determining their effects on the hosts [28]. Therefore, *Drosophila* acts as a useful model to study various human neuropathies and the interaction between the host and pathogens.

### **1.2.2 Genome-editing in *Drosophila***

As regards the powerful genetic model in *Drosophila*, genetic mutation has been successfully used for the purpose of genome editing as well as a potential therapy of genetic diseases. Genome-editing techniques allow the generation of mutant strains for each gene. Several technologies have been used. Frame shift mutations enable the possibility to investigate the function of interesting genes. Sequence insertions can fuse gene to epitope tags or other functional domain, like fluorescent proteins, which can point genes expression pattern directly. Point mutations can induce amino acid substitutions for disease modeling to correct defective genes for therapeutics. Efficient and targeted genetic replacement is vital for the cure of genetic diseases [29].

Clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 is the most promising gene-editing technology, which is the adaptive bacterial immunity against virus and plasmids through CRISPR RNAs (crRNAs) guiding, silencing the invading acids. In addition, CRISPR/Cas9 has lots of advantages, such as lower cost, ease of manipulation and flexibility, compare to the conventional methods: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Next, I will introduce the gene editing technologies I used in this thesis.

### **A. Knock out (KO)**

The type II CRISPR-associated endonuclease Cas9 and guide RNA (gRNA) can form a ribonucleoprotein (RNP) complex to produce double-strand breaks (DSBs), leading to modifications of the genome. The gRNA comprises 20 nucleotides (nt), complementary to a specific targeted DNA sequence, then binds to the DNA and cleaves it. In principle, the gRNA will be designed as close as possible to the start codon to abolish the target genes by producing premature stop codons. The process of site-specific cleavage happens at a location with base pair complementarity between the target protospacer DNA and the crRNA, as well as a short sequence known as protospacer adjacent motifs (PAM) in the target DNA [30].

DSBs can be repaired by two different pathways in nearly all cell types and organisms, non-homologous end-joining (NHEJ) and homologous-directed repair (HDR). NHEJ leads to insertion or deletion of various lengths as gene knockouts as targeted sites. However, NHEJ is considered to be error prone, which determines that it is not widely used in precise transgene insertion. Genetic replacement is regarded as homologous-directed repair (HDR), which comprises homologous recombination from double-stranded DNA (dsDNA) donor, and single-stranded template repair (SSTR) [31]. HDR can induce point mutations or insert specific sequence by recombination.

However, the off-target effects cannot be ignored. To improve this technology, multiple single guide RNAs (sgRNAs) have been developed to enhance CRISPR-based gene activation and decrease the presence of alleles that develop resistance [32]. A study in 2014 revealed that the off-target effects can be reduced by increasing specificity though truncating gRNAs by 2-3nt at the 5' end in order to decrease mismatch [33]. Gene editing

mediated by RNA-guided CRISPR technology has been extensively used, which provides a powerful tool to study the function of genes.

### **B. knock in (KI)**

HDR is involved in the knock in (KI) strategy by repairing DSBs [34]. When a double strand DNA break is generated, the broken part of the genome will perform HDR if a DNA repair template is provided. The repaired template contains homologous sequence (homologous arms), which are homologous to the upstream and downstream of the target gene. DNA repaired templates can be linear or double strand DNA, or double strand DNA plasmid.

However, HDR repair pathway occurs at a low rate, generally less than 10%; there is also a high frequency of random integrations [35]. Blocking the function of the NHEJ pathway in *Drosophila* can greatly improve the effect of the HDR pathway [36, 37]. Manipulation of the associated cell activity can change gene targeting results. In *Drosophila*, the *lig4* mutant enhances the efficacy of HDR. Moreover, regarding the injection of embryos, more than 1kb homologous arms in the donor plasmid leads to high efficiency in knock-in strategy, especially in the *lig4* mutant background [38].

Here, we generated knock in transgenic *Drosophila* with *mCherry* reporter gene. As noted above, we generated the donor plasmid with around 1kb homologous arms and with *mCherry* reporter. A study reported that a flanking tRNA with multiple sgRNAs can increase the efficiency of knocking in of targeting genes in *Drosophila* [39]. Thus, we constructed the *pCFD5* expression plasmid with sgRNAs, which are downstream of a single *U6:3* promoter. We successfully generated the KI mutant flies by co-injecting the expression and donor plasmids into *lig4* mutant embryos.

### **C. Ribosomal Nucleic Acid interference (RNAi)**

RNA interference (RNAi) is induced by double strand RNA (dsRNA), inhibiting the expression of targeted genes by blocking the transcription or translation of specific genes. RNAi was first identified by Fire and his colleagues that dsRNA can silence post-transcriptional gene expression in *Caenorhabditis elegans* [40]. They received the 2006

Nobel Prize in Physiology or Medicine. This discovery figured out the mystery of gene silencing in plants and fungi.

In plants, RNA is processed to a 25nt in length that correlates with post-transcriptional gene silencing [41]. In *Drosophila*, dsRNAs of 21-23nt in length cause RNA interference and are also known as small interfering RNAs (siRNAs) [42]. RNase III family members, such as Dicer-2 (Dcr-2) specially process dsRNAs. It has been experimental validated in *Drosophila* that Dcr-2 has the ability to yield 22nt fragments [43, 44]. This enzyme is able to digest dsRNA into siRNA, and is evolutionarily conserved from fly to human. Subsequently, siRNAs involved in the formation of an RNA-induced silencing complex (RISC), which acts on the homologous mRNA, leads to the degradation of mRNA. Argonaute2 (AGO2) is the main component in RISC, aiding in target recognition and cleavage during RNA. The activated RISC is guided by a single siRNA strand (guide strand), and the sequence specifically binds to the target mRNA and cuts off the target mRNA, resulting in specific decomposition of the target mRNA, blocking translation or causing post-transcriptional gene silencing.

RNAi is efficient and simple; it is another important genetic tool to investigate the function of genes. This technology does not cause permanent alterations, but reduces the mRNA levels in order to eliminate the function of the gene. As regards redundant genes in the genome, RNAi is more efficient to silence the targeted genes. The off-target effect cannot be ignored however. RNAi is cell-autonomous in *Drosophila*, and is used with UAS-GAL4 system, to achieve cell or tissue specific knock down or reduce the expression of the mRNA at any stages of development [45].

#### **D. Overexpression**

Loss-of-function strategies are useful and powerful to investigate gene function. However, this strategy may fail to reveal phenotypes for the functionally redundant genes. Thus, gain-of-function approaches, like overexpression and misexpression, are complementary to loss-of-function strategies.

Overexpression is one of the main technologies to improve the host defense against fungal pathogens [46]. Overexpression of transcription factors can enhance defense-related genes in plants, thus stimulating host defenses [47]. However, overexpression of

the Cecropin in plants did not increase resistance to bacteria, which might be due to protein instability [48]. Additionally, expression of new proteins may cause allergic or toxic effects.

The overexpression transgenic flies are used in conjunction with the UAS-GAL4 system (describe below). In addition, *P*-element system has a relatively moderate transformation efficiency, which makes it suboptimal for transgene generation [49]. In *Drosophila*, a UAS-ORFome library has been generated to control the expression of transgenes to avoid these limitations [50].

To further study the function of targeting genes, in this study, we generated UAS-ORF fly lines with the site-specific  $\Phi$ C31 (phiC31) integrase method [49, 51]. The bacteriophage  $\Phi$ C31 encodes an integrase, which mediates sequence-directed recombination between the bacterial attachment site (*attB*) and the phage attachment site (*attP*) [52]. This strategy of site-specific integration allows the efficient construction of large numbers of transgenic fly lines.

### 1.2.3 UAS-GAL4 system

The GAL4 activation system was first introduced into flies in 1993 [45], providing a powerful strategy to study the expression pattern of genes. Afterwards, multiple GAL4 lines have been generated. For example, some GAL4 lines might be expressed only in muscle cells, or nerves, or fat body, and so on, the so-called tissue specific GAL4. GAL4 is a yeast transcription factor and its spatial and temporal expression can be controlled thereby to direct the activity of target genes at specific cells and tissue, and also at specific developmental stages. GAL4-binding upstream-activating sequence (UAS) is placed in the front of target genes, crossing to the strain that contains GAL4. Therefore, their offspring express the target gene driven by the UAS-GAL4 system. For instance, the transgenic flies (RNAi or overexpression) are crossed to the GAL4-expressing flies, resulting in the target gene being transcribed in a specific GAL4-expressed pattern. In the absence of GAL4, the target gene remains silent, allowing the spatial and temporal expression. Every driver line can be used to drive target genes to perform large scale screens.

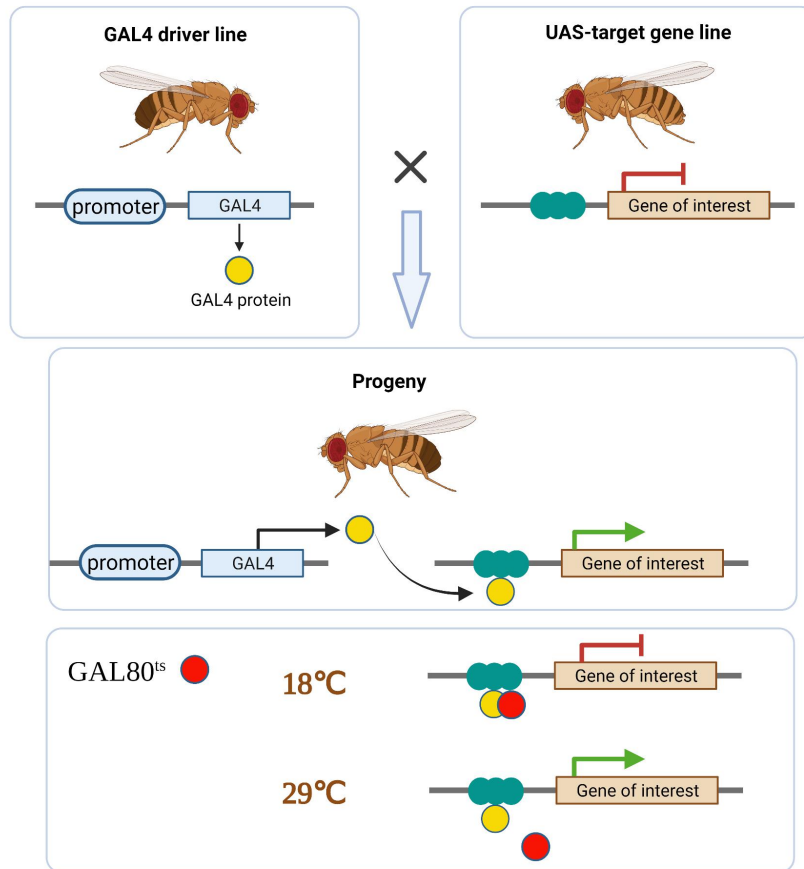
The activity of GAL4 can be inhibited by yeast GAL80 protein [53]. At thermo-sensitive (ts) allele of *GAL80* was found in yeast with a genetic screen and which was then introduced in *Drosophila* [54]. GAL80<sup>ts</sup> binds to GAL4, leading to the inactivation of UAS-GAL4 system at 18°C. GAL80<sup>ts</sup> fails to bind to GAL4 at 29°C [55, 56]. Heat shock allowed inducible but ubiquitous expression. The temporal expression of UAS-GAL4 system can be controlled by the temperature shifts. In addition, hormone-inducible GAL4 can also be used to achieve temporal expression, the Gene Switch GAL4 system for instance [56]. Gene Switch GAL4 responds to the synthetic steroid mifepristone (RU486). This system acts as powerful tool in the use of *Drosophila* as a model for investigating complex cellular and developmental process.

## 2. Immune response

To escape from predators, animals can effectively protect themselves through protective coloration and physical barriers such as keratinous epidermis, scales or carapace. In contrast, humoral immunity induces the production of highly effective immune molecules in the fat body that are capable of recognizing and removing foreign substances from the body to protect against microbial infection [20]. Epithelial immunity, that is, fighting against invading microorganisms at the level of the barrier epithelia cells, is now understood as a great contribution to the protection of fruit flies. Different pathogens induce different immune effects in insects, and the effective molecular mechanisms depend on host pathogen study and their route of infections.

*Drosophila* relies on multiple innate defense responses. The immune system of *Drosophila* can be divided into three major arms: systemic immune response, melanization and phagocytosis. The systemic humoral immune response is mediated by the Toll and IMD pathway, which are detecting microbes. Then, antimicrobial peptides (AMPs) will be produced against pathogens and hundreds of genes will be induced. The fat body of *Drosophila* is a composite of the mammalian liver and adipose tissue, it is a powerful organ and a major immune-responsive tissue that triggers the systemic response





**Figure 2. UAS-GAL4-GAL80<sup>ts</sup> system.**

When the GAL4 expresses, it targets the UAS and results in the expression of the gene of interest. When flies carry GAL80<sup>ts</sup> at 18°C, GAL80<sup>ts</sup> binds to GAL4 to inhibit its activities. When at 29°C, GAL80<sup>ts</sup> cannot prevent the activity of GAL4 that binds to the UAS, leading to the expression of the targeting gene.

that produces large amounts of humoral response molecules, such as serine proteases and AMPs during infection [20]. AMPs are produced by the fat body and secreted into the hemolymph, then attack bacteria and/or fungi. The cellular immune response refers to the direct activity of blood cells (hemocytes) in *Drosophila*, which are similar to mammalian monocytes/macrophages. Specialized hemocytes also possess a significant role by participating in the humoral immune responses such as the melanization reaction [19, 57]. *Drosophila* has innate immunity, and lacks an adaptive immune response compared to mammals. However, the main factors of this innate immune response are broadly conserved between mammals and fruit flies. Therefore, the fruit fly provides a useful model of innate immunity for dissecting the genetic interactions of signaling and effector function, as *Drosophila* does not have to deal with interference of adaptive immune mechanisms that may confuse results. Various genetic tools, protocols and detection methods have made *Drosophila* a classical and great model for studying the innate immune system [58].

The *Drosophila* genome encodes three NF- $\kappa$ B/Rel-like proteins. Dorsal, Dorsal-Related-Immunity Factor (Dif), and Relish. Dorsal and Dif contain an N-terminal Rel DNA binding domain and a C-terminal trans-activator domain. Relish is present as similar organization of p105, containing an N-terminal Rel domain as well as a C-terminal inhibitory ankyrin repeat domain [15, 59-61]. Genetic studies have revealed that the key roles of these transcription factors in regulating of AMP genes through two different signaling pathways, named as Toll and IMD pathways. Toll pathway deficient mutants are sensitive to the infections of most Gram-positive bacteria, dimorphic fungi, filamentous fungi or yeast, while IMD pathway mutants are susceptible to Gram-negative bacterial and some bacilli infections [19-21].

## **2.1 Humoral immune response**

### **2.1.1 The Toll pathway**

The Toll pathway was first described for the establishment of the dorso-ventral axis by regulating the nuclear localization of the transcription factor Dorsal during early embryogenesis [11]. Humoral responses are well characterized, and the hallmark is the

robust production of a battery of AMPs. The immune responses of Gram-positive bacteria and fungi are mainly mediated and regulated by the *Drosophila* Toll signaling pathway by regulating hundreds of effector genes in the fat body. Unlike the Toll-like receptors in mammal, Toll receptors in *Drosophila* do not interact directly with microbial products, but are activated by the cleaved form of cytokines Spätzle (Spz) [21]. The Spz processing enzyme (SPE) has a CLIP-domain, which has been documented to cleave Spz [62]. Two pathways have been shown to activate the SPE in genetic study: PRR (pattern recognition receptors) and Persephone (Psh) pathways. In the PRRs process, microbial ligands Lys-PGN can be recognized by GGBP1 and PGRP-SA after Gram-positive bacterial infection [63, 64], or GGBP3 binds to  $\beta$ -(1, 3)-glucans after fungi challenge [65, 66], leading to the activation of ModSP (modular serine protease), resulting in triggering Grass.

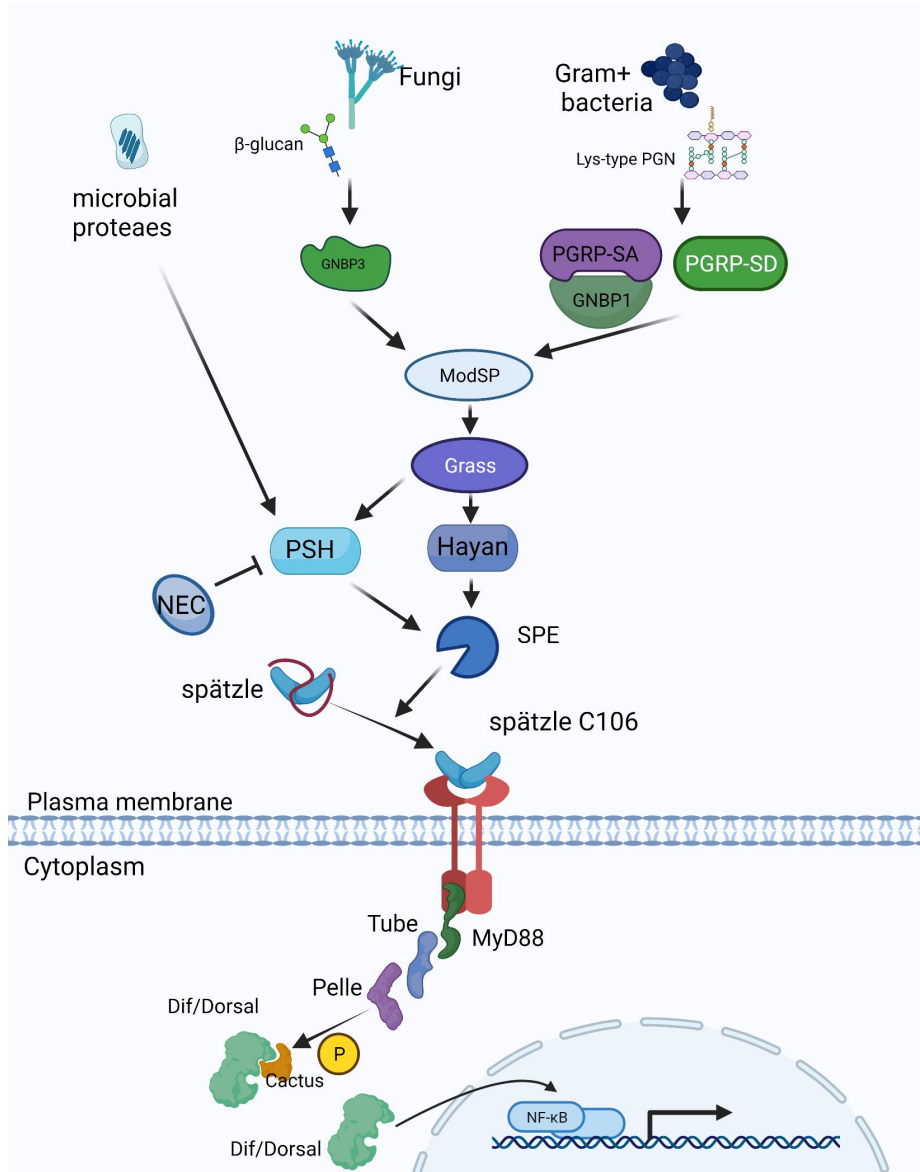
Microbial proteases can also stimulate Toll signaling pathway, which activates Psh, resulting in the activation of SPE [66-68]. Of note, serpin necrotic can inhibit the activity of Psh [69]. Research revealed that Hayan and Psh are close to each other (only 751bp apart) on the *Drosophila* X chromosome and encode highly similar proteins, resulting from the duplication of ancestral gene. The double mutant flies are more susceptible to *S. aureus* than wild type flies. After *Candida albicans* infection, Hayan and Psh double mutant flies failed to activate the Toll pathway. These data indicates that Hayan and Psh redundantly regulate the Toll pathway [70]. Psh also detects protease danger signals such as abnormal proteolytic activity [68]. The SPE then cleaves proSpz-1 into Spz-1. The cleaved cytokine Spätzle (Spz) protein forms a functional Toll ligand that can bind to the Toll membrane protein receptor Toll-1, inducing the intracellular domain of the Toll receptor to recruit the death domain-containing protein MyD88 [71], whose C-terminal phosphatidylinositol-binding domain recruits the Tube (ortholog to IRAK4 in mammal) [72, 73]. When Tube recruits Pelle (ortholog to IRAK1 kinases in mammal, which are associated with the Toll-like receptors and IL-1) [74], Pelle phosphorylates the target protein Cactus (homologous to the I $\kappa$ B protein of mammals), resulting in degradation of Cactus [75]. Ultimately Dorsal or Dif are released from Cactus and translocate into the nucleus, which allows them to enter the nucleus (Fig. 3). This process effectively induces the expression of AMPs and enhances the ability of the host to resist microbial invasion

[76, 77]. The Toll pathway is activated within hours, while the transcription of target genes persists for days, Drosomycin for instance [78].

The *Drosophila* Toll protein is a transmembrane protein receptor consisting of an extracellular ligand-binding region, a transmembrane region and a cytoplasmic region consisting of a leucine-rich tandem repeat and a cysteine-rich tandem repeat. The intracellular region resembles the structure of the interleukin receptor. Toll protein is associated with *Drosophila* development and its innate immunity [79]. However, Toll does not act as a pattern recognition receptor [80]. To date, nine Toll family genes have been identified in *Drosophila*. All of the Toll receptor proteins have been shown to share similar molecular structural features, with Toll/Toll-1 being the first receptor protein identified to directly mediate innate immunity [21, 81]. Toll-2/18-W regulates cell polarity in embryonic epithelial tissues via the Rho-GTPase pathway and is associated with morphogenesis [82]. Toll-5, which is evolutionarily closest to Toll-1, interacts with the intracellular domain of Toll-1 and Pelle to synergistically upregulate Dorsal-dependent transcriptional activation [83]. Toll-8, also named Tollo protein, is associated with the induction of glycosylation in *Drosophila* embryonic neurons [84]. Yagi *et al* [82] suggested that Toll-2/18-W, Toll-7 and Toll-8 regulate the growth and development in *Drosophila*. Toll-9 contains only a cysteine-rich region in its sequence, and its structure is very similar to that of the mammalian TIR, which constitutively expresses the antimicrobial peptide Drosomycin, it has no known function in innate immunity [85].

### 2.1.2 The IMD pathway

The IMD pathway was first identified with the *immune deficiency (imd)* mutation, that blocks the induction of antibacterial peptides during the immune response in *Drosophila* [86]. *imd* encodes a protein with a death domain, and plays a role in the activation of NF- $\kappa$ B and apoptosis. The protein is similar to the death domain of Receptor Interacting Protein (RIP) in mammal in tumor necrosis factor receptor (TNF-R) pathway, which is the pathway that shares similarity to the IMD pathway [87]. Moreover, overexpression of *imd* results in the transcription of antibacterial peptides but not antifungal peptide Drosomycin [87].



**Figure 3. The Toll pathway in *Drosophila*.**

Pathogen cell wall components from fungi or Gram-positive bacteria or virulence factors can trigger the Toll pathway. Persephone (PSH) or Hayan activates SPE to process pro-Spätzle to form the active form, leading to the activation of the NF-κB factor Dif or Dorsal and eventually resulting in the production of antimicrobial peptides and other immune genes.

Recognition of microorganisms is the first step that activates the IMD signaling pathway. A number of conserved proteins peptidoglycan recognition proteins (PGRPs) function as receptors for Gram-negative bacterial peptidoglycan (PGN). DAP-type PGN, which belongs to the inner cell wall of most Gram-negative bacteria, binds to transmembrane receptor PGRP-LC, both in gut and fat body after the infection of Gram-negative bacteria or some Gram-positive bacteria, like *Bacillus spp.* [88, 89]. However, PGRP-LC mutant can still respond to PGN in the gut [90]. Further research found that, PGRP-LE is secreted into hemolymph and binds to PGN. However, PGRP-LE is the only intracellular receptor has been found in *Drosophila* so far [91]. PGRP-LF acts as a negative regulator to block the IMD pathway by antagonizing the activation of PGRP-LC [92].

Once bound to PGN, these receptors dimerize or multimerize then stimulate the recruitment of a complex, including IMD, the adaptor protein dFADD (*Drosophila* Fas-Associated protein with Death Domain), and DREDD (Death-related ced-3/Nedd2-like protein, relates to vertebrate caspase-8) [86, 93-96]. Dredd is activated by ubiquitination. The active form of DREDD cleaves IMD by removing N-terminal fragment (30 aa). This process allows the recruitment and activation of the TAB2/ TAK1 (TAK1-associated binding protein / TGF- $\beta$  activated kinase 1) complex. TAK1 is also responsible for triggering the JNK pathway. The activated TAB2/ TAK1 complex is required for the phosphorylation and activation of IKK (inhibitor of  $\kappa$ B kinase) complex [97], which results in the activation of Relish [98], the third *Drosophila* NF- $\kappa$ B-related protein that is homologous to NF- $\kappa$ B1 (p105) in mammals [61]. Relish is cleaved during signal stimulation into N-terminal and C-terminal part by DREDD. The C-terminal fragment (Rel-49) remains in the cytoplasm, while the N-terminal fragment (Rel-68) is the active form [99]. Relish gets phosphorylated by the IKK (kenny/ Ird5) thereby activating its transcription activation function [98]. Relish N-terminal domain translocates to the nucleus. After the translocation in the nucleus, Relish regulates the transcription of different subset of antimicrobial peptide genes [100, 101].

The activation of the IMD signaling pathway is rapid after bacterial infection. Starting from the signal activation to Relish translocation, the cascade of the IMD pathway can occur and finish within minutes [102]. Transcription of target genes occurs within hours, like that of AMP genes [78]. Thus, the IMD pathway is considered to be the rapid

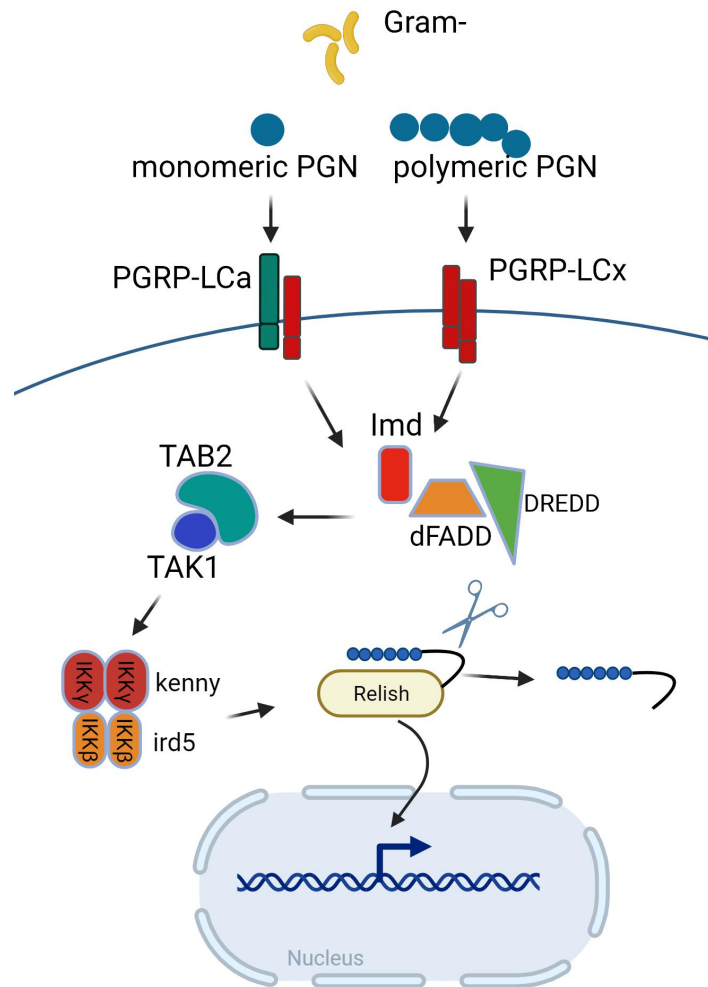
response against fast-replicating pathogens, such as bacteria. Besides bacteria, the IMD pathway also has been shown to be involved in the defense against some RNA viruses by a non-canonical pathway involving dSTING [103]. Mutations in *imd*, *dFADD*, *dredd*, *ird5*, *Tab2*, *relish* and *kenny* showed increasing replication of the Sindbis virus. These results were confirmed with the *Relish<sup>E20</sup>* null mutant, which had higher virus load than wild type flies [104].

### 2.1.3 The Pattern Recognition Receptors (PRRs)

The first step of the signaling cascades is to recognize the invading microbes. In innate immune responses, pathogen recognition relies on interactions between relatively invariant structure from microbe such as components of the cell wall and recognition proteins from the host. Once infection occurs, host defense relies on non-self-recognition. From the above mentions, fungi are sensed by glucans while bacteria can be sensed by PGN. The cell wall components from microbes are highly conserved molecular structures and patterns from insects to mammals, and are referred to as pathogen-associated molecular patterns (PAMPs). Detection of microorganisms by pattern recognition receptors (PRRs) are conserved in evolution. Ten TLRs function as PRRs in human. In *Drosophila*, the Toll signaling pathway can be triggered by fungi and Gram-positive bacteria. Unlike TLRs in mammal, Toll is not a PRR. It is activated by the Spätzle cytokine instead. Recognition of PAMPs relies on two PRR families, the PGN Recognition Proteins (PGRPs) and the Gram-negative Binding Proteins (GNBPs). GNBPs are known as  $\beta$ -Glucan Recognition Proteins ( $\beta$ GRPs) [20].

#### A. PGRP

As regard to bacteria, PGN serves as the PAMP in *Drosophila*. PGN is a major bacterial cell wall component. The Toll pathway is initiated by the detection of lysine type PGN (Lys-PGN), which is present in the cell wall of Gram-positive bacteria. The IMD pathway is activated by *meso*-diaminopimelic acid (DAP) PGN (DAP-type PGN), which



**Figure 4. The IMD signaling pathway in *Drosophila*.**

The IMD pathway is activated by the DAP-PGN of Gram-negative bacteria. PGRP-LC acts as the receptors, leading to the signaling cascade. The NF- $\kappa$ B transcription factor Relish translocates into nucleus, resulting in the production of AMPs.



in characteristic of all of Gram-negative bacteria and a few Gram-positive bacteria. These PGNs are recognized by PGRPs. PGRPs were firstly described in 1996 [105]. PGNs are components of the bacterial cell wall, conserved from insects to mammals. The PGRP protein found in the hemolymph from silkworm bound to Gram-positive bacteria and trigger the prophenoloxidase cascade, which forms melanin. There are 13 PGRP genes in *Drosophila* and are transcribed into at least 17 PGRP proteins [106]. Basic to the transcript size of PGRPs, it can be divided into short (S) PGRPs and long (L) PGRPs. Short PGRPs are extracellular proteins with signal peptides, while long PGRPs usually comprise extracellular, intracellular and transmembrane domains. *Drosophila* has seven short PGRPs and ten long PGRPs. The domain of PGRPs is in the C-terminal regions of long PGRPs, which are homologous to short PGRPs [107]. PGRPs can be found in immune organs such as fat body, hemocytes and gut [106, 108]. Detection of Lys-type PGN in the Toll pathway requires a combination of PGRP-SA and glucan binding protein 1 (GNBP1). PGRP-SA is important for the Toll pathway to recognize the Gram-positive bacteria. Mutant flies with a C80Y mutation in PGRP-SA resulted in high susceptibility to Gram-positive bacteria [63]. As in the IMD pathway, DAP-type PGN can be sensed by PGRP-LC or PGRP-LE. PGRP-LC has three alternative splice forms, PGRP-LCa, LCx, LCy, which are transmembrane proteins [109]. PGRP-LCa and PGRP-LCx are involved in the recognition of Gram-negative bacteria but not PGRP-LCy. The function of PGRP-LCy still remains unknown. PGRP-LCx binds to polymeric PGN [110]. PGRP-LCa and PGRP-LCx interact with the monomeric PGN, a disaccharide tetrapeptide known as tracheal cytotoxin (TCT) [111], which is secreted when bacteria proliferate: it is released during the remodeling of the cell wall during bacterial division.

## **B. Gram-negative Binding Proteins (GNBPs)**

GNBPs were firstly identified in the hemolymph of *Bombyx mori* for binding to Gram-negative bacteria (*Escherichia coli*), hence named as GNBP [112]. There are three GNBP genes (GNBP1, 2, 3) and three shorter related genes (GNBP-like1, 3, 4). GNBPs contain a N-terminal glucan binding domain and a C-terminal domain, similar to  $\beta$ -(1, 3)- and  $\beta$ -1, 4-bacterial glucanases, and  $\beta$ -(1, 3)-glucanase from the *Strongylocentrotus purpuratus* [113]; yet, a key catalytic residue is missing in the glucose domain

GNBP1 belongs to the  $\beta$ -Glucan recognition protein ( $\beta$ GRP) family. A complex including GNBP1 and PGRP-SA is required for the host defense against Gram-positive bacteria. Although both GNBP1 and PGRP-SA bind to PGN, just PGRP-SA binds to purified PGN fragments however [114]. Further research identified that GNBP1 functions as an enzyme, and is required to process and present Lys-PGN to PGRP-SA [115]. Without immune infection, the complex of GNBP/ PGRP-SA fails to generate a signal to activate the downstream cascade. When Lys-PGN is present in the hemolymph, the GNBP/ PGRP-SA complex can recognize and bind to it efficiently and rapidly [64, 115]. GNBP2 seems to play a role in the recognition of some Gram-positive bacteria that are not detected through the canonical GNBP1. GNBP1-GNBP2 double mutant flies are more sensitive to *Streptococcus pyogenes* than GNBP1 mutant flies, which indicates that GNBP2 might respond to *S. pyogenes* infection [116].

As for fungi, GNBP3 is the corresponding PRR able to recognize  $\beta$ -(1, 3)-glucans, which belongs to the  $\beta$ GRP family. Structure based mutagenesis experiments have identified that the N-terminal region of GNBP3 discriminates between short and long polysaccharides. GNBP3 N-terminal domain binds to long chains of  $\beta$ -(1, 3)-glucans [65]. GNBP3 mutant flies are unable to activate the Toll pathway and are susceptible to fungal infections [66]. Furthermore, GNBP3 can also activate melanization and initiate the attack complexes that target invading microorganisms [117].

The short related GNBP-like proteins are homologous to the N-terminal domain of GNBP3. A study in 2004 suggested that GNBP-like 3 (CG13422) is phylogenetically closer to GNBP3 [118]. Proteomic approach revealed that GNBP-like 3 has been highly up regulated (72 folds) in the adult flies after *Beauveria bassiana* challenge, but not Gram-positive or negative bacterial infection, which indicates that GNBP-like 3 might act as a specific antifungal protein [118]. However, Jessica Quintin found that GNBP-like 3 was strongly induced in the hemolymph after immune challenge with *E. coli* or *M. luteus*, which could remain at a high level for at least 24 hours [116]. Moreover, GNBP-like 3 is able to bind specifically to yeasts (unpublished data). A mRNA sequencing experiment claimed that GNBP-like 3 is required in neurons for long-term memory in *Drosophila*. In addition, GNBP-like 3 also has bacteriostatic activity [119].

#### **2.1.4 Innate immune effectors**

Pathogen recognition receptors detect pathogen associated molecular patterns (PAMPs) upon infection, which initiates the recognition of non-self that activates innate immune pathways [120]. An important output of the innate immunity is the production of AMPs [121]. AMP gene promoters from *Drosophila* and other insects contain conserved binding sites for NF- $\kappa$ B, which indicates that the AMPs inducible production responses to infection are evolutionarily conserved [14].

The activation of Toll and IMD pathways ultimately produce AMPs and up regulate more than 250 immune response genes. AMPs are also known as ribosomally synthesized antibiotics. AMPs are produced by fat body and released into the hemolymph after infection, then kill invading pathogens or inhibit their growth. In *Drosophila*, AMPs can be induced at epithelia locally [60, 122] or secreted into hemolymph systemically. AMPs in *Drosophila* can be divided into at least seven classes. They are of small size (<100 residues), secreted and cationic peptides, with a relatively broad spectrum of activity. Drosomycin and Metchnikowin are antifungal peptides. Diptericins (two genes), Drosocin and Attacins (four genes) are predominantly active against Gram-negative bacteria [3, 123, 124]. Defensin shows antibacterial activity to Gram-positive bacteria [125]. Cecropins (four inducible genes) show both antifungal and antibacterial activities [126].

AMPs after infection can accumulate close to millimolar range concentration (0.3mM). For instance, Drosomycin is active against *Neurospora crassa* infection at concentration of 100 $\mu$ M in hemolymph [127]. A genetic approach revealed the function of AMPs by ectopically expressing one or two AMP transgenes in a IMD/Toll pathway double mutant background, in which no AMP genes can be induced [128]. Immuno-compromised flies of *imd* and *spz* double mutant, which constitutively expressed Drosomycin or Defensin displayed a wild type resistance to certain microorganisms [129]. In addition, one copy of UAS-*Drosomycin* is enough to protect flies from *N. crassa*, while two copies are sufficient to induce full protection against *Fusarium oxysporum*. Upon some Gram-negative bacteria infection, there is a cooperative effect when two AMPs were co-expressed, which indicates that several AMPs may act in an additive way but not synergistic effect [129]. A study in 2019 directly addressed the function of AMPs in innate immune response *in vivo* in *Drosophila*. The idea was to knock out individual or

groups of AMP genes by CRISPR/ Cas 9, then challenge these mutant flies with various bacteria and fungi to examine survival phenotypes. When the AMPs genes regulated by from the IMD pathway were absent, the mutant flies displayed a sensitivity to Gram-negative bacteria similar to that of flies that lack a functional *imd* response, and succumb to bacteremia. These data indicate that AMPs play a role in resistance to Gram-negative bacteria and are important for flies to survive against bacteria. However, deleting most AMPs exhibit hardly any decreased resistance with the infection by Gram-positive bacteria [130].

Effectors regulated by the Toll pathway able to attack pathogenic yeasts or Gram-positive bacteria *in vitro* have not been described so far. In addition to seven well characterized AMPs, some innate immune inducible genes encoding small proteins in *Drosophila*, which have been identified that likely have antimicrobial activity [131]. 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) [118]. Some of them correspond to known AMPs whereas others belong to a family of 12 proteins that contain a domain known as the Bomanin (Bom) domain [132, 133]. Twelve related genes belong to the Bomanins family that all encode proteins comprising a short amino acid domain, the Bomanin domain. The deletion of ten Bomanin in the 55C locus leads to a phenotype almost as strong as mutants for the Toll signaling pathway to some fungi and bacteria infection, revealing that Bomanins play a crucial role as effectors of the Toll pathway [132]. Peptides from Bom family lack sequence similarity to AMPs. Boms are also important for infected flies to survive and showed a candidacidal activity after *Candida glabrata* infection [133]. A gene named *Bombardier (bbd)* is involved in the host defense by somehow stabilizing the short-form Bom peptides [134]. Furthermore, BBD is required for proper folding, secretion of short-form Boms. In addition, Daisho1 and Daisho2 have been shown to be involved in the defense against filamentous fungias likely AMPs [135].

Several DIMs are actually derived from a polyprotein precursor known as IMPPP and until recently their function was not understood. More than 30 peaks that found in the Mass-spectrometry in 1998, among them DIM5, 6, 8, 10, 12, 13, 24 are derived from IMPPP [118, 136]. Most of their peptides, except for DIM24, share sequence similarity to the form of 12 amino acid domain. This situation is akin to the formation of

neuropeptides that are also found initially in large precursor proteins. A recent study renamed this protein as BaramicinA (BaraA) [137]. *BaraA* encodes a precursor protein that can be cleaved into multiple peptides through furin-like sites. *BaraA* is strongly induced in the fat body. BaraA also can be observed in the head by BaraA driver that drives the GFP expression. Lacking BaraA show a susceptibility phenotype to the entomopathogenic fungus *Beauveria bassiana*, while overexpression of BaraA increased resistance to fungi. Moreover, IM10-like peptides show antifungal activity *in vitro* [137].

## 2.2 The developmental Toll pathway

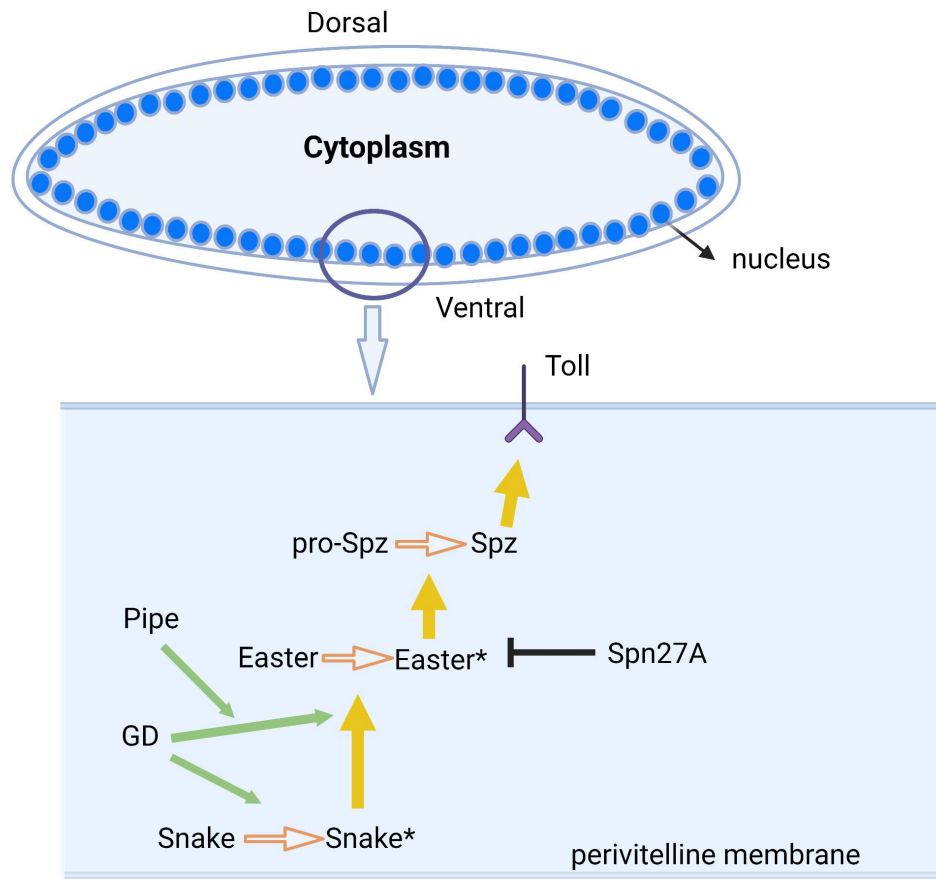
The Toll pathway has initially been identified and genetically delineated because of its role in the establishment of the dorso-ventral axis of embryos during the first three hours of their development [11, 138]. The dorsal group genes display a loss-of-function mutant phenotype in which embryos produce mostly dorsal structures and lack ventral structures, hence the name dorsal given to the first identified gene of this group after its phenotype. Ten further genes with a similar maternal effect phenotype have been identified throughout the years and include: *gastrulation-defective (gd)*, *dorsal*, *windbeutel*, *nudel*, *tube*, *pipe*, *snake*, *easter*, *Toll*, *spätzle*, and *pelle*. Of note, dominant gain-of-function alleles exist and display an opposite ventralization phenotype, *Toll*<sup>10B</sup> being the most notorious one. *Cactus* loss-of-function phenotype is also ventralized and is epistatic to all dorsal group genes, except for dorsal itself. These observations established Dorsal, a NF- $\kappa$ B transcription factor, as the key target of the dorso-ventral signaling cascade. Biochemically, the intracellular signaling cassette is similar to that found for the immune response, with two modifications. The Toll-MyD88 Weckle adapter functions only during development and Dorsal but not DIF mediates the dorso-ventral patterning of the embryo. As a result of a progressive activation of the Toll pathway along the dorso-ventral axis, Dorsal forms a gradient of nuclear localization, with all Dorsal transcription factor being located to nuclei in the most ventral parts whereas it remains cytoplasmic dorsally.

The activation of Toll is mediated by the binding of cleaved Spätzle to Toll. Spätzle is activated only on the ventral side, through a complex process that starts during oogenesis and leads to the deposition of spatially-restricted cue on the ventral side of the

perivitelline membrane, the inner layer of the egg covering. Briefly, the Pipe sulfotransferase gene is transcribed in the ventral follicular cells that surround the developing oocyte. It is thought that it will leave the spatially-restricted mark on an uncharacterized target likely carried by the perivitelline membrane. During early embryogenesis, this mark will be used to activate a protease activation cascade only on the ventral side, thus leading to the generation of an active Spätzle ligand only on the ventral side. This proteolytic cascade is distinct from the ones that were late identified to be involved in the immune response. Thus, Spätzle cleavage is not mediated by SPE in the embryo, but by the Easter (EA) protease. The premature activation of EA is inhibited by the SPN27A serpin, a serine protease inhibitor. EA is itself activated by Snake, which in turn gets activated by the Gastrulation defective (GD) protease. GD has also a direct input on the activation of Easter, which is indirectly regulated by the action of Pipe in the ventral follicular epithelium during oogenesis [139] (Fig. 5). Nudel is the most upstream protease of the signaling pathway, however, its exact function remains poorly understood to this date [140].

### **2.3 The cellular response: phagocytosis**

There are several kinds of hemocytes in *Drosophila*. Based on the function and structure, these cells can be divided into three types: plasmatocytes, crystal cells and lamellocytes [141]. Among the circulating hemocytes, 90-95% of them belong to plasmatocytes. Plasmatocytes are required to eliminate apoptotic cells and invading pathogens. This type of cells is high similar to macrophage in mammals. A 2-5% proportion of the cells are



**Figure 5. The developmental Toll pathway.**

The developmental Toll pathway has been identified to establish the dorso-ventral axis of embryos. The activation of Easter, Spz, and Toll is restricted to the ventral site, while the activation of Snake is occurring around the embryo. GD can directly process Snake, and facilitate the active form of Snake (Snake\*) to process Easter through a mechanism that requires Pipe activity.

crystal cells, which express proPOs and further mediate melanization, facilitating wound healing, the hypoxic response and innate immune response [142]. A third cell type are lamellocytes, which do not exist in embryos or adults, and are rarely found in healthy larvae. However, hemocyte precursors can differentiate into lamellocytes after wasp eggs infection [143]. Lamellocytes are large and flat cells that are primarily function in encapsulation following parasitization and only can be observed in larval stages [144].

Phagocytosis is mediated in *Drosophila* by plasmatocytes, which are analogous to the mammalian macrophages. Phagocytosis plays an important role in cellular immunity defense mechanism and is conserved throughout evolution process from insects to humans. Phagocytes are thought to be the only type of blood cell, which maintains in a monophyletic manner throughout evolution. Phagocytosis is a complex membrane-driven process that is driven by the actin cytoskeleton of the host phagocytes. Ligation of phagocytic receptors recognizes various pathogens by binding to phagocytic markers that present on the cell surface of the target pathogenic organism. Upon binding to target cells, the intracellular portion of phagocytosis receptor activates a signaling pathway, followed by the rearrangement, modification and internalization of the actin cytoskeleton. The plasma membrane of phagocyte then extends and surrounds its targets. Finally, the target cells are engulfed into phagocytes as phagosomes [145].

The first step of phagocytosis is microbial recognition. To date, in *Drosophila*, several proteins have been shown to act as phagocytic recognition receptor proteins. These proteins include Scavenger receptor class C, type I (Sr-CI), which is a pattern recognition receptor for bacteria and a scavenger receptor similar to mammalian class A scavenger receptors [146]. Peste (Pes) is a CD36 family member required for uptake of mycobacterial, but not of *E. coli* or *S. aureus*. The *Drosophila* CD36 homologue Croquemort is a phagocytic receptor for bacteria. Croquemort(Crq) has been documented to be expressed on all embryonic hemocytes and has been shown to be required for microbial phagocytosis and efficient bacterial clearance [147]. Previous research shows that Crq is also involved in phagosome maturation, and *crq* mutant flies lack the ability to clear bacterial infection efficiently [148] (Fig. 6).

Peptidoglycan recognition protein LC (PGRP-LC) [149], a member of the PGRP family which is important for AMPs induction, and has been involved in phagocytosis of Gram-negative but not Gram-positive bacteria. Its functional role acts as a phagocytic receptor



*in vivo* remains to be established. The IgSF-domain protein Down syndrome cell adhesion molecule (Dscam) is a single-pass transmembrane receptor and is expressed in larval hemocytes, fat body cells and brain tissue. Protein sequencing experiments suggested that it is possible that thousands of Dscam isoforms circulate in the hemolymph or fat body cells. Phagocytosis was significantly reduced in by genetic inhibition of expression in hemocytes and by blocking Dscam protein interactions. In addition, Dscam isoforms are also involved in opsonizing invading pathogens in the hemolymph [150]. The ten characteristic EGF-like repeat-containing proteins Nimrod C1 (NimC1) is a single pass transmembrane. Inhibiting the expression of NimC1 in plasmatocytes suppressed the phagocytosis of *Staphylococcus aureus* [151]. The EGF-domain protein Eater is well documented as a phagocytosis receptor for a broad range of bacterial pathogens in *Drosophila*. Eater is a trans-membrane protein, which is expressed in both mature and immature plasmatocytes. Knocking down the *eater* gene in macrophage cell line caused decreased internalization and binding of bacteria. Moreover, *eater* frame shift mutant flies neither affected the Toll nor IMD pathways but impaired the function of phagocytosis and died faster than wild type flies to bacterial infection in an intestinal infection model [152]. Bruno Lemaître and colleagues constructed a novel NimC1 null mutant and characterized the role of NimC1 in cellular immune responses, alone and in combination with Eater. The results indicated that Eater and NimC1 play a synergistic role in the initial step of phagocytosis, specifically adhesion to bacteria [153].

## **2.4 Melanization**

Melanization is an immediate immune response in *Drosophila*, which results in production and deposition of melanin on the cuticle at the injury site. The recognition of pathogens triggers a serine protease cascade that activates phenol oxidase (PO), the key enzyme in generating melanin and expresses in hemolymph [154]. PO can be activated instantly where melanization is required. Melanization requires the activation of prophenol oxidase (PPO), which is the inactive form of PO. Phenols are oxidized to quinones, which are toxic to microorganisms and also can form melanin. During the



generation of melanin, some products like Reactive Oxygen Species (ROS) are thought to be directly toxic to the microbes [156].

Recognition of invading pathogens or injury triggers the activation of serine protease cascade that leads to the cleavage of inactive PPO to active PO. The *Drosophila* genome includes three PPOs: PPO1 (CG5779), PPO2 (CG8193), PPO3 (CG2952). A study revealed that PPO1 and PPO2 are produced in crystal cells in larvae, whereas PPO3 is expressed in lamellocytes [157]. A genome-wide microarray study showed that the expression levels of PPOs are unaffected by bacterial or fungal infection [131]. PPOs do not have signal peptide for secretion. PPOs are stored in crystal cells and released into the extracellular environment by cell lysis [158]. Subsequent melanization and the rupture of crystal cells are dependent on the c-Jun N-terminal kinase (JNK) pathway and the TNF homolog Eiger, are prevented when RhoA GTPase is mutated [159].

Pattern recognition receptors (PRR) have been shown to activate PO in silkworm [105]. In *Drosophila*, PGRP-LE encodes a PGRP with affinity to DAP-type PGN. It proposed to be involved in microbial melanization. PGRP-LE mutant blocked PO activation after a challenge with *E. coli* while overexpression of PGRP-LE resulted in constitutive melanization [91]. In addition, PGRP domain deleted PGRP-LC caused massive melanization [160].

In crystal cells, proPO is inactive form. Haysan is the protease that is required to activate PPO1 and PPO2 through cleavage of these zymogens. *Haysan* transcription is Toll and IMD pathways dependent [161]. A null mutant of *Haysan* blocks melanization in adults [162]. Clip proteases play a crucial role in the regulation of Toll pathway activation in dorsal-ventral pattern and immunity in embryo [163]. Genetic studies have shown that two Clip proteases serine protease (SPs), Melanization protease 1 (MP1), and Melanization protease 2 (MP2, also known as Sp7 and PAE), and Serpin27A, have been identified that involved in the melanization cascade [164, 165]. Overexpressed MP1 or MP2, the level of melanization was high regulated, while knock down MP1 or MP2 caused inactivation of PO and knock down MP2 caused high lethality after fungal infection. A study has shown that two SPs, Haysan and Sp7 are required in melanization in different manners. Haysan responses to the blackening reaction after clean injury, which acts through PPO1 and PPO2. Melanin deposition can still be observed after *M. luteus* challenge in the absence of Haysan, while PPO1 and Sp7 are both required. These

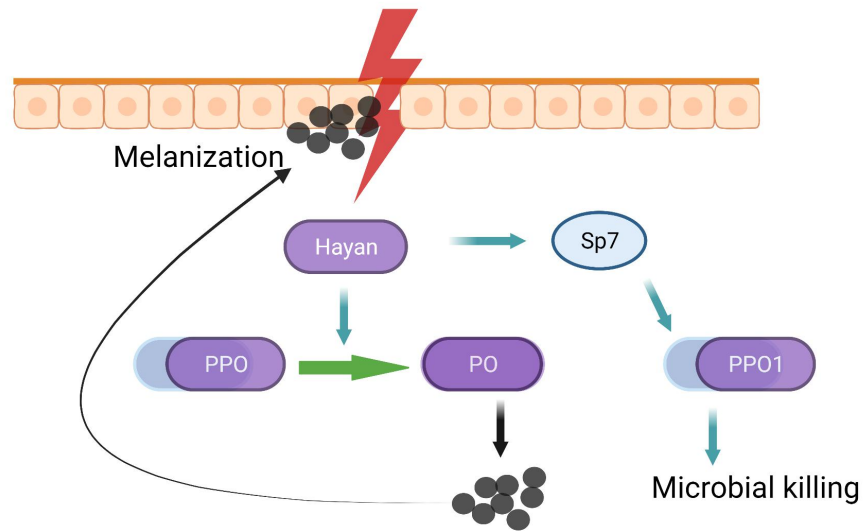
results indicate that two independent pathways, both *Sp7* and *Hayan* contribute to melanization in adults. The study also stated that *Hayan* and *Psh* evolved from a gene duplication and both genes encode high similar proteins. These two genes are required for melanization [70].

In addition, a loss of function of *Serpin27A* (*Spn27A*) led to excessive melanization, while overexpression of *Spn27A* inhibited the activation of PO, leading to a poorer survival rate than wild type flies, which indicated that *Serpin27A* is negatively regulating the melanization process. *Spn27A* plays a vital role in preventing spontaneous melanization. When the process of melanization is initiated, Toll pathway is required to down regulate *Spn27A*, which accelerates the activation of PO then facilitates the melanization response [81]. Melanization has been identified to be an important immune response in arthropods to fight against microorganism, which results in a darkening and hardening of damaged tissue.

## **2.5 Resistance and Resilience (disease tolerance)**

The immune system of plant is triggered by PAMPs, resulting in PAMP-triggered immunity (PTI) [166]. To increase virulence, pathogens produce virulence associated molecules to defense against the host immune responses. To combat virulence factors, plants introduce a second immune signaling, known as effector-triggered immunity (ETI) [166]. PTI and ETI are two kinds of mechanisms with different triggered components, however ultimately have many similar downstream reactions [167].

The aim to treat infectious diseases is to reduce the damage to the infected host. The immune system protects the host from infections by detecting and clearing the invaded pathogens. Yet there are variations between different host types in the ability to control pathogen load [168]. It has been argued that high rates of infection but low virulence should belong to host resilience (disease tolerance). These two different strategies are required to fight against pathogens, which determines disease severity. The definition of resistance and resilience has been well documented in plant immunity to assess plant health a century ago [169, 170]. This model helps us to understand defense mechanisms



**Figure 7. Scheme of melanization response pathway.**

Hayan is activated when the injury occurs in the cuticle, leading to the cleavage of PPO1 and PPO2 into the active form. This process leads to the production of melanin, which results in the blackening reaction at the wound site. Hayan can also activate the Sp7, which mediates microbial killing through PPO1 [162].

against various pathogens in animal. Many studies have demonstrated that this evolutionarily conserved host defense also exists in animals, including flies [171] and humans [172].

One theoretical way to discriminate the resistance and resilience is to monitor the pathogen burden. Resistance describes the host's ability to reduce the microbial load through detection, destruction and expulsion of pathogens, which means that acts on the pathogen itself. Unlike resistance, resilience describes the ability to decrease the negative impact of infectious host fitness without directly decreasing the microbe load, which means that resilience acts on the effect from pathogens without targeting them directly. In a study, 11 different mutants of *Drosophila* were infected with *Pseudomonas aeruginosa* to check whether the survival was correlated with bacterial load. However, they found variation between survival and pathogen burdens [173]. Moreover, in *Drosophila* intestinal tract, pathogen-damaged enterocytes are replaced by compensatory proliferation of intestinal stem cells (ISCs), which is regulated by multiple pathways [174]. These data indicated that besides resistance, resilience is also required in the host defense and the genetic variation exists. In other words, resilience is a property of individuals whereas tolerance refers to a population. In immunology, tolerance is generally considered to correspond to an attenuated immune response: if an individual does not develop an immune response to a particular antigen, it means that the host is tolerant to the pathogen. In the research of plants, the definition of tolerance is as the slope of host adaption against infection intensity, a property of populations, not individuals [175].

During infection, the host could have the problems of abnormal behavior, anorexia, et al. The defense system could counter these behaviors to protect the host against pathogen transmission through resilience strategy [176]. To favor the transmission, some bacteria, like *Salmonella enterica Typhimurium* promote host resilience. Microbes can activate the resilience in the host defense through several strategies. For instance, *Wolbachia* can activate the production of reactive oxygen species in the dengue virus mosquito vector *Aedes aegypti* and activate the oxidative stress response. After activation, some genes like catalase can be up regulated, leads to resilience to intestinal bacterial infections in *Drosophila* [177]. The evidence for resilience in the host defense of animals has been shown in a study that a monogenic hemoglobin disorder in humans could protect the host

from malaria. Infection intensities of *Plasmodium falciparum* in heterozygous or homozygous mutants are not lower than wild type. However, the degree of anemia is reduced when the host was in high infection intensity, resulting in lower malaria mortality. Tissue damage of the host was associated with the pathogen load. These findings suggested that there are two types of defense in the host and indicated the epidemiology and evolution of infectious diseases [163]. The evolution of resistance is harmful to the prevalence of infectious diseases while resilience has a neutral or positive impact. Therefore, resistance and resilience have different effects on the epidemiology of infectious diseases [178]. Resistance and tolerance can be seen as complementary, exclusive and interchangeable components based on mechanistic details. Accordingly, studies already demonstrated that resistance and resilience were negatively correlated [163].

Some regulators do not affect pathology directly but block signaling pathways. For example, TNF is regulated to activate immune cells to provide the resistance function. Meanwhile, immune effectors like other targets of TNF can cause tissue damage, which finally reduce the health of the host. Accordingly, studies have indicated a trade-off between resistance and resilience [179]. When resilience is functioning, the host defense can limit the tissue damage, which provide the higher possible endurance to pathogens. A study addressed that the *Drosophila* regulator, PGRP-LC-interacting inhibitor of IMD signaling (PIMS), function at suppressing the IMD signaling when infected by commensal bacteria. Resident bacteria can stimulate strong local expression of AMPs in the absence of PIMS. Moreover, PIMS interacts with PGRP-LC leads to the interruption of IMD pathway. PIMS is required to limit the immune reaction in ingested bacteria. These data suggest that PIMS is required to set up the threshold of activation of the IMD signaling pathway, establishing immune tolerance to the gut microbiota, akin to immune tolerance in mammals below this threshold [180]. Another study reported that in the absence of *CrebA* gene in *Drosophila* causes high susceptibility to bacterial infections without altering bacterial load. Strikingly, mutant flies were killed by a much lower dose of bacteria than wild-type or Toll pathway mutant flies, as determined by the bacterial number upon death (BLUD) parameter [181]. Indeed, *CrebA* is a gene that regulates the expression of genes involved in secretion. In its absence, flies likely succumb to

endoplasmic reticulum stress taking place in the fat body. These data suggested that the transcription factor CrebA is a novel regulator of infection resilience [182].

Understanding the mechanisms is important for treating diseases. Despite mechanism-based partitions of host defense strategies are known for many purposes, a more conceptual definition into resistance and resilience is more helpful to understand the consequences of infection and interactions between hosts and pathogens [170].

### **3. Pathogenic microorganisms**

#### **3.1 *Enterococcus faecalis* (*E. faecalis*)**

*E. faecalis* belongs to *Enterococci*, and is a Gram-positive bacterium, a facultative anaerobic coccus, and is a commensal organism of the gastrointestinal tract, also associated with endodontic and systemic infection. *E. faecalis* is an opportunistic pathogen in immunocompromised patients and ranks as a leading pathogen in nosocomial infections, as well as root canal infections in the field of dentistry. *E. faecalis* is regarded as one of the most frequently encountered bacterial species in the wounds, including diabetic foot ulcers, surgical sites [183]. Moreover, *E. faecalis* can slow healing during wound infection [184]. It is common in clinical infections that *E. faecalis* develops resistance to a range of antibiotics and heavily colonizes patients after antibiotic therapy [185].

*E. faecalis* is found to naturally colonize the *Drosophila* intestine. *Drosophila* is widely used as a model to study virulence factors of pathogenic microorganisms, and determining their effects on the hosts [28]. It has been studied that fly can be killed after septic injury with *E. faecalis* and triggers Toll pathway and phagocytosis [186, 187]. Pathogenic bacteria can express effector molecules (virulence factors), which modify host defense mechanisms. These molecules contribute to the pathogenic potential. Cytolysin, a toxin from some *E. faecalis* strains, are significantly more virulent to both flies and mammals, contribute to serious infection [188].

Bacteria produce antimicrobial substances used in microbial warfare, including organic acids, hydrogen peroxide, and bacteriocins that are AMPs. Bacteriocins exhibit potent



inhibitory activity against sensitive strains of bacteria. There are four classes (I–IV) of bacteriocins [189, 190]. (i) Class I are lantibiotics (<5kDa). (ii) Class II bacteriocins (5–10 kDa) are heat-stable unmodified peptides comprising four subgroups: class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), class IIc (circular bacteriocins), and class IId (linear and non-pediocin-like bacteriocins) [191]. (iii) Large heat labile proteins (>30kDa). (iiii) Macromolecular complexes. Bacteriocins are considered to be devoted to self-protection or immunity. One of mechanisms for bacteriocins to cause cell death is of that target membrane insertion and pore formation [192].

The *E. faecalis* bacteriocin known as Enterocin O16 (also called EntV) is active on some lactic bacteria strains and has been shown to inhibit the formation of *Candida albicans* biofilm and hyphae as well as to interfere with *C. albicans* infections in the nematode *Caneorhabditis elegans* model [193, 194]. Additionally, Teixeira et al at 2013 showed that deletion of the *ef1097* locus led to a reduction of virulence of *E. faecalis* in *Drosophila* [195]. EF1097 protein, found by Bourgogne et al. 2006 to be dependent on Fsr (*Enterococcus faecalis* sensor regulator) regulation in *E. faecalis* [196]. EF1097 is conserved in all *E. faecalis* strains. Enterocin O16 was shown to be encoded by *ef1097*. The molecular mass of the purified Enterocin O16 antimicrobial peptide was determined to be 7,231 Da [197].

In BaramicinA chapter, we studied the interaction between virulence factors from *E. faecalis* and the host, to further study the host tolerance mechanisms to infections.

### **3.2 *Metarhizium robertsii* (*M. robertsii*)**

Entomopathogenic fungi such as *Metarhizium* are an important class of fungi in the study of pathogenicity and can be used for biological control of insect pests [198]. Importantly, their pathogenesis is similar to mammal pathogenic fungi. The insect cuticle provides physical barrier or occasionally through wounds against infection. In fact, most pathogens infect the host through the oral route, only pathogenic fungi such as *Metarhizium robertsii* can invade via cuticle directly. They are able to invade the body cavity by penetrating through the insect cuticle via a series of mechanical pressure of the appressorium, and a combination of enzymes [199]. After invading to the hemocoel, the

dimorphism of fungus can go through from hyphae to blastospores. When fungi successfully colonize in the hemocoel of the host, and the infected host can be killed by fungal growth and toxins. Ultimately, the fungus that grows in the hemocoel emerges from the cadaver. *Metarhizium robertsii* has been showed to have the ability to respond to insect innate immunity, finally affecting the synthesis of AMPs [200].

This organism produces secondary metabolites through polyketide synthase and nonribosomal peptide synthetase pathways. A genetic study showed that blocking these two pathways resulted in failing to produce the nonribosomal peptides, including destruxins, serinocylins and others, affecting the virulence and fitness in *Metarhizium robertsii* [201]. Destruxins (Dtxs), are secondary metabolites produced by entomopathogenic fungi like *M. anisopliae* and *Aschersonia spp.* They are considered to be essential virulence factors accelerating the death of insects [202-204]. Destruxins were firstly reported in 1961 from *Oospora destructor* [205]. Five analogues have been isolated, Destruxin A-E. Chemically, Destruxins have a typical composition, containing  $\alpha$ -hydroxy acid and five amino acids, forming a cyclic hexapeptide. Until now, 39 analogs of destruxins have been extracted from various fungal species [206-208]. Among them, few destruxins such as Destruxin A, Destruxin B, and Destruxin E have exhibited significant insecticidal activities against various insects [202, 209-211]. It was reported that Destruxin A is able to damage midgut, Malpighian tubules, and hemolymph. In addition, Dtxs are thought to induce flaccid paralysis, visceral muscle contraction in insects, and cytotoxic effects, which are demonstrated that  $Ca^{2+}$  ion balances and vacuolar-type ATPase are involved in insects hemocytes [211, 212]. A study showed that Dtx A can deplete intracellular ions, forming transient ion channels [213]. Furthermore, Dtxs can destroy encapsulation and phagocytosis [214, 215]. Further study revealed that, Dtxs defective *Metarhizium* strains had less virulence to silkworm and were unable to escape from hemocytes [216]. Additionally, destruxins are also reported to affect the immune system of insects. *Drosophila melanogaster* systemic innate immune response has been reported that of which suppressed by Destruxin A [217]. However, whether immune related proteins can counteract the action of Destruxins still remains unknown.

### **3.3 *Candida glabrata* (*C. glabrata*)**

Candidiasis caused by *Candida* species has increased significantly, since the widespread use of antibiotics. *Candida albicans* is the leading cause of candidiasis; nevertheless, among non-*albicans* species, including *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*, which are now widely identified as human pathogens, *Candida glabrata* is the most common cause of invasive candidiasis [218]. Indeed, *Candida* species are common members of the human microbiota, which can be found in skin, gastrointestinal tract, genitourinary tract and also in the environment.

The pathogenicity of *Candida* is promoted by a number of pathogenic virulence factors, such as adherence and biofilm formation on host surfaces and medical devices. They escape host defenses and secretion of hydrolytic enzymes. Hyphae play a vital role in tissue invasion by dimorphic *Candida* such as *C. albicans*. In addition, biofilm formation acts as a virulence factor, due to its ability to develop resistance to antifungal therapy [219, 220]. A study revealed that a bacteriocin, EntV, from *E. faecalis*, shows the ability to reduce the virulence of *C. albicans* through inhibiting the formation of hyphae and biofilm [194].

*Drosophila* is extensively used as a model to study the interaction between the host and *Candida* [221]. *C. glabrata* can activate the Toll pathway through GGBP3 in *Drosophila*. However, the Toll pathway is required to control the proliferation of *C. glabrata* but not eliminate it [222]. Until now, among the seven well documented AMPs, none of them has been reported to display activity against yeast. A study reported that Bomanins show killing activity to *C. glabrata* [133]. Additionally, an experiment *in vitro* showed that IM10-like peptides (BaraA derived peptide) show antifungal activity against *C. albicans* [223].

## PhD objectives

In *Drosophila*, fungi and most of Gram-positive bacteria can trigger the Toll pathway while Gram-negative bacteria activate the IMD pathway. During fungal infections, the PRR GGBP3 recognizes the PAMP  $\beta$ -(1, 3)-glucans. The activated proteolytic cascades lead to the cleavage of SPZ and subsequently to Toll signaling transduction. The Toll pathway activation results in the transcription of a large numbers of immune related genes, including antimicrobial peptides (AMPs), IMPPP (immune induced molecule prepropeptide), GGBP-like3 and Bomanins. The well-known AMPs so far are more the antifungal or antibacterial peptides. To date, there are still no identifies AMPs active against yeasts.

In 1998, an approach based on a differential mass spectrometric analysis on single fly hemolymph to detect immune effectors following *Micrococcus luteus*, a Gram-positive bacterial infection, leading to the detection of more than 30 peaks and named as *Drosophila* immune-induced molecules (DIMs) [136]. Among them, four peptides correspond to well documented antimicrobial peptides. The other peptides like DIM5, 6, 8, 10, 12, 13 and 24 were characterized as IMPPP by peptidomic approach subsequently [118]. They are encoded by a gene. The polyprotein precursor is cleaved at furin-like sites [224]. However, either knocking down or overexpressing this cluster did not show any sensitivity or protection phenotypes to various microbial infections [118]. Since then, the functions of this family have not been studied. In our laboratory, RNA-seq data showed that IMPPP is up-regulated following a fungus *Metarhizium robertsii* infection [225].

During my PhD study, one of my projects was to study the function of this cluster. In agreement with Mark Hanson, we renamed this polyprotein as BaramicinA (BaraA).

*Aspergillus fumigatus* is ubiquitous in the environment and can reproduce in the soil and decaying organic matter. Due to the small size of its conidia, it can penetrate deeply in the lungs, producing tuberculosis-like symptoms and can cause fatal systemic infections in immuno-compromised patients. It is a deadly opportunistic pathogen that causes high morbidity and mortality. It also produces toxins, which contribute to its virulence, including restrictocin, verruculogen, ergot alkaloids and others. In our laboratory, Dr. Rui Xu has demonstrated that when *A. fumigatus* conidia are injected in *Drosophila*, the host

do not die from the proliferation of the fungus, but succumb to its secreted toxins. Interestingly, he found that protection required a non-canonical Toll pathway and was mediated partly by specific Bomanins depending on the nature of the injected toxins (Rui Xu, submitted). According to Rui Xu's work, I focused on whether BaraA-derived peptides play a role in resilience, to protect the host from virulence factors of pathogens. In chapter I, I will present the work of BaraA that plays a role in counteracting toxins from two distinct pathogens, *Enterococcus faecalis* and *Metarhizium robertsii*. The objectives of chapter I were to understand how BaraA works, improving our understanding to BaraA in effector-trigger immunity (ETI) to protect *Drosophila* against pathogen infections. This manuscript is currently in revision for PNAS journal.

A protein identification assay reported that only after a *Beauveria bassiana* fungal infection, the most strongly induced protein is GGBP-like 3, which is phylogenetically close to GGBP3 [118]. Jessica Quintin, a former PhD student in Dominique Ferrandon's team, started to study the functions of GGBP-like 3. However, unlike the result from Levy *et al* [118], Jessica found that GGBP-like 3 can be induced after both fungal and bacterial infections. Furthermore, she also observed that GGBP-like 3 protein can bind to *Candida* directly. However, RNA interference to reduce the expression of GGBP-like 3 failed to reveal any sensitivity phenotypes to *Candida*. To continue studying the gene's functions, we successfully generated a *GGBP-like 3* frame shift mutant with the genome-editing technology CRISPR/Cas9 in SHFI platform, creating a premature stop codon that produces a truncated protein.

In chapter II, based on results from Jessica Quintin, I will describe the results on the investigation of the functions of GGBP-like 3 against *Candida glabrata*. We aimed to explore the anti-yeast role of GGBP-like 3 and to figure out the mechanisms. Of note, a recent study suggested that that some Bomanin genes are involved in the host defense against *C. glabrata* [133]. I therefore investigated possible interactions between GGBP-like 3 and Bomanins.

This work led us altogether toward a renewed understanding of the function of secreted peptides as effectors of the innate immune response.

**Chapter I\_A *Toll pathway effector protects Drosophila specifically from distinct toxins secreted by a fungus or a bacterium.***

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**Author Contributions:** JH, YL, ZL, SL, and DF designed, performed and analyzed experiments. PB performed and analyzed the mass spectrometry analysis. JL and RJ generated the CRISPR/Cas-9 mutant flies reported in this study. JH and DF wrote the manuscript with inputs from all authors

**Competing Interest Statement:** The authors have declared no competing interests.

**Classification: Biological sciences; Immunology & Inflammation** Paste the major and minor classification here. Dual classifications are permitted, but cannot be within the same major classification.

**Keywords:** BaramicinA, microbial toxins, Destruxin A, Enterocin O16, resilience/disease tolerance.

**This PDF file includes:**

Main Text

Figures 1 to 5

**Abstract**

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 (1, 2). 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 BaramicinA (BaraA) (3-5). Unexpectedly, the deletion of a cluster of ten Bomanins phenocopies the Toll mutant phenotype of susceptibility to infections (6). Here, we demonstrate that *BaraA* is required specifically in the host defense against *Enterococcus faecalis* and against the entomopathogenic fungus *Metarhizium robertsii*, albeit the microbial burden is not altered in *BaraA* mutants. BaraA protects the fly from the action of distinct toxins secreted by Gram-positive and fungal pathogens, respectively EnterocinV and

DestruxinA. The injection of DestruxinA leads to the rapid paralysis of flies, whether wild-type or mutant. However, a larger fraction of wild-type than *BaraA* flies recovers within five to ten hours. BaraA 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.

### **Significance Statement**

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 a novel 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.

### **Main Text**

### **Introduction**

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 by antimicrobial peptides (AMPs) (1, 7-9). 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 (10-12). 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 (13-15). 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) (16, 17).

In *Drosophila*, the Toll pathway is one of the two NF- $\kappa$ B 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 (14, 18-21). A few AMPs active against filamentous fungi have been identified (Drosomycin, Metchnikowin, Daisho) (22-24). 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) (3, 4). 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 (4, 6). Ten such *Bomanin* genes are located at the 55C locus, including DIMs 1 to 3 now referred to BomS1 to S3. 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* (6). Some short Bomanins that essentially contain only the Bomanin domain may be active against *Candida glabrata in vivo* (25).

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 was not understood (3, 4). A recent study renamed this protein as BaramicinA (BaraA) and proposed that some of the derived peptides function as antifungal AMPs (5). 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 microbial burden does not appear to be altered in the mutants, from the beginning to the end of the infections. Our data indicate that the major function of *BaraA* is in the resilience against distinct toxins, Destruxin A (DtxA), a pore-forming toxin and EnterocinV (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 (26, 27) (Fig. 1A, 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 (26, 27) (Fig. S1A, 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. 1C), in keeping with previous data at the transcriptional and peptide levels (5, 28). In contrast, the *CG18278* gene does not appear to be induced by any of these challenges (Fig. S2A) and also not upon natural infection (Fig. S2B).

### ***BaraA contributes to the host defense against Enterococcus faecalis***

In this work, we have generated two independent CRISPR-Cas9-mediated KO lines, a *mCherry* knock-in (KI) line, and used also an RNAi line for knock-down (KD) experiments (Fig. S1A,C, D). In these lines, the induction of *BaraA* expression by an immune challenge is hardly detected, both at the transcriptional (Fig. S1E) and protein level, even though some minor peaks can still be observed in the KO2 line (Fig. S3; 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 (Fig. S2E-F). In contrast, we observed a significant susceptibility of *BaraA* mutant lines isogenized in the wild-type  $w^{A5001}$  background after the injection of this Gram-positive bacterial strain (Fig. 2A). Contrary to the *MyD88* line, measurements of the bacterial burden at any time did not reveal any difference between the KO/KI lines and the isogenic  $w^{A5001}$  control line, even within 30min after death (Bacterial Load Upon Death : BLUD (16); Fig. 2B-D).

We conclude that the *BaraA* mutant lines display an intermediate sensitivity to *E. faecalis* infection and do not show a significantly altered bacterial burden.

### ***The BaraA mutant is susceptible to Metarhizium robertsii infection only in the septic injury model***

The *BaraA* KO and KI lines consistently exhibited a moderately enhanced sensitivity to the injection of 50 *M. robertsii* conidia (Fig. 3A). As for *E. faecalis*, we did not observe 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. 3B, C). Interestingly, no susceptibility to *M. robertsii* in the natural infection model was observed, even though the *BaraA* gene is induced by this challenge (Fig. S4A, A'). We have also tested a panel of other bacterial and fungal strains and did not observe any sensitivity to those infections (Fig. S4).

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 microbial burden was not altered in the *BaraA* mutants for both infections, which indicates that *BaraA* is not required in the resistance against these pathogens.

### ***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 several pathogens yet rescued the *BaraA* sensitivity phenotype to *M. robertsii* and to *E. faecalis* (Fig. S5A-E).

We next tested the overexpression of *BaraA* in a sensitized *MyD88* background. The *BaraA* transgene partially rescued the sensitivity of *MyD88* flies to respectively *M. robertsii* and *E. faecalis* (Fig. S5F-G), suggesting that BaraA can function in the absence of Toll-induced Bomanins. We also checked by 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 Fig. S5H 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 pathway-dependent Bombardier activity needed to stabilize the expression of short Bomanins is not required for the stability of DIM10, DIM12, and DIM13 (29).

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 feed-back 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 *DIM1 (BomS1)* (2, 30, 31). As shown in Fig. S6, we did not observe any influence of the isogenized *BaraA* KO or KI null mutations over 48 hours on their expressions.

### ***BaraA protects Drosophila from the action of secreted microbial toxins***

Our data thus far are not compatible with a function for *BaraA* in resistance against *E. faecalis* or *M. robertsii*. 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 (Xu *et al.*, submitted). As mycotoxins, namely Destruixins, have been described as important virulence factors from generalist *Metarhizium* entomopathogenic fungi (32, 33), we therefore injected DtxA into wild-type and *BaraA* flies. Interestingly, *BaraA* KO and KI mutant as well as *MyD88* flies reproducibly succumbed to a larger extent than wild-type flies to the injection of DtxA (Fig. 4A), a result confirmed in axenic flies (Fig. S7A). We next determined that *BaraA* mutants are not more susceptible than wild-type flies to a challenge with a *Dtx* mutant *M. robertsii* strain (33) (Fig. 4B). 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 Destruixins 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. 4C). Filtration experiments allowed us to determine that the toxic component of the supernatant can be recovered in a three to ten kD fraction (Fig. 4D). 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 (Fig. S7B, C). Interestingly, it has been reported that the bacteriocin enterocin O16 is an *E. faecalis* virulence factor in *Drosophila* (34). 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 biofilm formation of *C. albicans* (35, 36).

We therefore asked whether the toxic activity in the supernatant is still present when using a bacterial *entV*<sup>-</sup> strain. We observed that the supernatant from the complemented *E. faecalis* strain *entV*<sup>+</sup>/*entV*<sup>-</sup> 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*<sup>-</sup> *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. 4E). Similarly, the direct infection with the *entV*<sup>-</sup> mutant *E. faecalis* strain did not differentially kill *BaraA* and *MyD88* mutants as compared to wild-type flies submitted to the same challenge (Fig. 4F). As expected, the complemented *E. faecalis* strain *entV*<sup>+</sup>/*entV*<sup>-</sup> behaved as the wild-type bacterial strain and killed the immuno-deficient *MyD88* and *BaraA* flies more than the wild-type control flies. The EntV peptide derives from the open reading frame found in the *efl1097/entV* gene, which encodes a preproprotein. This precursor protein gets cleaved by the GelE protease into a 7.2 kDa active peptide (36, 37). As expected, a *gelE* *E. faecalis* mutant strain also did not kill *BaraA* flies faster than wild-type flies (Fig. S7D). We conclude that *BaraA* protects the flies from the action of the EntV bacteriocin.

Taken together, our data suggest that the 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 recover their activity within five-ten hours of DtxA injection in contrast to less than 40% for *BaraA* KO flies (Fig. 5A-B; Supplementary movies 1-3). 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 have validated an RNAi KD line molecularly and in survival experiments using an ubiquitous Gal4 driver (Fig. S1E and Fig. S8). We then 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. 5C-F). In the case of DtxA, the effect was as strong as that observed upon using a ubiquitous driver (Fig. 5C, 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. 5H).

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.

## Discussion

Our analysis of the *BaraA* mutant phenotype revealed a susceptibility 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 the 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 *Aspergillus fumigatus* restrictocin (Xu *et al.*, submitted), *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 (Fig. S9)(5).

A recently published study proposed that BaraA is involved in resistance to infection to entomopathogenic fungi as an AMP since, besides being sensitive to *Beauveria bassiana* and *Metarhizium rileyi*, *BaraA* mutants exhibit an increased *B. bassiana* load 48 hours after infection (5). In addition, BaraA-derived IM10-like peptides synergize with a membrane-active antifungal compound to kill *Candida albicans in vitro* (5). 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 (5). Because BaraA encodes a polyprotein precursor, we cannot formally exclude such an AMP function for one or several of these BaraA derivatives, 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 (Xu *et al*, submitted), it is known that at least some *Bomanin* genes are required for resistance to *E. faecalis* (6), 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 specifically against the microbial species documented 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. In our view, the finding of a loss-of-virulence of bacterial and fungal toxin mutants in *BaraA* mutant flies supports the concept that BaraA's major function is to neutralize or counteract the action of specific secreted microbial toxins in the case of *E. faecalis* or *M. robertsii* infections, especially since we did not detect an enhanced microbial load of these pathogens in *BaraA* mutants.

Interestingly, several studies have shown that besides their direct antimicrobial functions, mammalian  $\alpha$ -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.  $\alpha$ -defensins are constituted by amphipathic  $\alpha$ -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 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 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 (27). The expression of *BaraB* and *BaraC* has been reported not to be induced by an immune challenge (27), 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 (27). BaraB function is essential in neurons whereas BaraC appears to be expressed in glial cells (27). Interestingly, a *BaraA* expression fluorescent reporter is detected in brain tissues (5). 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 opens 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 years ago and they appear to act as ionophores that deplete cellular ions such as H<sup>+</sup>, Na<sup>+</sup>, and K<sup>+</sup> through the formation of pores in the membranes in a reversible process (44). 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* (35). 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 indirectly alters 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 (Xu *et al.* submitted).

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 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 (Xu *et al.*, submitted), 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 a novel 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°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.

$w^{A5001}$  (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<sup>ts</sup>* (*Ubi-Gal4*, *Gal80<sup>ts</sup>*), *repo-Gal4*, and *elav-Gal4* transposon were crossed to males carrying an UAS-RNAi 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<sup>ts</sup>* driver were performed at 25°C for three days, then the progeny was left to develop at the non-permissive 18°C temperature. The hatched flies were kept at 29°C for five days 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 five to seven day old at the beginning of each experiment.

To generate axenic flies, standard fly media was autoclaved. Antibiotics were added (Ampicillin 50ug/mL, Kanamycin 50ug/mL, Tetracyclin 50ug/mL, Erythromycin15ug/mL) when it cooled down to 50-60°C. The embryos were bleached then cultured on the sterilized media. The sterility of axenic flies (20 days old) was checked on LB, BHB, YPD, and MRS plates.

## ***Pathogen infections***

The bacterial strains used in this study include the Gram-negative bacterium *Pectinobacterium carotovorum carotovorum 15* (strain *Ecc15*, OD<sub>600</sub>=50) and the Gram-positive strains *Enterococcus faecalis* (ATCC 19433) (OD=0.1), *Micrococcus luteus* (OD=200) and *Staphylococcus albus* (OD=10), as well as *entV<sup>-</sup>* and complemented *entV<sup>+</sup>/entV<sup>-</sup>* and, which are derivatives of the wild-type *E. faecalis* OG1RF strain (OD=0.5) (kind gifts of Profs. Garsin and Lorenz, Houston, USA) (35). The fungal strains we used include filamentous fungi, *Aspergillus fumigatus* (5x10<sup>7</sup> spores/mL, 250spores in 4.6nL), *Metarhizium robertsii* (ARSEF2575, 1x10<sup>7</sup> spore/mL, natural infection 5x10<sup>4</sup>/mL), *DestruxinSI* mutant strain (1x10<sup>7</sup> spore/mL), a kind gift from Prof. Wang, Shanghai, China (33). Besides, we used yeast as well, *Candida albicans* (pricked) and *Candida glabrata* (1x10<sup>9</sup> yeasts/mL). The following media were used to grow the strains: Yeast extract- Peptone-Glucose Broth Agar (YPDA, *C. albicans* and *C. glabrata*) or Luria Broth (LB - all others) at 29°C (*Ecc15*, *M. luteus*, *C. albicans*, *C. glabrata*) or 37°C, *entV<sup>-</sup>*, *entV<sup>+</sup>/entV<sup>-</sup>* *E. faecalis*, BHI medium, 37°C overnight, Rifampicin 100ug/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 one weeks or three weeks (*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 5ml 0.01% tween-20 solution containing *M. robertsii* conidia at a concentration of 5x10<sup>4</sup>/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). Flies were anesthetized with light CO<sub>2</sub> for about three minutes during the injection procedure and were observed 3h 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.

## ***Pathogens Load Quantification***

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 *E. faecalis* 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  $\mu$ l PBS with 0.01% tween20 (PBST) or PBS. Homogenates were diluted serially (ten-fold dilutions, checked by five-fold 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.

### ***Gene Expression Quantitation***

We followed the protocol as described (49) using primer pairs displayed in Table S4.

### ***Survival tests***

Survival tests were performed using 20-25 flies per vial in biological triplicates. Female adult flies used for survival tests were 5–7-day old. For survival tests using RNAi-silencing genes, flies were crossed at 25°C for 3 days for laying eggs then transferred to 18°C; after hatching, flies were kept for at least 5 days 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 (MCE) was resuspended in high-quality grade DMSO and was diluted in PBS to a 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 III microinjector (Drummond). Restrictocin (Sigma) was resuspended in PBS to the concentration of 1mg/ml, 4.6nl was injected. Beauvericin (Sigma) was resuspended in high-quality grade DMSO, 20mM, 9.2nl was injected.

### ***Collection and preparation of E. faecalis supernatants***

Filter-sterilized supernatants phases were obtained from 10ml overnight cultures grown in LB medium that were collected by centrifugation at 4,000 rpm for 10min. The supernatants were sterilized by passing through a 0.2- $\mu$ m-pore-size sterile syringe filter. The sterilized supernatants were centrifuged through a 15mL Amicon Centricon filter (Millipore) to separately collect the molecules larger or lower than 10kDa. 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<sub>2</sub>O and thus concentrated 10 to 20-fold. The solution was filtered on 3kDa Amicon Centricon filter columns (Millipore) 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. All experiments were performed at least three times.

### ***Statistics***

Data are expressed as means  $\pm$  SEM. 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. Details are included in the legend of each figure. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

### **Acknowledgments**

We thank our colleagues at the Sino-French Hoffmann Institute for help and advice at various steps of this work. We are grateful to Dr. Wenhui Wang and Prof. Erjun Ling for providing antibodies, to Prof. Chengshu Wang for the *M. robertsii Destruxin* mutant strain, to Profs. Danielle Garsin and Michael Lorentz for the *entV* and *gelE* mutant strains (kindly sent by Melissa Cruz, Murray laboratory), to Dr. Lucas Waltzer for the PPO2-Gal4 stock, and the Bloomington and Tsinghua stock Centers for fly stocks. We would

like to thank Sébastien Voisin from the BioPark for his help in mass spectrometry and Yongfang Zheng for expert technical help. We are indebted to Mrs Chuping Cai for help with some experiments. We are indebted to the Guangzhou Drosophila Resource Center for the generation of CRISPR-Cas9 KO lines. We also thank Bruno Lemaitre and Mark Hanson for their cooperation and discussions on the *BaraA* gene.

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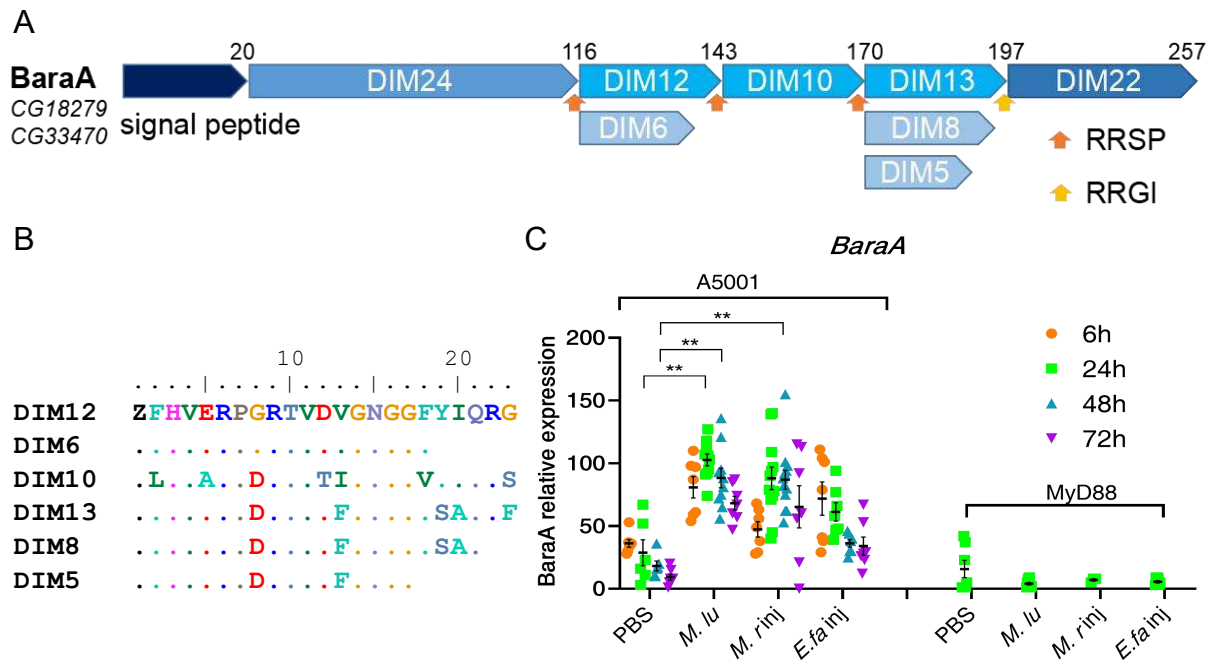
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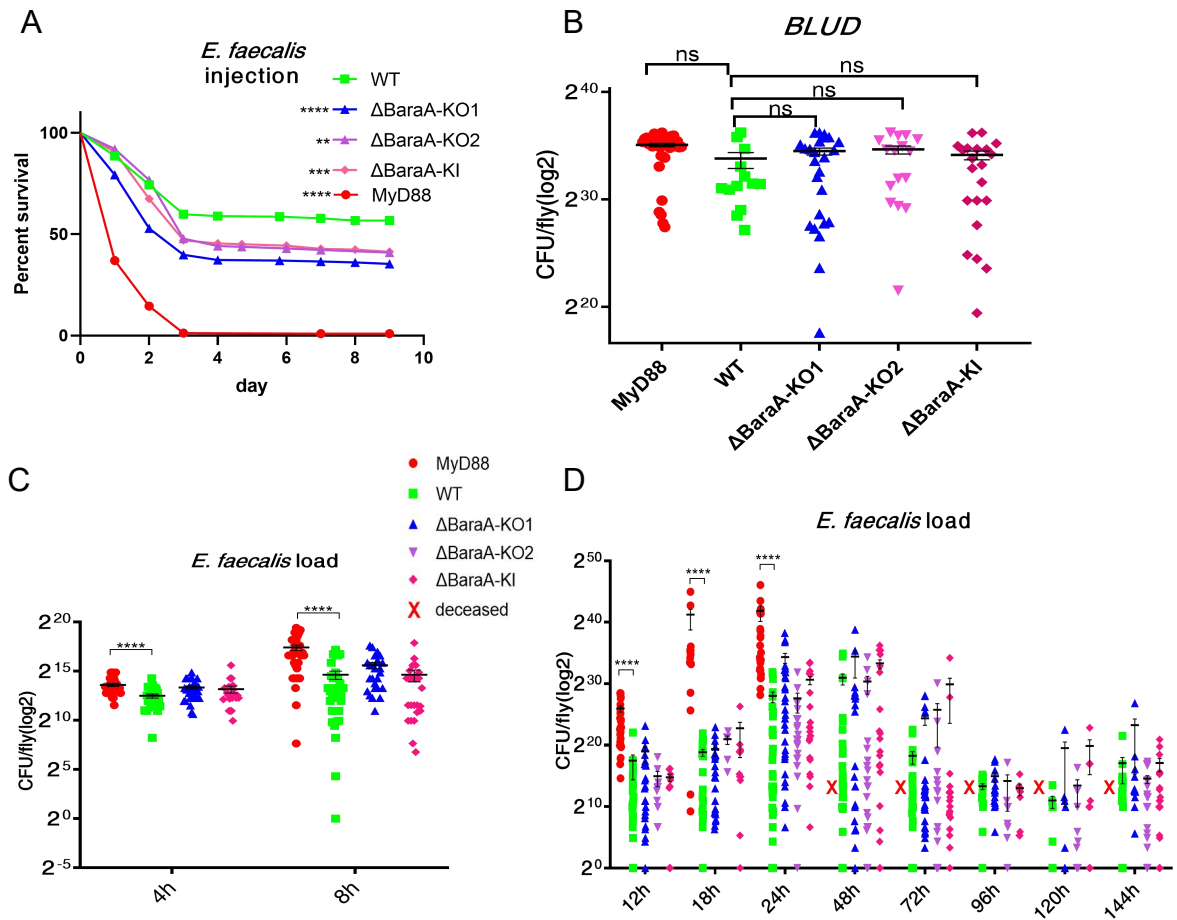
## Figures

Fig. 1



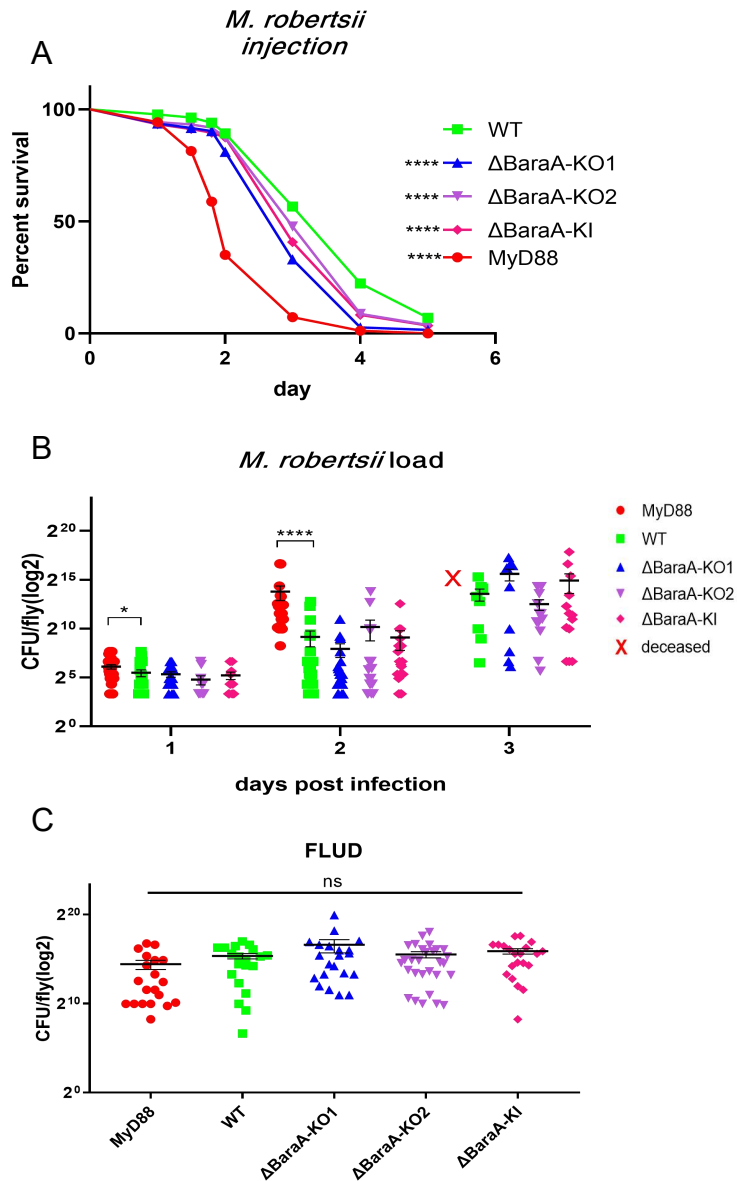
**Figure 1. Structure of the BaraA precursor protein and induction of *BaraA* expression by an immune challenge.**

Schematic structure of the BaraA polyprotein. The name of the peptides derived from the processing of the precursor upon furin cleavage are 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 RTqPCR at various time points after the injection of the indicated microbes; *M. lu*: *M. luteus*; *M. r*: *M. robertsii*; *E. fa*: *E. faecalis*. The measured expression of *BaraA* 24 hours after a *M. luteus* challenge is taken as a reference for all other data points and given a 100% value. The means  $\pm$  SEM are shown in black. Pooled data from three independent experiments, \*\*  $p < 0.01$ .



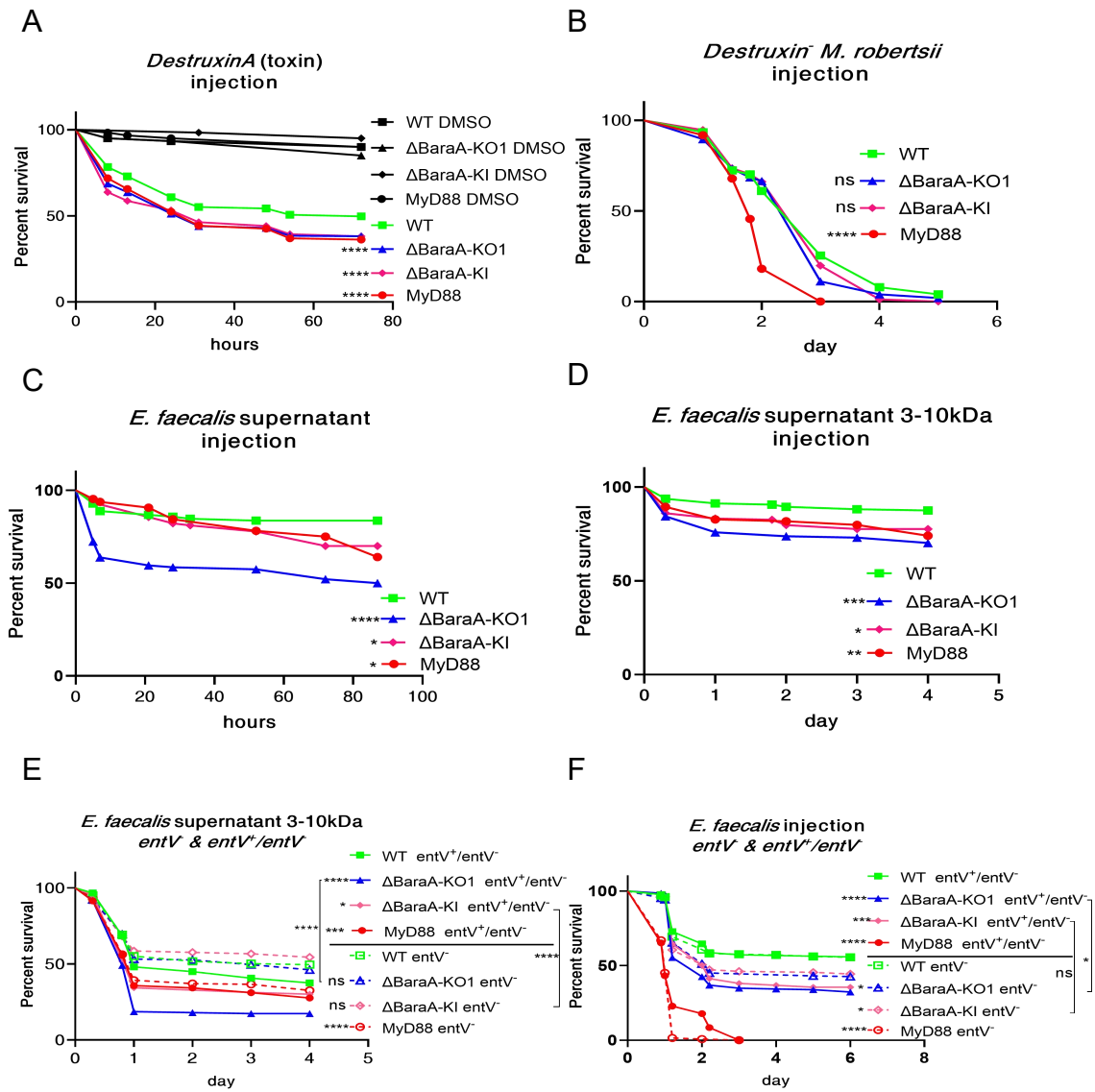
**Figure 2. Susceptibility of *BaraA* mutant flies to *E. faecalis* infection.**

(A) Survival curves of the isogenic *BaraA* KO and KI flies infected with *E. faecalis*. 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* in WT and *BaraA* KO and KI lines. Pooled data from three independent experiments. (C, D) Bacterial load of *E. faecalis* in WT and *BaraA* KO and KI lines from early time points to six days after infection. No significant differences were detected between WT and *BaraA* mutants at each time point. *MyD88* was significantly different from WT, \*\*\*\*  $p < 0.0001$ . Caption applies to panels C-D. Pooled data from three independent experiments. Data are expressed as means  $\pm$  SEM.



**Figure 3. Susceptibility of *BaraA* mutants to *M. robertsii* infection.**

(A) Survival of isogenic *BaraA* mutants injected with 50 *M. robertsii* conidia. Pooled data from ten independent experiments. Statistical significance between WT and the KO or KI mutants: \*\*\*\*  $p < 0.0001$ . (B) Kinetics over three days 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 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, C). Data are expressed as means  $\pm$  SEM.

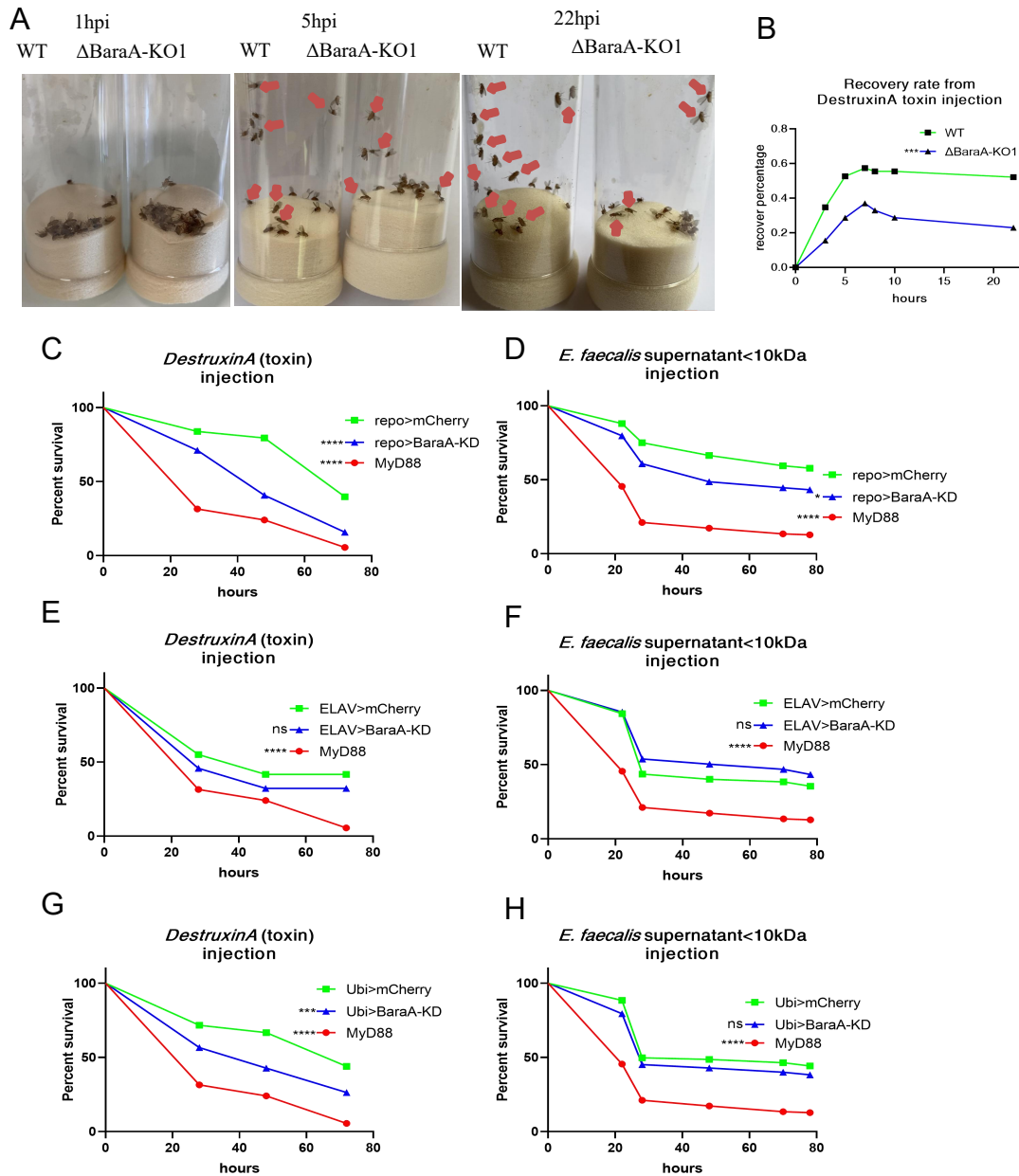


**Figure 4. *BaraA*-dependent protection of *Drosophila* flies from the noxious effects of microbial toxins.**

Mutant flies were injected with 4.6nl, 8mM 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 *DestruxinSI*-*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* 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 three to ten kDa. Pooled data from four independent experiments, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . **(E)** The 3-10kDa fraction supernatant from *E.*

*faecalis* was collected from *entV<sup>-</sup>* and *entV<sup>+</sup>/entV<sup>-</sup>* strains. The supernatant from the *entV<sup>-</sup>* strain killed *BaraA* mutants at the same rate as wild type flies whereas *BaraA* KO1 mutants were killed by the complemented *entV<sup>+</sup>/entV<sup>-</sup>* supernatant significantly faster than wild-type flies. For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies submitted respectively to the same challenge for statistical analysis. Pooled data from eight independent experiments, \* p<0.05; \*\*\* p<0.001; \*\*\*\* p< 0.0001. **(F)** 0.5 OD, 4.6nl of the mutant and rescued *E. faecalis* strains were injected. Rescued strain *entV<sup>+</sup>/entV<sup>-</sup>* strain killed *BaraA* KO1 faster than wild-type flies. Significant differences between wild type and *BaraA* mutant flies were detected upon *entV<sup>-</sup>* infection. *BaraA* KO1 infected by *entV<sup>+</sup>/entV<sup>-</sup>* strain were killed faster than *BaraA* KO1 infected by *entV<sup>-</sup>* strain, while no such significant difference was observed in the case of the *BaraA* KI line. For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies for statistical analysis. Pooled data from four independent experiments, \* p<0.05; \*\*\* p<0.001; \*\*\*\* p< 0.0001





**Fig. 5 BaraA counteracts the paralysis induced by exposure to DestruxinA and is required in glial cells**

(A) Wild type flies or BaraA-KO1 mutant flies have been injected with 4.6nl, 8mM Destruxin A toxin. Pictures were taken at one, five, and 22 hours post infection (see also the corresponding Supplementary Movies 1-3). After one hour post infection, all flies were paralyzed. At five hours post infection, 11 wild type flies woke up whereas only six BaraA-KO1 mutant flies woke up from toxin injection. At 22 hours post infection, 12 wild type flies woke up while four BaraA-KO1 mutant flies woke up. (B) Quantification of A). Pooled data from two independent experiments, \*\*\*  $p < 0.0001$ . (C, D) BaraA-KD flies were silenced in glial cells (*repo-Gal4*) and injected respectively with 4.6nl, 8mM Destruxin A toxin (C) or 23nl of *E. faecalis* supernatant

<10kDa (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, F)** BaraA-KD flies were silenced in neurons (*elav-Gal4*) and injected respectively with 4.6nl, 8mM Destruxin A toxin (E) or 23nl of *E. faecalis* supernatant <10kDa (F). BaraA-KD flies showed no significant difference from wild type control flies. Pooled data from four independent experiments. **(G, H)** BaraA-KD flies were silenced ubiquitously (*ubi-Gal4*) and were injected respectively with 4.6nl, 8mM Destruxin A toxin (G) or 23nl of *E. faecalis* supernatant <10kDa (H). Ubi>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 while *E. faecalis* supernatant injection. Pooled data from four independent experiments.

**Supplementary Information 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 3<sup>rd</sup> chromosome using *y<sup>l</sup> 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<sup>Cas9</sup>)) 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 (Gibson 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 ratio pCFD5:pSK=3:1, was injected into recipient  $y^l$  M{Act5C-Cas9.P.RFP-}ZH-2A  $w^{1118}$  DNAlig4<sup>169</sup> 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 DH5a 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  $y^l$  M{vas-int.Dm}ZH-2A  $w^*$ ; M{3xP3-RFP.attP}ZH-86Fb embryos and missing constructs were reinjected alone.

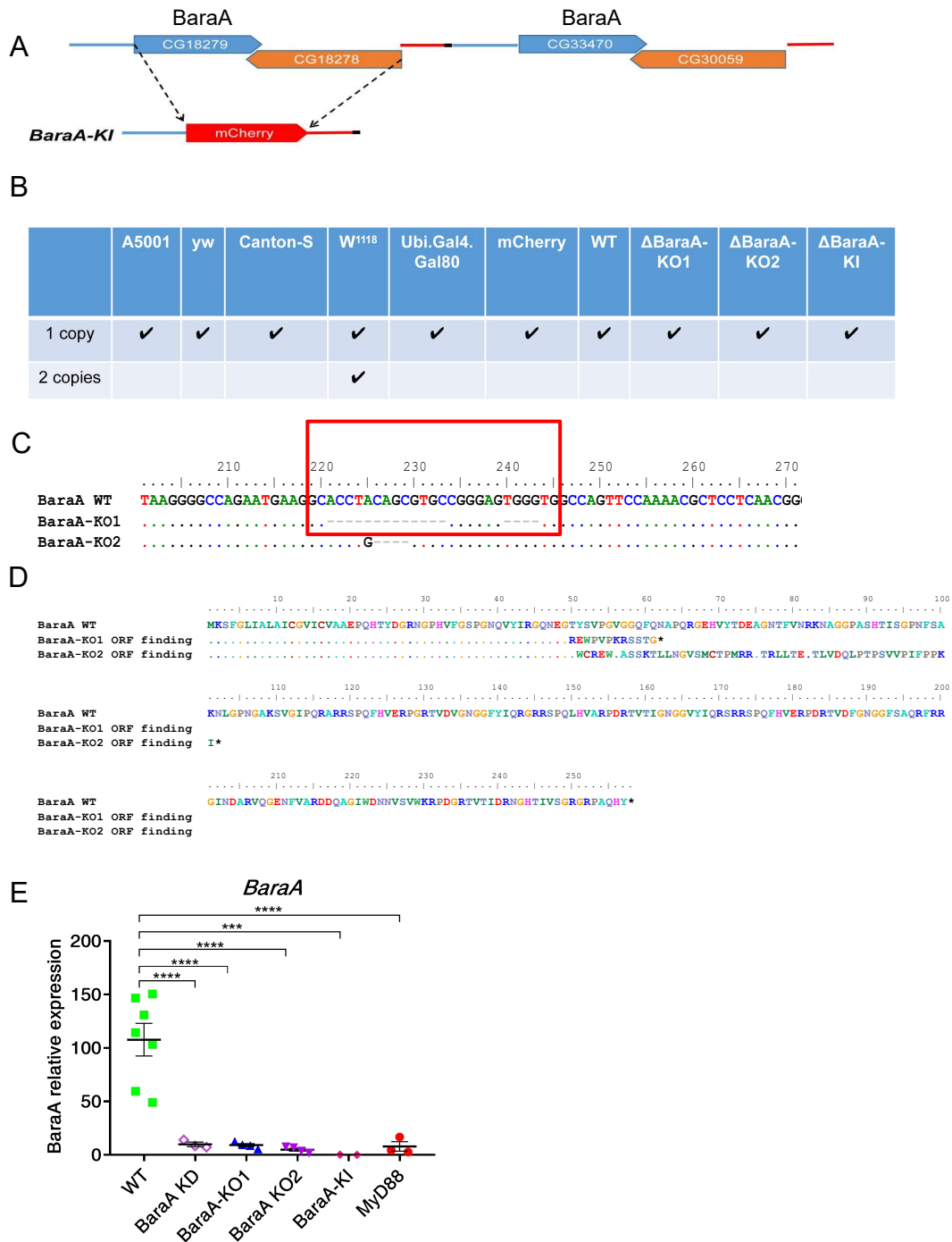
### *Molecular mass fingerprints by MALDI MS*

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**

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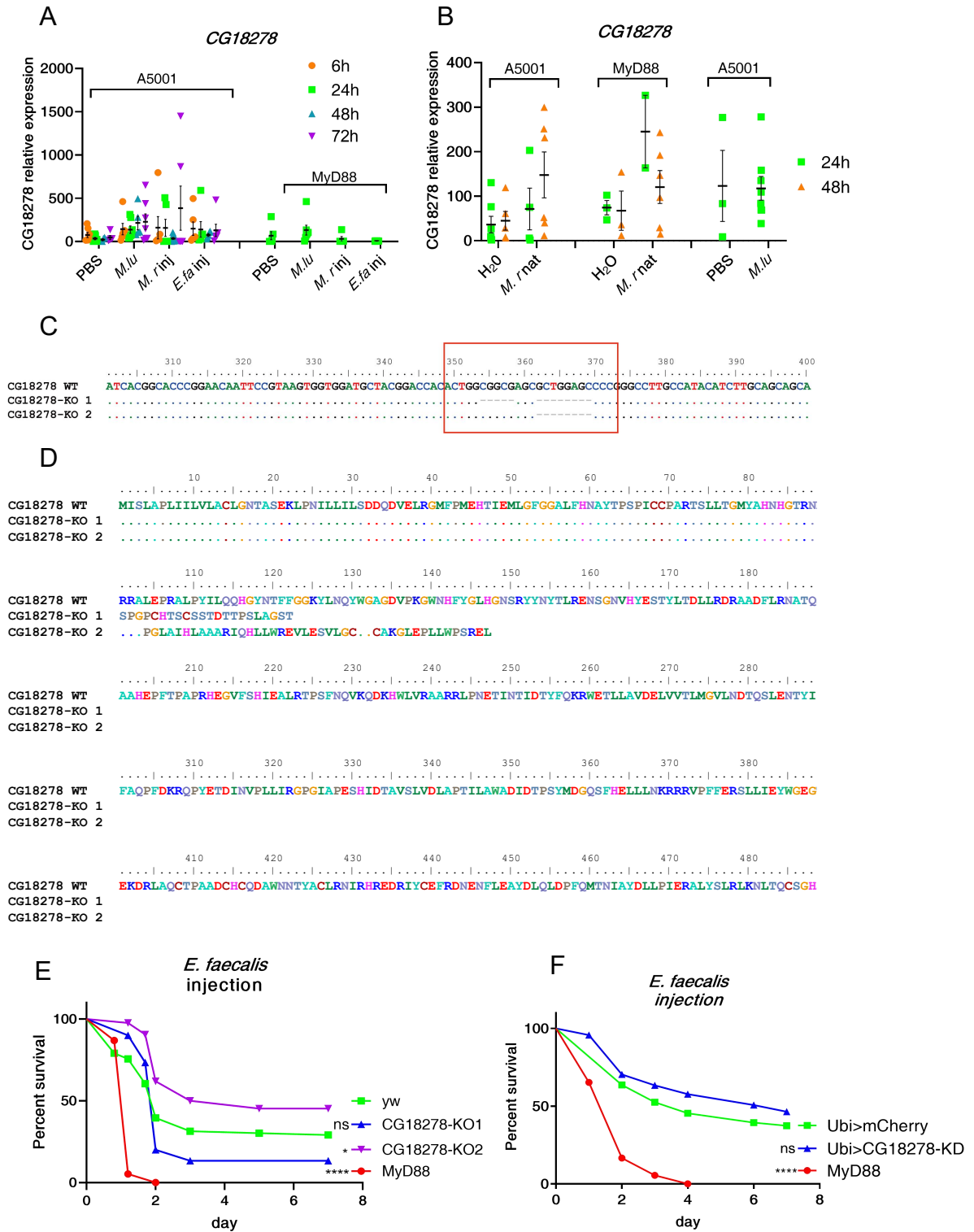


**Figure S1. Mutants affecting the *BaraA* locus.**

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

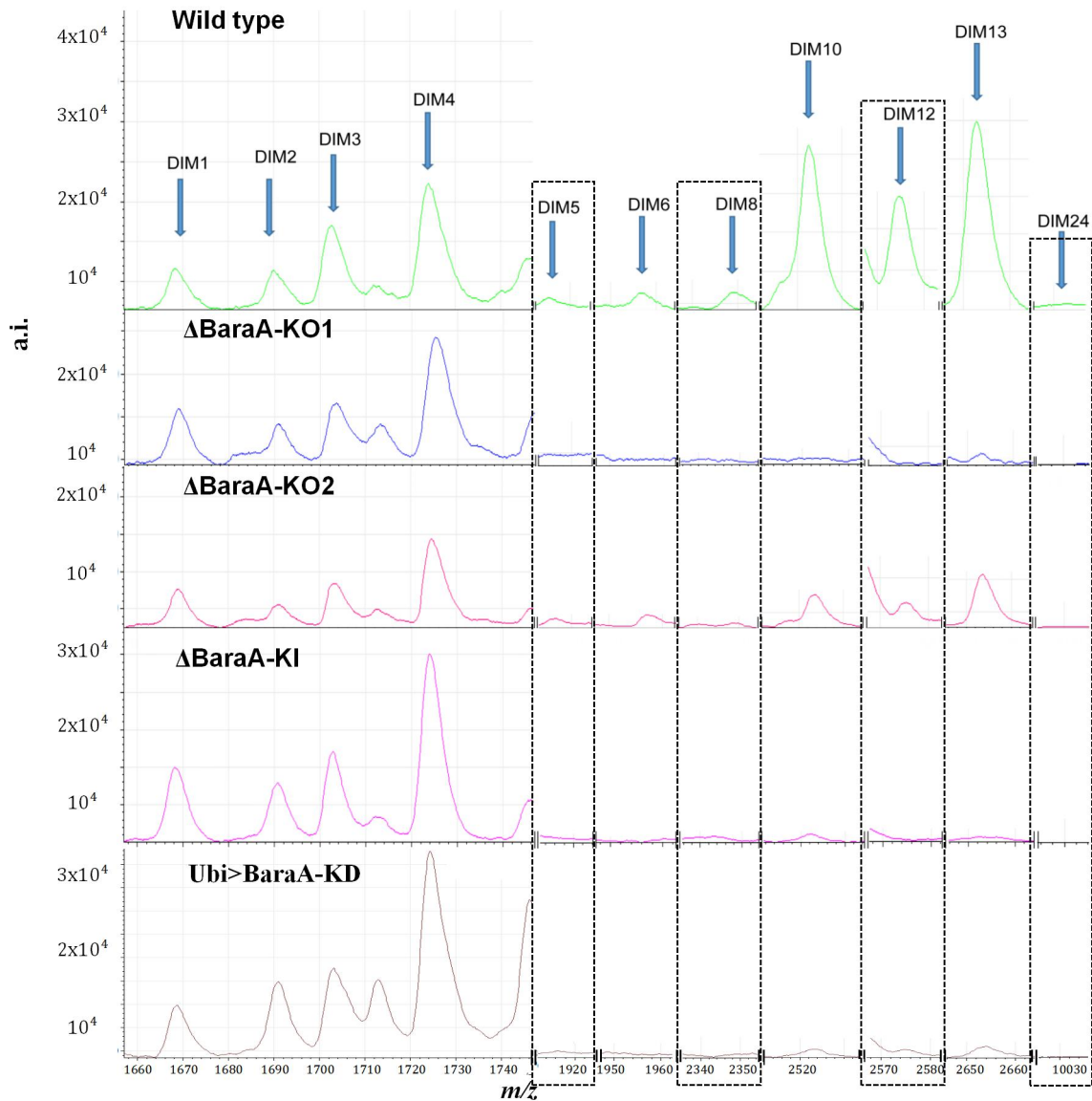
fly line, the two genes (*CG18279*, *CG18278*) were replaced by *mCherry* coding sequence after the START codon from *CG18279*. **(B)** Table recapitulating the tested strains and the presence of the duplication. The KI line was originally generated in a *yw* background with only one copy of the locus. **(C)** CRISPR Cas9 knock out mutants of *BaraA*: KO1 has a complex deletion pattern removing 17bp in total while the KO2 has a 4bp deletion and one point mutation. **(D)** The small deletions found in the KO lines lead to frame shift mutations which generate early stop codons. **(E)** *BaraA* expression level measured by RTqPCR in wild-type, knock down (KD), knock out (KO), knock in (KI), and *MyD88* flies, 24h after a *M. luteus* challenge. Data are expressed as means  $\pm$  SEM. Pooled data from two independent experiments, \*\*\*\*  $p < 0.0001$ .





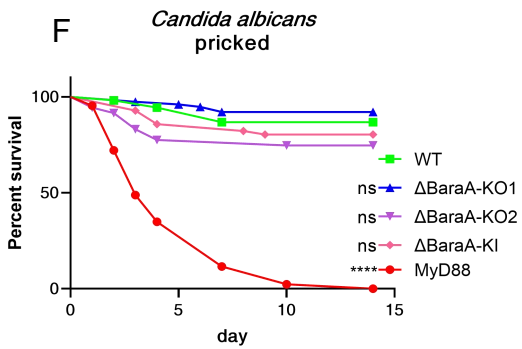
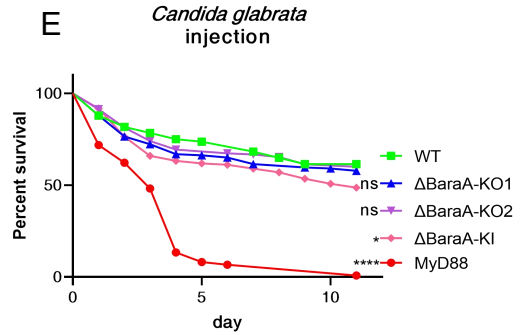
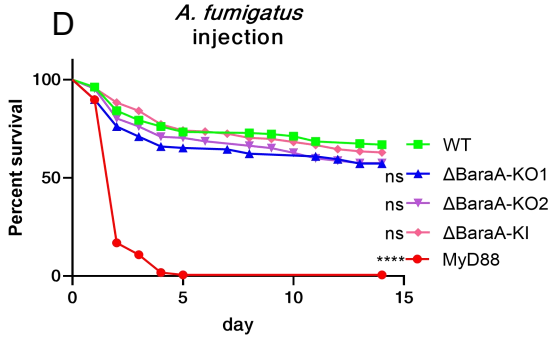
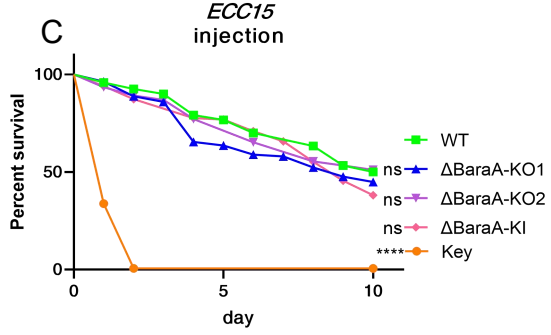
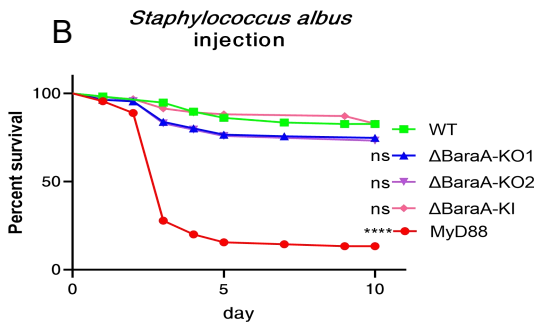
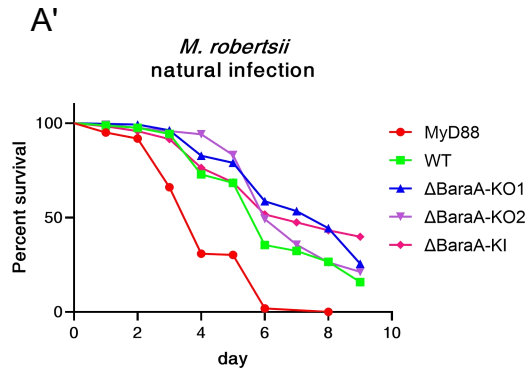
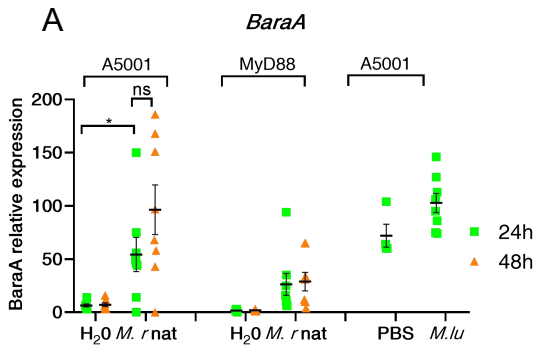
**Figure S2. The *BaraA* neighboring gene *CG18278* is not involved in host defenses against *E. faecalis*.**

(A) Expression of the *CG18278* gene monitored by RTqPCR in wild-type  $w^{45001}$  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  $\pm$  SEM. The measured expression of *BaraA* 24 hours after a *M. luteus* challenge is taken as a reference for all other data points and given a 100% value. (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  $w^{45001}$  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  $\pm$  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 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* while *CG18278* KO2 mutant showed slightly 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* similar to that of the wild type. Two independent experiments have been pooled.



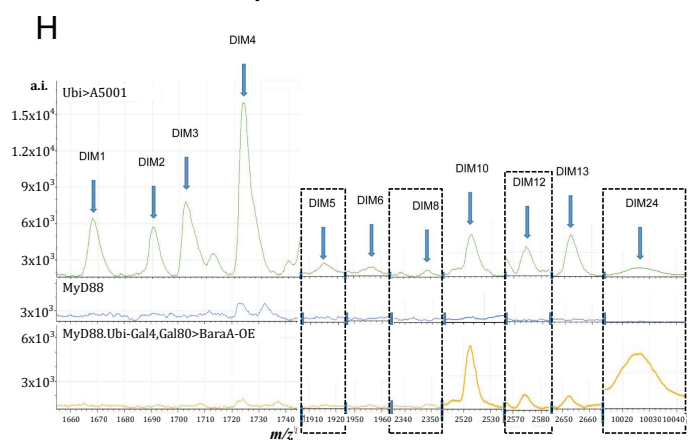
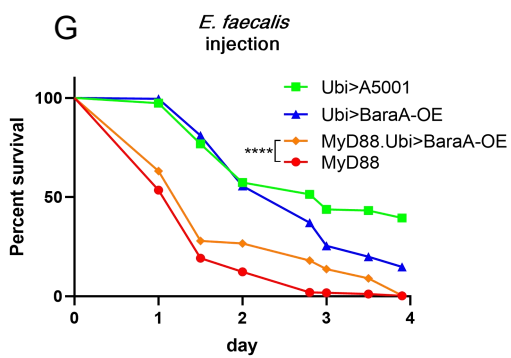
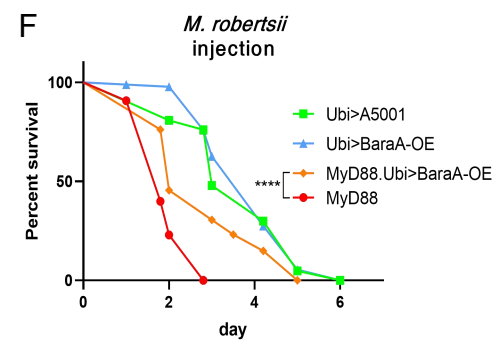
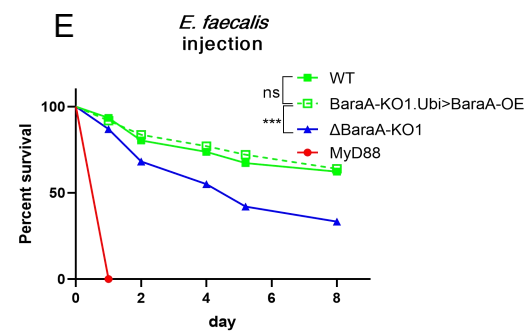
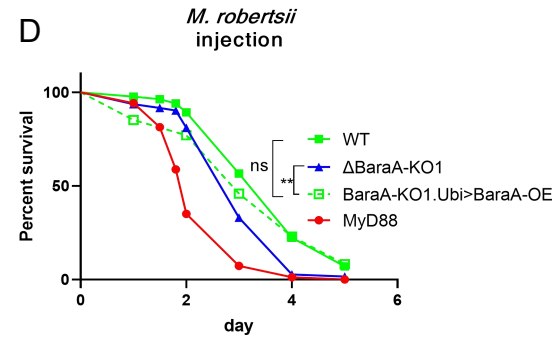
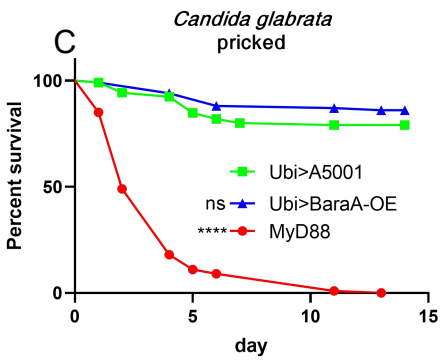
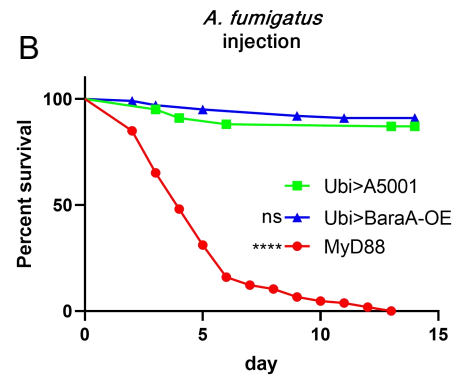
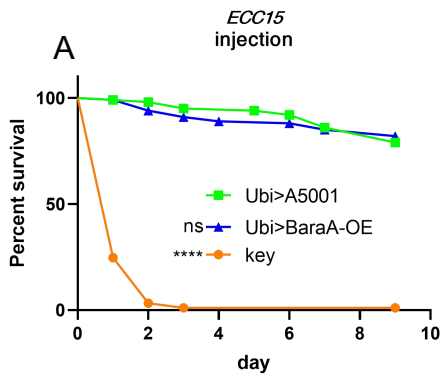
**Figure S3. Detection of *BaraA*-derived DIM peptides in wild-type and *BaraA* KD, KO, and KI mutants by mass-spectrometry analysis.**

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. Unexpectedly, in *BaraA*-KO2, DIM6, DIM10, DIM12, and DIM13 or unrelated peptides of similar molecular weight appear to be somewhat expressed. Unless otherwise specified, the notation *BaraA* KO refers to the KO1 line. See also Table S1 for quantification of the peaks. a.i. : absolute quantitation.



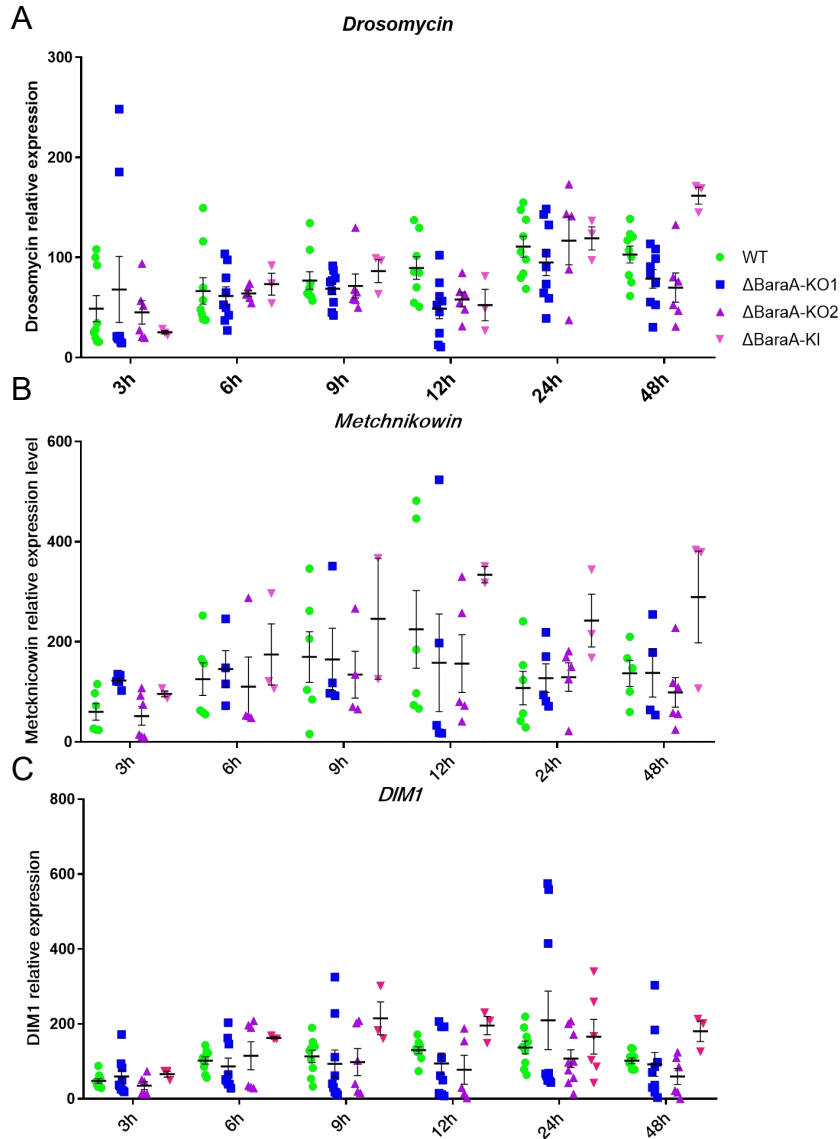
**Figure S4: *BaraA* mutants survive as well as wild-type (WT) flies to different types of infection.**

(A) Expression of *BaraA* steady-state transcripts during *M. robertsii* natural infection as monitored by RTqPCR. Pooled data from three independent experiments; data are expressed as means  $\pm$  SEM. (A', B-F) Survival experiments after the indicated infectious challenge in the septic injury model and natural infection model are presented and are representative of at least two independent experiments. The appropriate positive 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. (C, F) Pooled data from two independent experiments; other survival curves correspond to pooled data from three independent experiments. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ .



**Figure S5: *BaraA* overexpression in *MyD88* but not WT background confers an enhanced protection against *E. faecalis* and *M. robertsii* infection *in vivo*.**

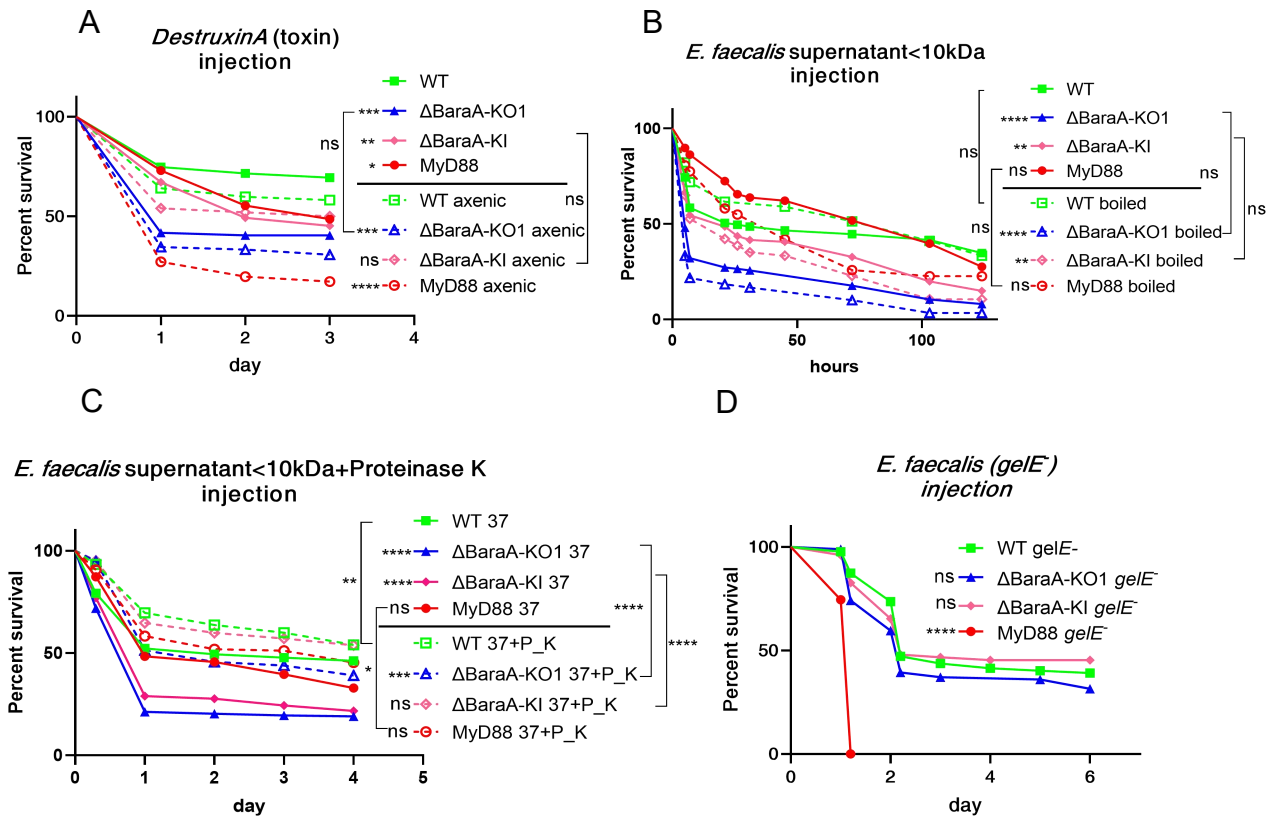
(A-C) BaraA ubiquitous overexpression in WT background did not enhance the protection against *ECC15* (A), *A. fumigatus* (B), *Candida glabrata* (C) infection. (D, E) Rescue of the nonisogenic *BaraA* KO1 mutant with a *BaraA* expressed under the control of a p*Ubi*-Gal4<sup>ts</sup> driver (cross performed at 18°C and induced at the adult stage at 29°C) after *M. robertsii* (D) or *E. faecalis* injection (E). Three independent experiments with each pathogen have been performed and pooled. \*p < 0.05. (F, G) BaraA overexpression in *MyD88* background enhanced the protection against *M. robertsii* (F) and *E. faecalis* (G) infection compared to *MyD88* flies. Pooled data from three independent experiments, \*\*\*\* p<0.0001. (H) 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. See Table S2 for quantification.



**Figure S6. Toll-mediated activation of some of its target effector genes is not altered in *BaraA* KO or KI flies.**

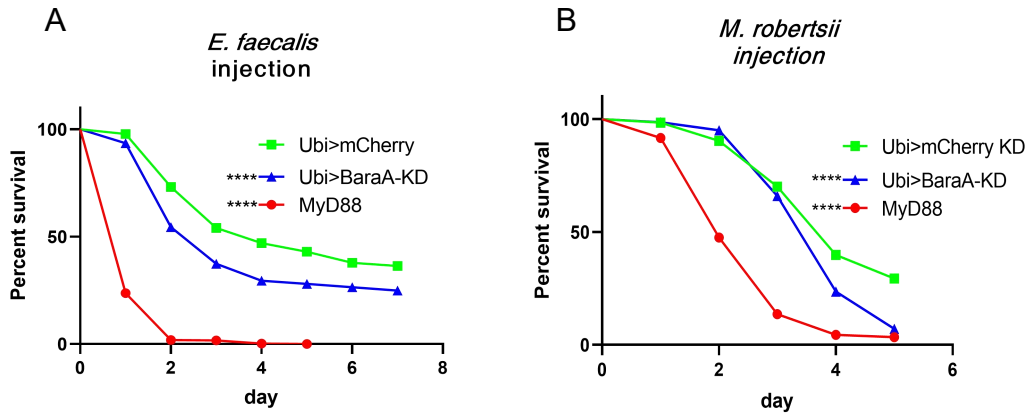
Steady-state transcript levels of *D. melanogaster* Toll pathway-regulated genes were measured by quantitative RT-PCR at different time points after a *E. faecalis* challenge: *Drosomyacin* (A), *Metchnikowin* (B), and *DIM1*=*BomS1*(C). These experiments are representative of three independent experiments. Gene expression was normalized against *rp132* 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 Kruskal-Wallis multiple comparisons test has been used. Data are expressed as means  $\pm$  SEM.





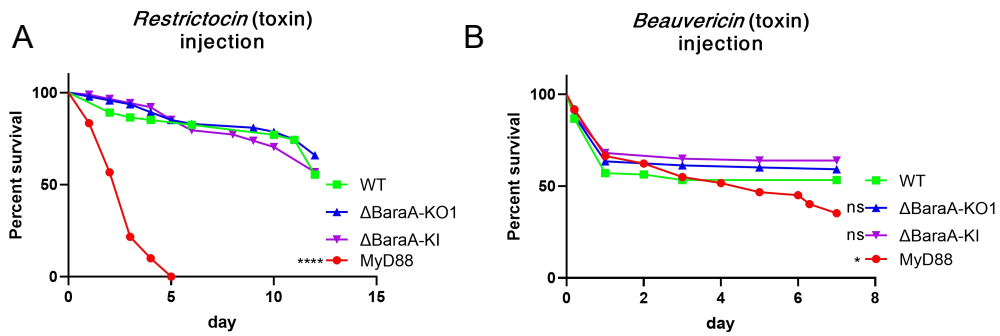
**Figure S7. Further characterization of the effects of toxins secreted by *M. robertsii* or *E. faecalis***

(A) 4.6nL of 8mM DestruxinA were injected into axenic (dashed lines) or conventionally-raised flies. Axenic *BaraA* mutants showed no significant difference from control conventionally-raised *BaraA* mutant flies. Pooled data from two independent experiments, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . (B) Supernatant from *E. faecalis* was boiled at 95°C for 5min. 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 respectively 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. S8 *BaraA* KD flies are sensitive to *E. faecalis* and *M. robertsii* infection.**

**(A, B)** Ubiquitously-silenced *BaraA* KD flies were infected with *E. faecalis* (A) or *M. robertsii* (B) and displayed significant differences in their survival rates compared to control wild type flies. Pooled data from eight independent experiments (A) or seven independent experiments, \*\*\*\*  $p < 0.0001$



**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 ( <i>m/z</i> )		Wild type		Δ <i>BaraA</i> -KO1		Δ <i>BaraA</i> -KO2		Δ <i>BaraA</i> -KI	
	Theoretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
<b>DIM1(BomS1)</b>	1667	1668	67258	16767	62061	12083	56542	13367	103407	21980
<b>DIM2(BomS2)</b>	1690	1690	40706	11767	61520	11580	42637	9723	82204	16824
<b>DIM3(BomS3)</b>	1704	1703	80236	20008	72387	15006	67951	16488	121265	25515
<b>DIM4(Dso1)</b>	1723	1724	134713	26255	260151	44055	102276	21136	289787	52637
<b>DIM5</b>	1914	1915	47815	15539	1863 (0)	2276 (0)	7557	23505	3125 (0)	5929 (0)
<b>DIM6</b>	1955	1957	121705	30592	-	-	19619	6534	-	-
<b>DIM8</b>	2348	2349	46950	18659	2182 (0)	1672 (0)	8293	6944	-	-
<b>DIM10</b>	2521	2523	257018	51458	2738	1797	68802	16807	9619	6569
<b>DIM12</b>	2573	2575	165811	36743	-	-	39942	12085	-	0
<b>DIM13</b>	2651	2653	355175	68504	10071 (0)	2287 (0)	83105	20125	-	6020
<b>DIM24</b>	10031	10034	141464	4599	-	-	71869	2573	-	-
<b>Drosomycin (Drs)</b>	4890	4892	2065642	168320	155898	13213	1253321	108291	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 ( <i>m/z</i> )		Ubi-A5001		MyD88		BaraA-OE (MyD88)	
	Theoretical	Measured	Peak area	Peak intensity	Peak area	Peak intensity	Peak area	Peak intensity
<b>DIM1(BomS1)</b>	1667	1668	39814	5291	2262	283	-	105
<b>DIM2(BomS2)</b>	1690	1691	36729	4492	-	111	-	136
<b>DIM3(BomS3)</b>	1704	1703	70522	10659	1113	283	-	108
<b>DIM4(Dso1)</b>	1723	1724	120283	15820	6007	1337	2387	510
<b>DIM5</b>	1914	1915	6829	1223	-	-	-	-
<b>DIM6</b>	1955	1957	10952	1874	-	-	-	-
<b>DIM8</b>	2348	2349	13152	2049	-	-	531	175
<b>DIM10</b>	2521	2523	154384	20693	685	164	26381	4382
<b>DIM12</b>	2573	2575	104939	14020	-	-	4563	926
<b>DIM13</b>	2651	2653	169357	24429	-	-	4211	828
<b>DIM24</b>	10031	10033	29166	1054	-	-	134314	4227
<b>Drosomycin (Drs)</b>	4890	4892	184661	14427	9057	977	7168	844

Quantitation of Fig. S5H

**Table S3:** Primers used for cloning

BaraA Fw	AAAAAGCAGGCTTCAACATGAAATCGTTTGGATTGATTGC
BaraA Rv	AGAAAGCTGGGTCTTAACTTTTTGGAGGCATATGA
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
Drosomyein Fw	TACTTGTTCCGCTCTTCG
Drosomyein Rv	GAGCGTCCCTCCTCTTGC
IM1 Fw	CAATGCTGTCCACTGTTCGC
IM1 Rv	CGTGGACATTGCACACCCTG
Metchnikowin Fw	CGTCACCAGGGACCCATTT
Metchnikowin Rv	CCGGTCTTGTTGGTTAGGA
BaraA Fw	GGTGAGCATGTGTACACCGA
BaraA Rv	GGCGGAAAAATTGGACCAC
CG18278 Fw	GCCCATGAACCCTTCACTCC
CG18278 Rv	CACCAACCAGTGCTTATCCTGC

## **Complementary results to BaramicinA manuscript**

### **BaraA-derived DIM peptides display an antimicrobial activity *in vitro*.**

As previous data showed, overexpressing *BaraA* in wild type or *MyD88* background failed to display a protection phenotype to different pathogen infections in survival experiments. We also performed *in vitro* experiments with synthetic BaraA-derived peptides and *E. faecalis* to assess a potential antimicrobial activity of BaraA-derived DIM12 or DIM 13. Some activity that limited *E. faecalis* proliferation was observed when using DIM12 concentrations at or above 4 mM and not at lower concentrations or when using a DIM13 scrambled peptide at 5 mM (Complementary Fig. 1.). One experiment performed with DIM13 yielded a similar result. These data establish that some of the DIM peptides inhibit *E. faecalis* growth at high concentrations, which are however unlikely to be found *in vivo* in the hemolymph.

### ***BaraA* is required for efficient melanization**

Another important humoral immune response is melanization, which depends on the cleavage of prophenol oxidase proteins (proPO) into mature PO enzymes that catalyze several steps in the chemical reactions that lead to the formation and deposition of melanin [70, 162]. The PPO cleavage is initiated by proteolytic cascades that activate the Haya protease, which cleaves PPOs [117, 161, 226]. We challenged flies by injecting *M. luteus* and monitored PPO cleavage on Western blots using a PPO1 antibody (a kind gift from Prof. E. Ling). Complementary Figure 2A show that PPO is not cleaved as efficiently in isogenic *BaraA* KO and KI mutants as in wild-type flies, a result obtained in three out of four experiments. These data suggest that *BaraA* is required for efficient melanization.

### ***BaraA* plays a role in the host defense mediated by plasmatocytes**

In the *BaraA* KI line, the endogenous gene is replaced by the *mCherry* gene and is thus under the control of *BaraA* upstream regulatory sequences. Of note, because the KI line we obtained also removes the neighboring *CG30059* gene (Fig. S1A), *mCherry* expression would not be regulated by any sequence located downstream of the *BaraA* gene; the KI may not fully reproduce the expression pattern of the endogenous *BaraA*

gene. We detected fluorescence emitted by the *mCherry* reporter KI in hemocytes collected from the hemolymph, the fluorescence signal was significantly increased by *E. faecalis* challenge (Complementary Fig. 2B). Knocking down *BaraA* expression by Ubi Gal4 showed sensitivity phenotype to *E. faecalis* infection (Complementary Fig. 2C). Interestingly, knocking down *BaraA* expression in most plasmatocytes resulted in an enhanced sensitivity to *E. faecalis* infection (Complementary Fig. 2C'). Of note, a subset of hemocytes express the *PPO2* gene [57]. When we inactivated *BaraA* in *PPO2*-expressing cells [57], no susceptibility to *E. faecalis* (and to *M. robertsii*) was observed (Complementary Fig. 3A-B). We therefore tested whether phagocytosis was affected in *BaraA* mutants using killed pH-RODO *E. faecalis*. As shown in Complementary Fig. 2D, phagocytosis did not appear to be affected; nevertheless, a more definitive conclusion would require testing live bacteria.

Next, we determined that *BaraA* is required for the phagocytic uptake of live *M. robertsii* conidia using a differential permeabilization antibody staining protocol [227] (Complementary Fig. 2E-F). Knocking down *BaraA* expression by Ubi Gal4 showed significant sensitivity phenotype to *M. robertsii* infection (Complementary Fig. 2G). However, we observed no susceptibility of flies in which *BaraA* expression was knocked down in hemocytes (Complementary Fig. 2G'). This result can be explained if some of the BaraA-derived peptides supplied by other tissues function as opsonins. Indeed, we detected some opsonization activity provided by BaraA when conidia were incubated in wild-type hemolymph prior to injection back into either wild-type or *BaraA* KO recipients (Complementary Fig. 2H-I). In keeping with these results, knocking down *BaraA* expression in the fat body, where its expression is induced (Complementary Fig. 3C), led to a mild susceptibility to the injection of *M. robertsii* conidia, whereas a variable sensitivity was observed upon an *E. faecalis* challenge depending on the driver line used (Complementary Fig. 3D-G).

We conclude that *BaraA* plays distinct functions in the cellular host defense, depending on the pathogen. Its function is required in hemocytes against *E. faecalis* but does not appear to involve phagocytosis. In contrast, BaraA-derived proteins secreted from the fat body, and likely to some extent from hemocytes, opsonize *M. robertsii* conidia and thereby enhance their phagocytic uptake.

### **BaraA does counteract specific toxins**

From the data we showed before, we found that BaraA is counteracting fungal Destruxin A and bacterial Enterocin O16. We next addressed whether BaraA can protect the host from other toxins. Restrictocin is a ribotoxin from *Aspergillus restrictus*. However, when we injected the toxin into *BaraA* mutant flies, the flies did not show reproducible susceptibility phenotype to Restrictocin compared to wild type flies (Complementary Fig. 4A). A study revealed that *BaraA* mutant flies are sensitive to *Beauveria bassiana* [223]. We then checked whether BaraA plays a role against Beauvericin. BaraA did not display a susceptibility phenotype to Beauvericin injection, like wild type flies, while Beauvericin killed around 70% *MyD88* mutant flies in 7 days (Complementary Fig. 4B). This result adds one more toxin to a list of toxins protected by the Toll pathway that includes restrictocin, ergot alkaloids, verruculogen/fumitremorgins from *A. fumigatus*, DestruxinA from *M. robertsii* and EntV from *E. faecalis*. BaraA does not protect flies from the action of Beauvericin exhibits that protective function is limited to specific toxins.

In addition to Destruxin A, we also wondered whether other effectors from *M. robertsii* are counteracted by BaraA. We performed survival experiments with different mutants of *M. robertsii* strain, whether from *M. robertsii*\_2575 or \_23 background. As shown in Complementary Fig. 4C, the virulence function of phosphatidylinositol/phosphatidylglycerol transfer protein (PITP) mutant strain disappeared. *BaraA* mutant flies were as sensitive as wild-type flies, and not more sensitive to the *M. robertsii* PITP mutant strain. PITP family has a wide range of biological effects, including the binding/transport of phosphatidylinositol or phosphatidylcholine. Extracellular signal molecules bind to the cell surface G protein-coupled receptors to activate phospholipase C (PLC-B) on the plasma membrane and hydrolyze 4, 5-diphosphate phosphatidylinositol (PIP<sub>2</sub>) to 1,4, 5-triphosphate inositol (IP<sub>3</sub>) and diacylglycerol. IP<sub>3</sub> binds to the IP<sub>3</sub> ligand calcium channel in the endoplasmic reticulum to open the calcium channel and increase the intracellular Ca<sup>2+</sup> concentration, activating various calcium dependent proteins [228]. Whether PITP is required for the secretion of virulence factors such as Destruxins remains to be established.

### **BaraA has influence on the Outer membrane vesicles (OMV)**



Pathogens can secrete vesicles, which deliver virulence factors or mediate communications between cells. Vesicles in Gram-negative bacteria are derived from the outer membrane; hence they are named as outer membrane vesicles (OMVs). OMVs may contain different kinds of proteins, DNA and RNA [229]. To figure out whether BaraA is involved in host defense against OMVs, we extracted the OMVs from *Pseudomonas aeruginosa* (PAO1) and *Serratia marcescens* (Db11 and 21C4 strains). We observed that after OMV injection, both *BaraA* KO1 and KI mutant flies showed a sensitivity phenotype compared to wild type flies (Complementary Fig. 5A-B'). To figure out if BaraA is involved in the generation of ROS required for OMV pathogenicity, we co-injected Vitamin C (VitC) and OMVs into flies. Survival results showed that co-injection of VitC and OMVs caused more resistance in both wild type flies and *BaraA* mutant flies, but *BaraA* mutant flies with OMV injection alone still exhibited a higher susceptibility than wild type flies (Complementary Fig. 5C). These data suggest that the enhanced pathogenesis of OMVs on *BaraA* mutant flies does not involve ROS. *Serratia spp.* are able to generate multiple effectors as virulence factors that attack the host. A study of *S. marcescens* has reported that the metalloprotease serralyisin, also named as PrtA, exists in OMVs [230]. Furthermore, PrtA has been demonstrated to act as a virulence factor that causes keratitis induced by *Serratia* [231]. Following this information, we asked whether *BaraA* mutant flies died faster due to the PrtA in OMVs. To this end, we injected the OMVs from PrtA *S. marcescens* mutant strain. However, the mutant PrtA OMVs still killed *BaraA* mutant flies faster than wild type flies (Complementary Fig. 5E). Of note, this latter result was obtained only with 30-fold concentrated OMVs. At a normal concentration PrtA OMVs, which did not kill much wild type flies or *BaraA* mutant flies. For further confirmation, we injected the purified PrtA peptide into flies. We still observed a sensitivity phenotype of *BaraA* mutant flies (Complementary Fig. 5F). These data suggest that, PrtA from *S. marcescens* OMVs is the major pathogenic factor counteracted by BaraA but is not the sole target of BaraA in OMVs. AprA is a metalloprotease from *Pseudomonas aeruginosa* and is homologous to *S. marcescens* PrtA. It has been reported that AprA cleaves Monalysin from pro-toxin form to active toxin in *Drosophila* adults [232]. Thanks to Dr. Jing Chen, we obtained a PAO1 strain in which AprA can be induced with L-Arabinose. With infection of AprA overexpression (OE+) strain, we observed that the bacteria killed wild type and *BaraA* KI faster than the group

in which AprA is not overexpressed, while *BaraA* KO1 displayed very slight difference from 22h to 24h as it displayed already a strong sensitivity phenotype even in the absence of AprA overexpression (Complementary Fig. 5G). We monitored BaraA transcript levels after infection by PAO1 and noted a trend toward a high expression of *BaraA* when AprA was overexpressed by injection PAO1 (Complementary Fig. 5H). Interestingly, we observed a significantly stronger expression of *BaraA* in IMD pathway mutant flies (key) upon a challenge with PAO1 as compared to a mock challenge (Complementary Fig. 5H). This likely results from a higher microbial burden in key mutant flies.

Taken together, these data suggest that BaraA protects the host from action of pathogenic bacterial OMVs, likely mediated mostly by a metalloprotease family found in *P. aeruginosa* and *S. marcescens*.

### **BaraA expression in several tissues/ cell types is required for the protection against the action of toxins**

As chapter I showed, BaraA is counteracting the action of toxins. We next asked in which tissues its expression is required. We injected the supernatant from *E. faecalis* or Destruxin A respectively into *BaraA* KD flies driven by different tissue specific drivers. As a result, we found that flies in which *BaraA* is silenced in hemocytes, or nervous system are sensitive to either *E. faecalis* supernatant or Destruxin A (Complementary Fig. 6). Silencing *BaraA* in the fat body led to an enhanced sensitivity to the *E. faecalis* or to Destruxin A. Of note, the results obtained with Destruxin A were rather variable (Complementary Fig. 6F). Mark Hanson has documented a BaraA expression in the brain [223] and following the results we have in nervous system, we next checked which brain cell type(s) is involved. We used different drivers to drive *BaraA* KD in different type of brain cells and the results showed that *BaraA* KD driven by cortex glia Gal4 (GMR77A03 Gal4) and perineurial glia Gal4 (NP6293) are more sensitive to *E. faecalis* and Destruxin A compared to wild type control flies (Complementary Fig. 6). These data suggest that BaraA protection is mediated by sub-types of glia cells, whether it also functions in the blood brain barrier remains unclear at present.

### **Non-canonical pathway is involved in counteracting the toxins**

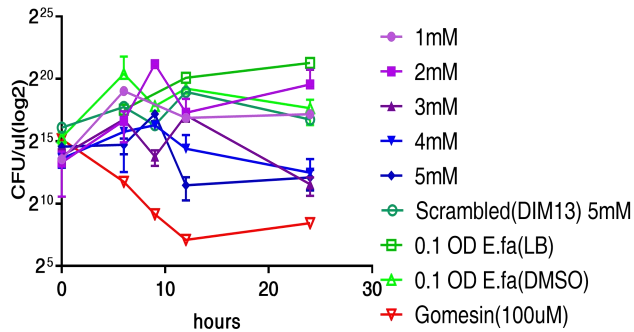
According to Rui Xu's work (submitted), a non-canonical Toll pathway is involved in the host defense against mycotoxins from *A. fumigatus*. The immune-activated proteolytic cascades are not required for the Toll dependent pathway against *A. fumigatus* secreted mycotoxins. Whereas the use of *easter* mutant led to a very mild sensitivity to *A. fumigatus* infection. Overexpression of *Spn27A* led to a more pronounced phenotype. Some *gd* alleles led to a very strong sensitivity phenotype. Taken together, these data suggested that at least some part of the Toll developmental protease cascade are involved in the Toll non-canonical pathway. In addition, the protection against *A. fumigatus* mycotoxins does not required *tube*, *pelle*, *dorsal*, *Dif*, nor *Cactus*. Some specific Bomanins are required to counteract mycotoxins. We then wondered whether the non-canonical pathway also plays a role against the toxins. We first checked whether AMPs are involved. We injected supernatant from *E. faecalis* or Destruxin A into AMP-deficient mutant flies, in which all well-characterized AMPs have been deleted [130]. AMP mutant flies displayed a sensitivity phenotype to *E. faecalis* supernatant, while they exhibited a sensitivity to Destruxin A that was as strong as *MyD88* mutant (Complementary Fig. 7A-B). We then injected supernatant from *E. faecalis* or Destruxin A into *Dif* mutant flies. With injection of *E. faecalis* supernatant, *Dif* mutant flies had the same survival rate as wild type flies. As regard to Destruxin A injection, *Dif* mutant flies exhibited protection compared to wild type flies (Complementary Fig. 7C-D). However, in this series of experiments, the *Dif* control (= wild-type flies) flies (*yw DD1 cnbw*) succumbed like *MyD88* flies. Additionally, we checked whether *tube* and *pelle* are involved in counteracting toxins, we injected *E. faecalis* supernatant or Destruxin A into mutant flies, which did not show any susceptibility phenotype (Complementary Fig. 7E-F). In embryo, Gastrulation defective (*gd*), *Nudel*, and *Snake* proteases can process *Easter* into an active form, which then cleaves pro-Spätzle, ultimately activating the Toll pathway [233]. Therefore, we injected the *E. faecalis* supernatant or Destruxin A into *gastrulation defective (gd\_1)* mutant flies, yielding the results that *gd\_1* mutant flies are sensitive to *E. faecalis* supernatant and Destruxin A (Complementary Fig. 7G-H). In addition to *gd\_1*, we also tested *spz* mutant flies with *E. faecalis* supernatant or Destruxin A. We observed that *spz* mutant flies were sensitive to *E. faecalis* supernatant or Destruxin A (Complementary Fig. 7I-J).

Taken together, these experiments suggest that the non-canonical pathway is involved in the protection of toxins from *E. faecalis* and Destruxin A toxin.

## Complementary figures

Complementary Fig. 1

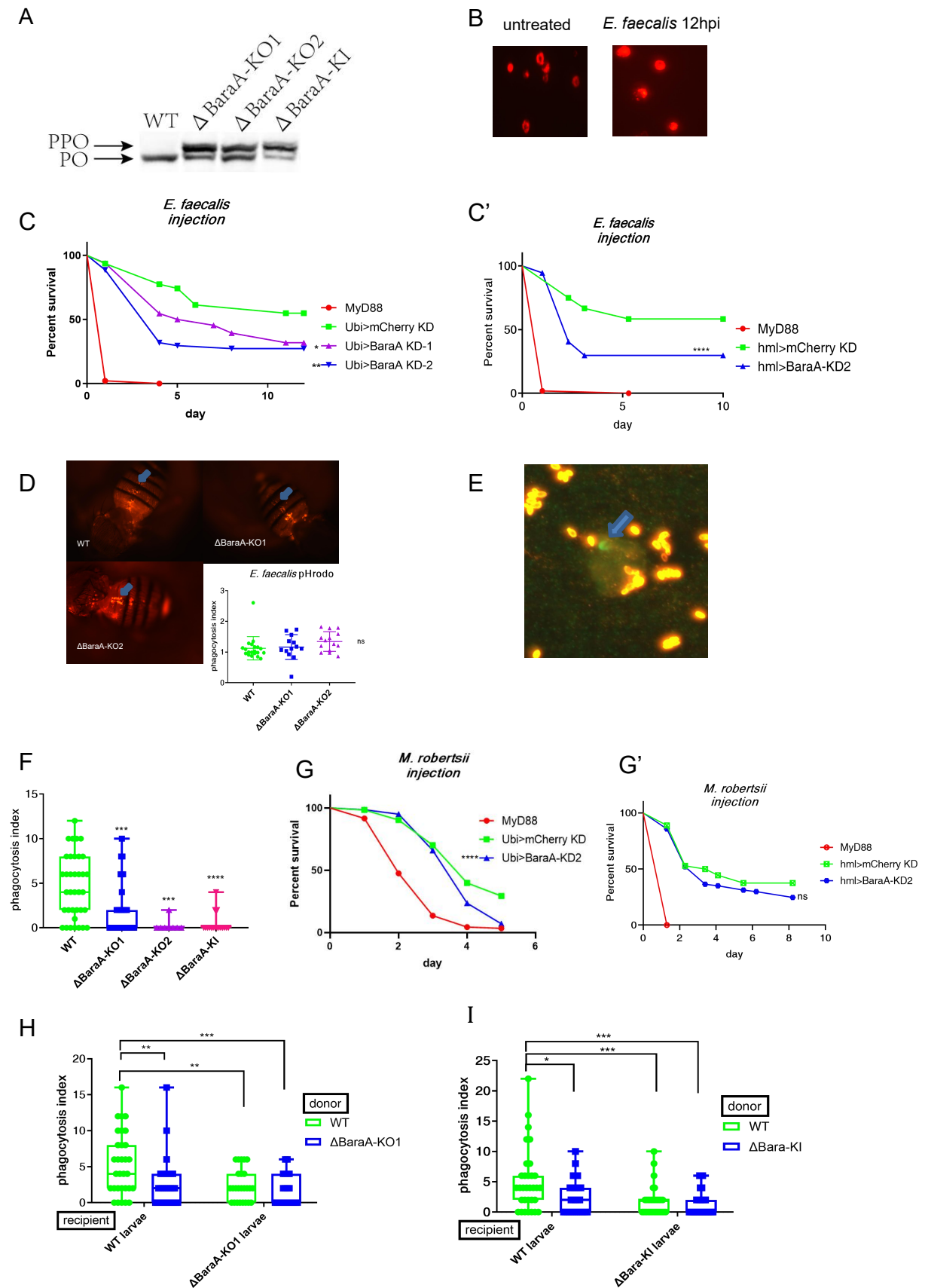
*DIM12 in vitro assay*



**Complementary Figure 1. High concentrations of the DIM12 synthetic peptide can protect the flies from *E. faecalis* infection *in vitro***

DIM12 synthetic peptide incubation with *E. faecalis in vitro* shows that the minimal inhibitor concentration is 4mM. Peptides were mixed with *E. faecalis*, in a total volume of 10 $\mu$ l in a microplate and incubated at 37°C with soft shaking. 0.5 $\mu$ l aliquots were plated at 0, 6, 9, 12, 24h to plate. 100uM Gomesin was used as a positive control.

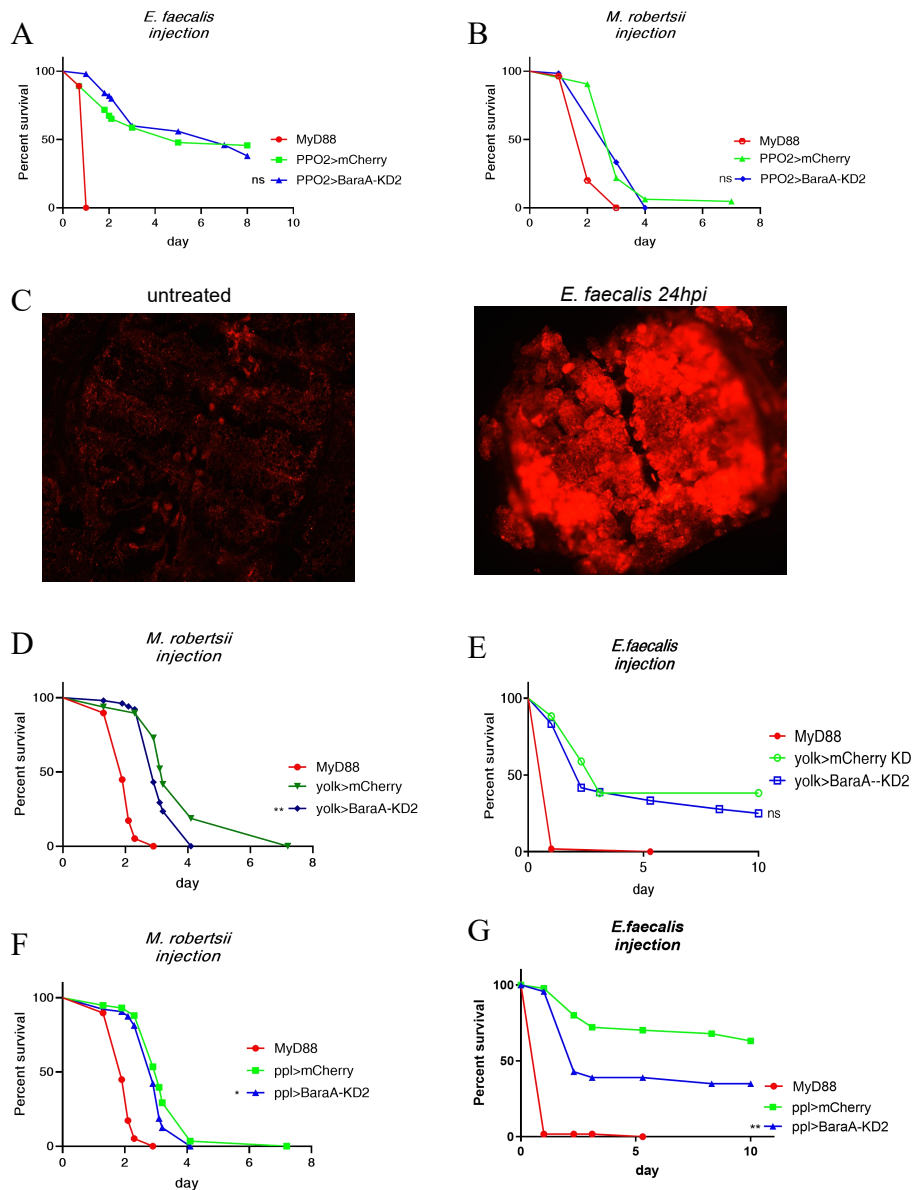
## Complementary Fig. 2



**Complementary Figure 2. BaraA interferes with the maturation of proPPO1 into an active melanization enzyme and plays a role in the host defense mediated by hemocytes against *E. faecalis*.**

**(A)** The cleavage of the prophenoloxidase was analyzed 4h after *M. luteus* septic injury by Western blotting using an anti-PPO1 antibody. This blot is representative of 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. **(C, C')** Survival of Ubi Gal4>*UAS-BaraA* RNAi KD2 (C) and *hmlGal4*>*UAS-BaraA* RNAi KD2 (C') after an immune challenge with *E. faecalis*. Statistical significance between wild type and KD flies (five out of five experiments in C, three out of four experiments in C'), LogRank test, \* p<0.05, \*\* p < 0.01, \*\*\*\*P < 0.0001. **(D)** 4.6nl pHrodo-labeled *E. faecalis* were injected into flies, which were observed 30-45min post infection. The quantification by ImageJ of the pHrodo fluorescent signal corresponding to bacteria present within an acidic compartment in hemocytes did not reveal any significant difference between WT and *BaraA* isogenized KO mutants. **(E)** Image of conidia stained by a polyclonal antibody raised against *M. robertsii* using a differential permeabilization procedure; conidia stained in yellow correspond to noningested conidia whereas green conidia (arrow) are found within plasmatocytes. **(F)** Phagocytosis index of the WT and *BaraA* larval hemocytes. 5000 *M. robertsii* conidia were injected into third-instar larvae, which were incubated for two hours 2 hours incubation at 29°C before being bled. The hemocytes were then stained using the differential permeabilization procedure described above; 50 hemocytes were scored for each sample. **(G, G')** Survival of Ubi Gal4>*UAS-BaraA* RNAi KD2 (G) and *hmlGal4*>*UAS-BaraA* RNAi KD2 (G') after an immune challenge with *M. robertsii*. Four independent experiments have been performed and yielded the same results (G'). (G) 4 out of 7 experiments showed significant difference between *BaraA*-KD2 and control. Pooled data from seven independent experiments. \*\*\*\*P < 0.0001 **(H-I)** Opsonization in the WT and *BaraA*-KO1 and KI. *M. robertsii* conidia were first incubated with third-instar larvae (donor), 5000 treated *M. robertsii* conidia were injected into third-instar larvae (recipients, X axis) then incubated for 2 hours at 29°C before being bled. These experiments were performed followed the differential permeabilization procedure described above. Two-way ANOVA was used. The pooled data from three independent experiments are shown.

### Complementary Fig. 3

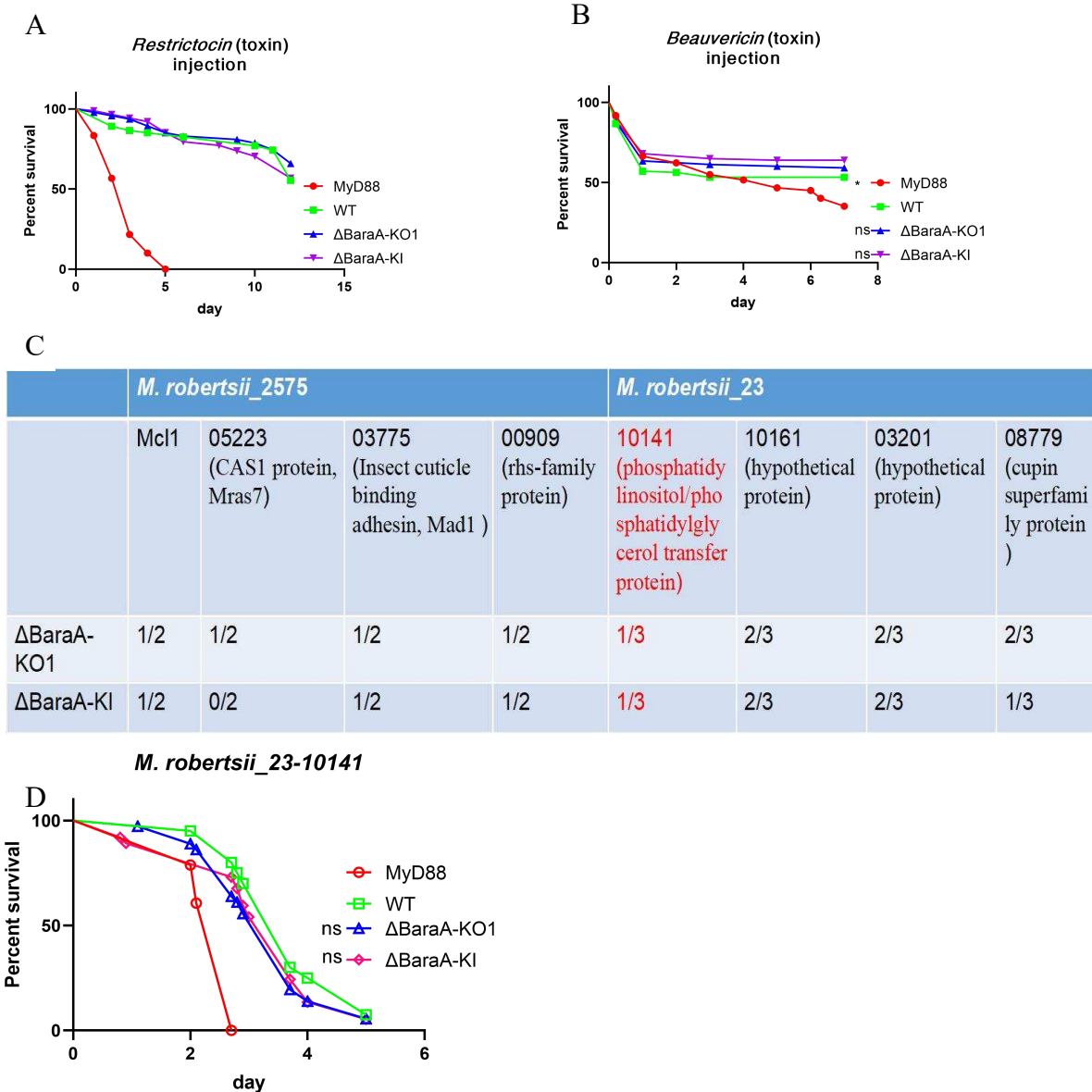


### Complementary Figure 3. Expression and role of *BaraA* against *E. faecalis* infection in the fat body.

(A, B) Flies for which *BaraA* RNAi KD was driven by *PPO2*-Gal4 were not distinctively sensitive to *M. robertsii* or *E. faecalis* infection. Two independent experiments have been performed with similar results. (C) Fat body was taken from adult flies infected by *E. faecalis* 24 hours post infection or from untreated flies. After dissection, the samples were observed by fluorescence microscopy at 10X magnification. (D, F) Flies for which *BaraA* RNAi KD was driven by *yolk*-Gal4 (D) or *ppl*-Gal4 (F) were weakly sensitive to *M. robertsii* infection (two experiments for *yolk*-Gal4 and one out of two for *ppl*-Gal4). (E, G) in three out of four experiments flies for which *BaraA* RNAi KD was driven by *yolk*-Gal4 (E) or *ppl*-Gal4(G) were not sensitive to *E. faecalis* infection.



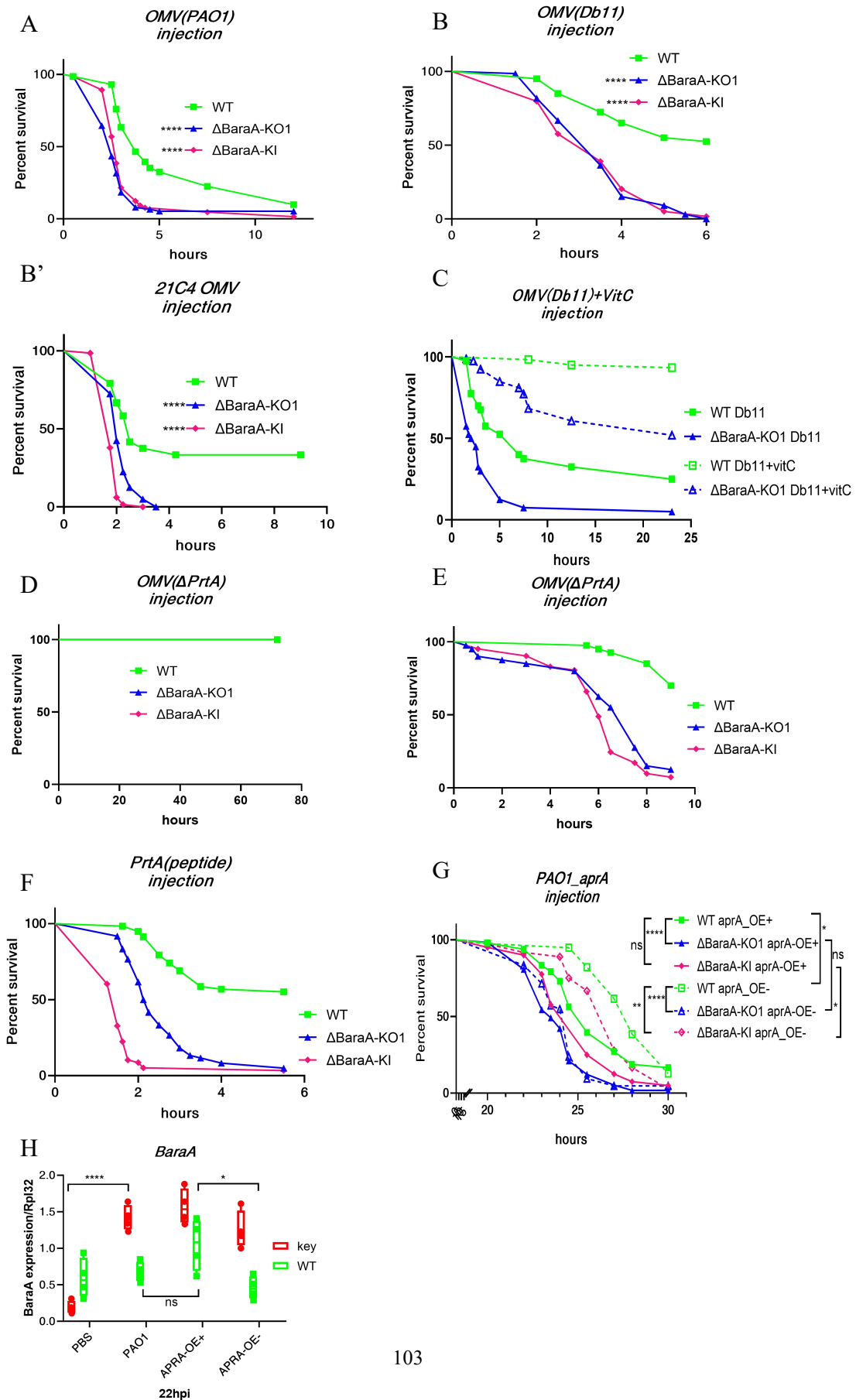
## Complementary Fig. 4



### Complementary Figure 4. BaraA is specifically counteracting toxins.

(A) Survival of *BaraA* mutants following Restrictocin injection. No statistic difference between wild type flies and *BaraA* mutant flies. (B) Upon Beauvericin injection, *MyD88* and not *BaraA* mutants displayed sensitivity to this challenge. (C) Injected different *M. robertsii* strains into *BaraA* mutants. Numbers in the table represents times of significance/total times of infection. Mutated phosphatidylinositol/ phosphatidylglycerol transfer protein strain of *M. robertsii* kills *BaraA* mutants at the same rate as wild type flies (marked in red). (D) Survival experiments of *BaraA* mutant flies infected with *M. robertsii*\_23 10141 mutant strain, 50 spores were injected. Two out of three experiments showed no significant difference compared *BaraA* mutant flies to wild type flies.

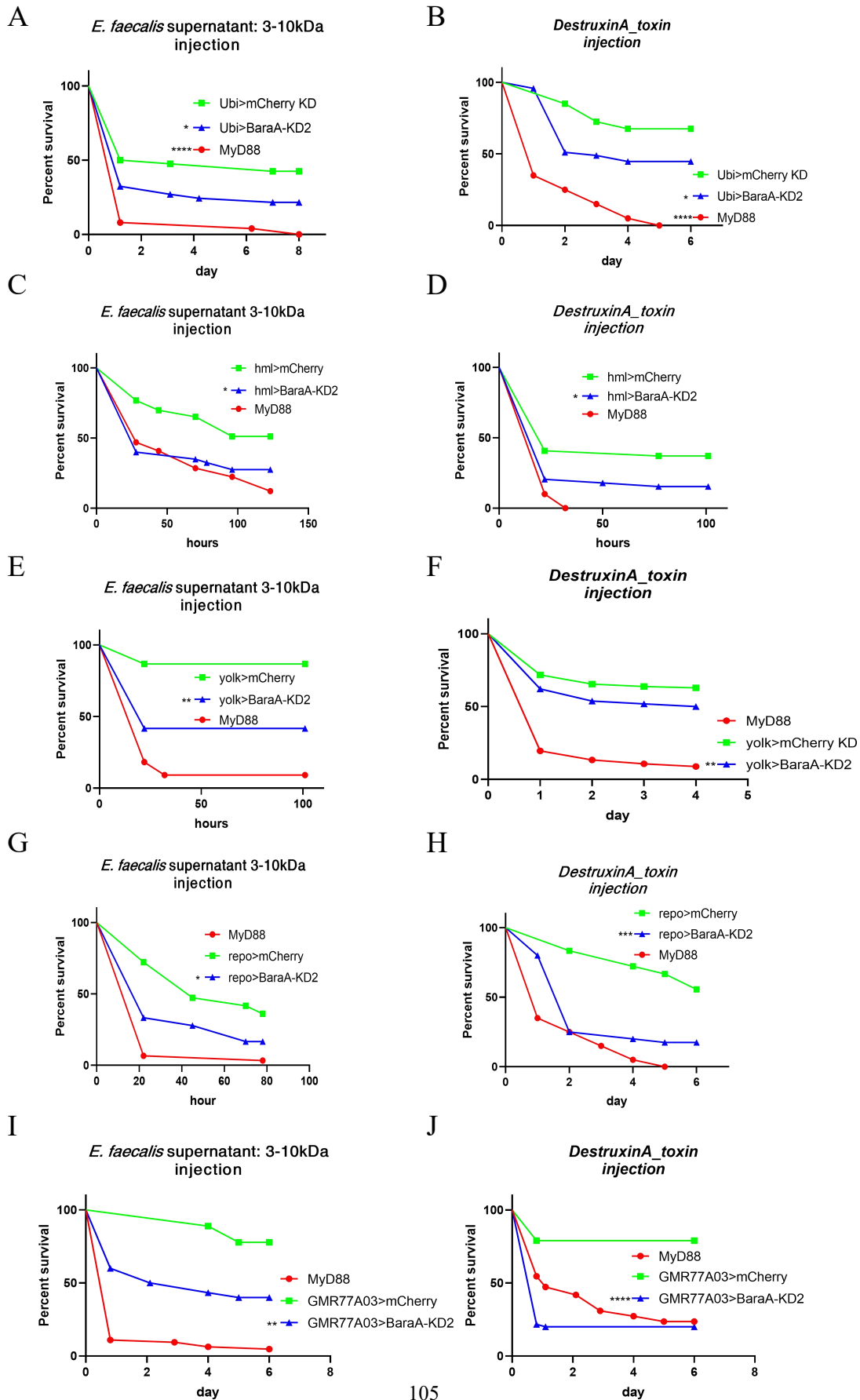
### Complementary Fig. 5



**Complementary Figure 5. *BaraA* mutants succumbed faster than wild-type flies to OMV injection**

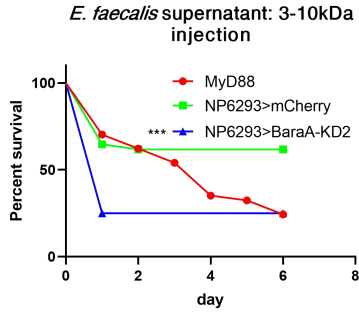
(A) 69nl OMV from *P. aeruginosa* was injected into single flies. Compared to wild type, *BaraA* mutants displayed significant difference in survival, \*\*\*\*  $p < 0.0001$ . (B, B') 69nl OMV from *Serratia marcescens* Db11 (B) or 21C4 (B') was injected into single flies. *BaraA* mutants showed significant difference compared to wild type flies, \*\*\*\*  $p < 0.0001$ . (C) 20mM VitC (as an antioxidant), was co-injected with OMV. VitC-OMV co-injected groups died slower than the group was only injected OMV. (D, E) The OMV was extracted from PrtA mutant *S. marcescens* strain. 69nl OMV was injected, (D) the concentration of OMV was equal to (B); (E) 30fold concentrated OMV was injected, yielding the results that *BaraA* mutant flies died significant faster than wild type flies. (F) 0.27 mg/ml, 69nl purified PrtA peptide was injected, which made *BaraA* mutant flies succumb to death faster than wild type. (F) The overexpression of AprA (OE) is induced by the exposure to arabinose during infection. 20 CFU in 13.8nl was injected. Upon AprA overexpression, wild type and *BaraA* KI mutant flies died faster, while no significant difference in *BaraA* KO group. \*  $p < 0.05$ . The *BaraA* KO1 line was reproducibly more sensitive than *BaraA* KI to PAO1 injection, whether overexpression AprA or not. (F) After aprA\_OE- (without AprA overexpression) challenge, *BaraA* expression in wild type was downregulated compared to aprA\_OE+ (with AprA overexpression). At least three independent experiments have been performed.

## Complementary Fig. 6

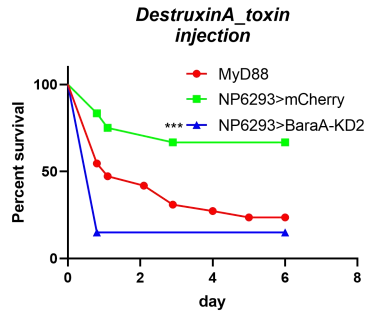


Complementary Fig. 6

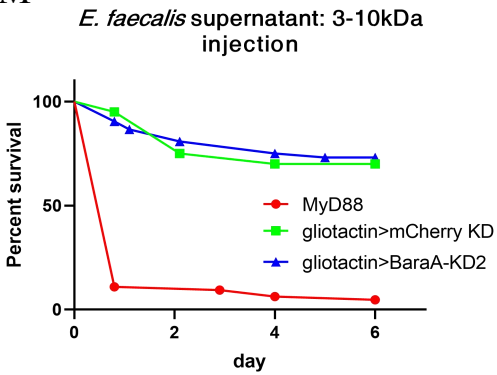
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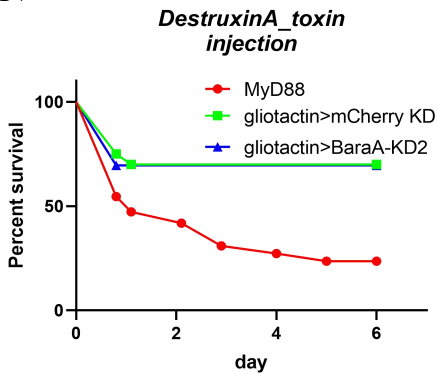
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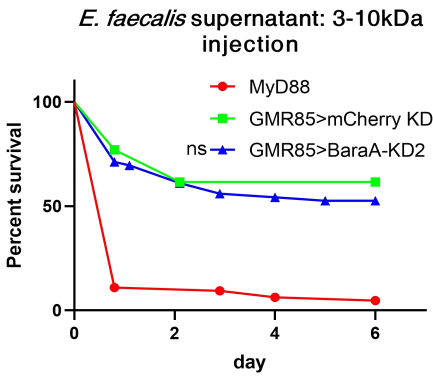
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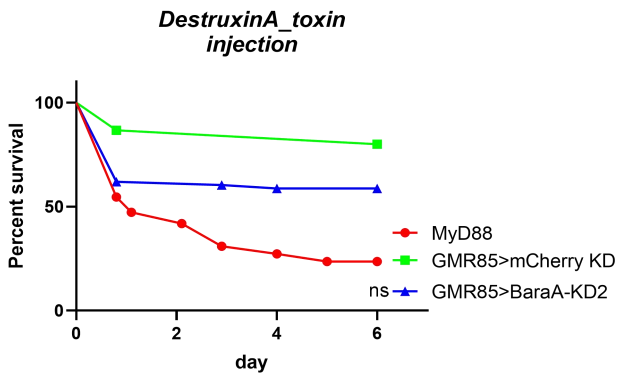
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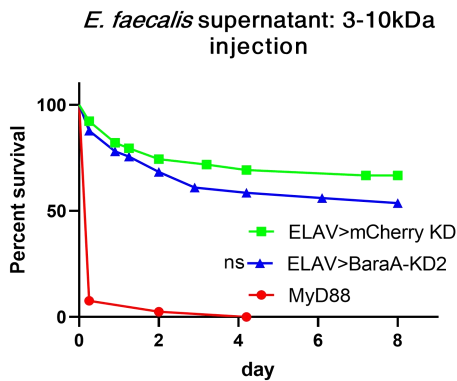
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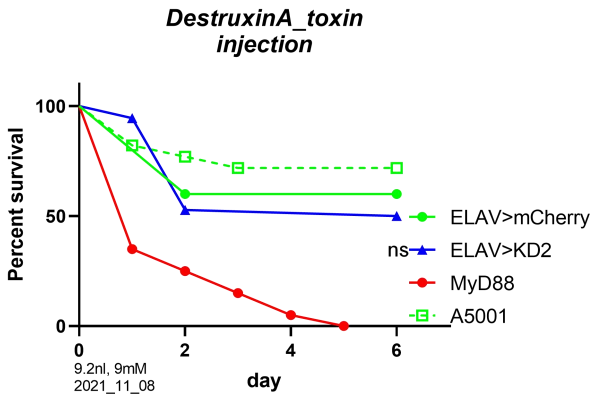
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## Complementary Fig. 6

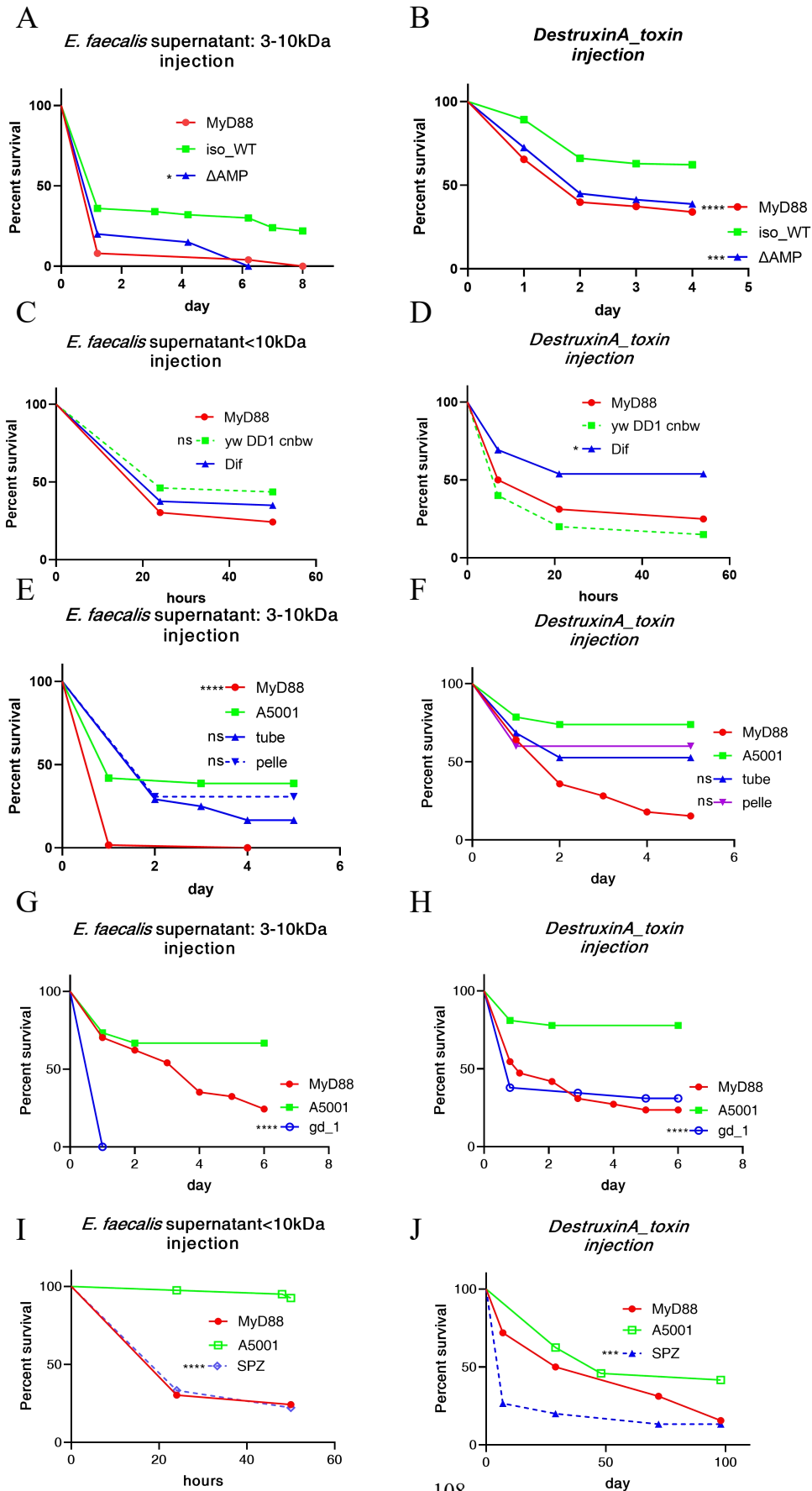
S

UAS-Gal4	expression pattern	<i>E. faecalis</i> supernatant	DestruxinA (toxin)
hml	Hemocytes	3/4	2/2
yolk	Fat body	3/5	2/6@
repo	All glia (CNS and peripheral)	2/3	4/4
GMR77A03	Cortex glia	2/3	2/3
NP6293	Perineurial glia	2/2	3/3
gliotactin	Subperineurial glia and very few neurons	0/3	0/3
GMR85G01	Perineurial glia	0/3	0/3
BL50472	Subperineurial glia	1/3	2/3
moody	Subperineurial glia	1/4	2/3
ELAV	Neurons	0/4	1/4

### Complementary Figure 6. *BaraA* expresses in the hemolymph, fat body, and nervous system are required to defense against toxins

*BaraA* KD flies were crossed to different drivers. 23nl o *E. faecalis* supernatant and 4.6nl of 8mM Destruxin A was injected respectively. \*  $p < 0.05$ ; \*\*  $p < 0.01$ , \*\*\*  $p < 0.005$ , \*\*\*\*  $p < 0.0001$ . At least three independent experiments have been performed and yielded the same results. (F) Pooled data from six independent experiments. (S) Table of expression patterns of different Gal4 lines. Times of sensitivity phenotypes/ total experiments for which a statistically significant difference was measured. @ Only 2/6 INDIVIDUAL experiments displayed a statistically significant difference. However, a trend was observable in the four other experiments, which was confirmed by pooling the data (shown in 6F).

# Complementary Fig. 7



**Complementary Figure 7. Non-canonical pathway is involved in counteracting the toxins.**

**(A, B)**  $\Delta AMP$  represents seven AMPs have been deleted, which is sensitive to *E. faecalis* supernatant injection. **(B)** Pooled data of four independent experiments. One out of four showed sensitivity phenotype, while the other three times displayed a trend of susceptibility phenotype. **(C, D)** *Dif* deficiency flies display susceptible to Destruxin A, while died at the same rate as control flies. Three independent experiments have been performed. **(E, F)** *tube* and *pelle* mutant flies did not show any significant susceptibility to Destruxin A and *E. faecalis* injections. **(G, H)** *Gastrulation defective* flies (*gd\_1*) displayed sensitivity phenotypes to Destruxin A and *E. faecalis* infections. **(I, J)** *spz* mutants showed susceptibility phenotypes to Destruxin A and *E. faecalis* infections. \*  $p < 0.05$ , \*\*\*  $p < 0.005$ , \*\*\*\*  $p < 0.0001$ . Three independent experiments have been performed.





# **Chapter II\_ Investigating the function of the short Gram-Negative Binding Protein like 3 in *Drosophila* innate immune response against *Candida glabrata***

## **Foreword**

The present chapter is a preliminary version for a scientific article. The present data were obtained by Jessica Quintin during her Ph. D thesis and myself. A future version will include further contributions from Alain Roussel's group on the structure from GGBP-like3 gained from X-ray diffraction experiments and from Vishu Kumar Aimanianda on the binding properties to  $\beta$ -(1-3) glucans of GGBP-like3 as compared to GGBP3.

## **Introduction**

*Drosophila melanogaster* is extensively used as a powerful organism model for studying the interaction between pathogenic microorganisms and the host defense and to reveal their effects on the host. *Drosophila* relies on humoral and cellular responses to fight against invading pathogens [19]. One aspect of host defense restricted mostly to protostomes is melanization. It is mediated by prophenoloxidasases and leads to the deposition of melanin around the wound and also fights against bacterial and fungal infections through a killing activity that may be distinct from the melanized plug at the injury site [70]. Additionally, phagocytosis functions through hemocytes engulfing invading microorganisms. A major arm of the humoral defense is the Toll pathway, which provides a systemic response to Gram-positive bacterial and fungal infections by generating antimicrobial peptides (AMPs) secreted into the hemolymph by the fat body [234].

*Drosophila* host defense against fungi relies on directly recognizing  $\beta$ -(1,3)-glucans via the GGBP3 pattern recognition receptor (PRR).  $\beta$ -(1,3)-glucans represent a major constituent of the cell wall of fungi. Their detection through GGBP3 activates proteolytic cascades that lead on the one hand to the activation of the melanization response and on the other hand to the cleavage of the Spätzle cytokine and thus to the subsequent activation of the Toll pathway, resulting ultimately in AMPs production [70, 117]. Complementing the PRR activation branch of the Toll pathway, the Persephone (PSH)

protease can be activated by virulence factors, such as the *M. anisopliae* protease PR1, which are secreted by entomopathogenic fungi or many pathogens [66-68].

GNBPs comprise two main domains: a N-Terminal  $\beta$ -(1,3)-glucan binding domain and a C-terminal domain, which shares sequence homology with bacterial  $\beta$ -glucanases, although the catalytic residues have not been conserved. Therefore, the recognition of sugars may be the sole function of the proteins that has been selected during evolution. The GGBP family is composed of three canonical members and three shorter proteins called GGBP-like, which appear to be *Drosophila* specific. GGBP-like genes code only for the  $\beta$ -(1,3)-glucan binding domain.

Levy et al. had performed a proteomic analysis of induced proteins in the hemolymph of *Drosophila* by Gram-negative bacteria (*E. coli*), Gram-positive bacteria (*M. luteus*), and fungi (*Beauveria bassiana* in a “natural” infection model in which the fungus invades the host through the cuticle). They had shown that GGBP-like 3 (CG13422) is strongly induced (72 fold) in the hemolymph of *Drosophila*, apparently only after *B. bassiana* [118]. This high induction factor together with the expected  $\beta$ -(1-3) glucan-binding properties suggested that GGBP-like 3 could act as a specific antifungal factor. To date, the AMPs in *Drosophila*, such as Drosomycin and Metchnikowin are considered to be active on filamentous fungi. However, no AMP defense against yeasts have been found so far, except Cecropins that can act against the *Saccharomyces cerevisiae* yeast *in vitro* [126, 235]. Of note, the *in vivo* activity of Cecropins against monomorphic pathogenic yeasts such as *Candida glabrata*. In addition to being induced at the protein level by *B. bassiana* natural infection, oligonucleotide arrays of mRNA expression revealed that GGBP-like 3 is also up-regulated after septic injury by the combination of *E. coli* and *M. luteus* [131]. A recent study reported that GGBP-like 3 may also regulate long-term memory in *Drosophila* and displays Gram-negative bacteria killing activity *in vitro* [119].

## Results

### Expression pattern of GGBP-like 3 protein

Most “long” *GGBP* genes encode more than 400aa, while GGBP-like 3 codes for 152aa. GGBP-like 3 protein is closely related to GGBP3, only encoding the N-terminal glucan-binding domain (Fig.1A). *Drosophila* GGBP-like proteins are phylogenetically closer to

GNBP3 than the two others canonical GNBP, which are highly divergent, suggesting that they might have a similar conserved function in dealing with fungi based on their expected ability to bind to their cell wall (Fig. S1A). Protein identification of 2D gel spots had shown that GNBP-like 3 is strongly induced (72-fold) in the hemolymph of *Drosophila* after *B. bassiana* infection, but not Gram-positive or -negative bacteria six hours after a challenge [118]. This high induction factor suggested that GNBP-like 3 could act as a specific antifungal factor. In addition, *GNBP-like 3* expression is also induced by a septic injury with a mixture of *E. coli* and *M. luteus* [131]. These data were confirmed and extended by performing Western blots with hemolymph from adult flies challenged with fungus or bacteria using a specific antibody.

A strong induction of GNBP-like 3 protein was observed two days post infection and the protein was still strongly expressed in the hemolymph for at least four days following natural infection with the entomopathogenic fungi *B. bassiana* (Fig. 1B). GNBP-like 3 was induced in the hemolymph starting from six hours after a *M. luteus* or *E. coli* septic injury challenge and remained at a high level for at least 24 hours (Fig. 1C). In *spz* mutants, the expression of GNBP-like 3 was totally blocked after a Gram-positive bacterial challenge, while a reduced amount of GNBP-like 3 protein in the hemolymph was detected in mutants of the Toll pathway transcription factor DIF after a *B. bassiana* challenge. Similarly, after a challenge only with the Gram-negative bacterium *E. coli*, in mutants of IMD pathway, *imd* or *key* mutants, the protein expression of GNBP-like 3 was entirely blocked (Fig. 1C, Fig. S1B). These results indicate that GNBP-like 3 protein expression pattern is IMD pathway dependent as well as Spätzle-dependent according to the elicitor of the systemic immune response.

### **Expression of *GNBP-like 3* mRNA steady-state transcripts**

We monitored the expression levels of *GNBP-like3* transcripts by RTqPCR after several types of immune challenges. In keeping with previously published microarray analysis results [131], we found that *GNBP-like3* was induced by *B. bassiana* natural infection and septic injuries with needles dipped into *M. luteus*, *E. coli*, *C. albicans* or *C. glabrata* (Fig. 2A). This induction was blocked in *spz* mutants challenged by a Gram-positive mutant or by *Candida* whereas its induction by *E. coli* was abolished in *key*

mutants. Thus, the induction of GGBP-like3 results likely from the induction of the transcripts of its gene.

To further decipher how *GGBP-like3* expression is induced by *C. glabrata*, we tested several mutants of the Toll pathway. Like that of *Drosomycin*, the induction of *GGBP-like3* was severely hampered in *spz*, *MyD88* and *Dif*. Unexpectedly, its induction was somewhat reduced in *psh* mutants and possibly not in *GGBP3* mutants. It was however abolished in *psh-GGBP3* double mutants (Fig. 2B).

The Toll pathway can be ectopically activated in the absence of infection by either the expression of a constitutively-active allele of *Toll* (*Toll<sup>10B</sup>*) or by the overexpression of either *GGBP3*, *GGBP1/PGRP-SA*, or *psh* [64, 66]. Both the gene and its protein products were induced in the absence of an immune challenge (Fig. S2).

Taken together, these results indicate that the Toll pathway regulates GGBP-like 3 expression upon challenge with Gram-positive bacteria or fungi.

### **GGBP-like 3 does not act as an opsonin**

In collaboration with the laboratory of Alain Roussel in Marseilles, we succeeded to obtain the crystal structure of GGBP-like 3. It revealed that the structure of GGBP-like 3 is closely related to that of the N-terminal domain of GGBP3 [65]. Moreover, GGBP-like 3 recombinant protein, obtained in *E. coli*, had similar binding properties to yeasts as the recombinant GGBP3 N-terminal protein. Indeed, GGBP-like 3 binds specifically to paraformaldehyde (PFA)-treated yeasts and not to Gram-positive or -negative bacteria (Fig. S3A). Since GGBP-like 3 binds to dead *C. albicans* and *C. glabrata in vitro*, this protein may function as an opsonin. However, hemocytes from *GGBP-like 3* loss-of-function flies (G13-KO, see below) engulfed *C. glabrata* at a similar rate as wild type control hemocytes (Fig. S3B). These results suggest that GGBP-like 3 likely does not function as an opsonin since it is not required for the phagocytosis of *C. glabrata* live yeasts.

### **Overexpression of GGBP-like 3 may inhibit the function of melanization**

In addition to phagocytosis, we next ask whether GGBP-like 3 plays a role in melanization. A septic injury triggers several proteolytic cascades, one of which leads to the deposition of melanin at the cuticular wound site [154]. The melanization processes

are catalyzed by the phenoloxidase (PO) enzyme, which becomes active after proteolytic cleavage of its Pro-domain. GGBP3 and the Toll pathway are required for triggering the prophenol-oxydase (PPO) cascade following bacterial challenge [81]. Therefore, we tested the possible role of GGBP-like 3 in PPO cleavage. To address this, we firstly collected the cell free hemolymph from *GGBP-like 3* KO mutant after *C. glabrata* challenge. Compared to wild type flies, the efficiency of PPO cleavage ratio in *GGBP-like 3* KO mutant flies did not show significant difference (Fig. S4A-B). Similar results were obtained upon a *M. luteus* challenge (Fig. S4C-D).

In contrast, the overexpression of GGBP-like 3 in flies through the *UAS-GGBP-like 3* transgenic construct inhibited PPO cleavage induced by a challenge with *M. luteus*, as compared to wild-type flies (FigS4E-F). This inhibition mimicked the inhibition obtained by overexpressing Serpin27A, a negative regulator of the PPO activation cascade [81] (Fig. S4F). However, in contrast to Serpin27 A, GGBP-like 3 is hardly detectable in the hemolymph of unchallenged adult *Drosophila*. Moreover, loss-of-function of *GGBP-like 3* through a RNAi construct driven by hspGal4 did not lead to constitutive PPO cleavage nor extend the kinetics of cleavage (Fig. S4E; see Fig. S5 for the validation of the RNAi construct). This suggests that GGBP-like 3 might play a role in a negative feedback loop affecting PPO cleavage after an elicitation of the Toll pathway by septic injury.

### **The *GGBP-like 3* KO mutant is susceptible to *Candida glabrata* infection**

As we failed to detect an enhanced sensitivity phenotype to a variety of immune challenge in ubiquitously silenced *GGBP-like 3* flies except for an inconstant susceptibility to *C. glabrata*, we generated a null CRISPR-Cas9 mutant allele in which no *GGBP-like 3* transcripts were detected by RTqPCR, possibly as a result of nonsense-mediated RNA decay (Fig. S6A-B). The null mutants were viable; however, males displayed a reproducibly slightly-impaired fitness (Fig. S6C).

We next injected *Candida glabrata* into *GGBP-like 3* KO mutant flies and monitored their survival. The *GGBP-like 3* KO mutant flies displayed an intermediate susceptibility phenotype to *C. glabrata* injection, which was rescued by the ubiquitous expression of the *UAS-GGBP-like 3* transgene (Fig. 3A). We confirmed the RNAi data as regards a lack of susceptibility to other infections (Fig. S7). Of note, we did detect an enhanced sensitivity of nonisogenized *GGBP-like 3* KO mutant flies to injected *M. robertsii*; this

phenotype was however lost after isogenization even though we performed the experiment four independent times.

To understand the function of GGBP-like 3 in the host defense against *C. glabrata*, we then monitored the fungal burden of the flies after the inoculation from day1 to day3. We detected significantly increased fungal loads at the day1 and day2 time points in the mutant as compared to wild type flies (Fig. 3B). The distribution at day 3 is bimodal: flies with a higher burden are likely fated to die whereas those with a load similar to that of wild-type flies (Set Point Fungal Load: SPFL) will remain alive [181]. Additionally, we also measured the fungal load upon death (FLUD). Unexpectedly, we obtained increased FLUD in *GGBP-like 3* and *MyD88* mutant flies as compared to that of wild type flies (Fig. 3C).

A possibility to account for an increased fungal burden in the *GGBP-like 3* mutants would be that GGBP-like 3 is required for Toll pathway activation by *C. glabrata* like GGBP3, leading to a decreased immune response to this opportunistic pathogen. Fig. 3D indicates that it may not be the case. Note however that the induction of *Drosomycin* was more variable.

In conclusion, the *GGBP-like 3* KO mutant flies succumbed to *C. glabrata* infection due to the proliferation of the fungus, suggesting that GGBP-like 3 plays a resistance role against this fungal infection.

### **The susceptibility phenotype to *C. glabrata* can be rescued by GGBP-like 3 recombinant protein**

The genetic overexpression of *GGBP-like 3* in *GGBP-like 3* KO background rescued the *C. glabrata* sensitivity phenotype. We next wondered whether an injected recombinant protein can protect the mutant flies from *C. glabrata* challenge. We co-injected a His-tagged recombinant GGBP-like 3 protein and the yeast into flies. The *GGBP-like 3* KO mutant flies with protein survived better than the flies without protein injection (Fig. 4A). The *MyD88* *C. glabrata* susceptibility phenotype was mildly yet significantly improved by the injection of the GGBP-like 3 recombinant protein. To figure out whether the recombinant protein can help the host to eliminate the *C. glabrata*, we monitored the fungal burden at day3 according to the LD50 in survival curve. We observed a

significantly decreased fungal burden in *GNBP-like 3* KO mutant flies after co-injection of the protein and yeast as compared to the mutant flies injected only with yeast (Fig. 4B). We next incubated the GNBP-like 3 protein with *C. glabrata* *in vitro* and observed a dose-dependent inhibition of *C. glabrata* proliferation (Fig. 4C). In contrast, the positive AMP control gomesin killed the yeast within 24 hours.

Taken together, these data suggest that GNBP-like 3 may act as a fungistatic peptide.

### **GNBP-like 3 may act independently from Bomanins in the host defense against *Candida glabrata***

We found that the recombinant protein binds to *C. albicans* *in vitro*, likely at the only site where  $\beta$ -glucans are exposed (budding scar) (Fig. S3A) [65, 236]. This observation opens the possibility that GNBP-like 3 through its  $\beta$ -(1-3)-glucan binding properties may address an attack complex to this potentially weak site of *C. glabrata*.

No *Drosophila* AMP with an activity against *C. glabrata* *in vitro* has been identified so far. As regards Toll pathway effectors, a family of 12 secreted peptides known as Bomanins appear to be required in the host defense against *C. glabrata*. Indeed, 10 *Bomanin* genes are clustered at the 55C locus and the deletion of the locus leads to a high sensitivity to this challenge [132]. Interestingly, it has been reported that the *Bom<sup>455C</sup>* phenotype can be rescued by the genetic overexpression of Bomanins encoding mostly the conserved Bomanin domain that defines the family and known as BomS, especially BomS3 [133]. However, no *in vitro* activity of BomS peptides against *C. glabrata* could ever be detected with synthetic BomS peptides (Philippe Bulet, personal communication) [133]. The collected hemolymph can be fungicidal, in a BomS-dependent manner [133]. Thus, it is likely that BomS peptides act in conjunction with another Toll-regulated effector.

To test the hypothesis of the formation of an attack complex between BomS peptides and GNBP-like 3, we constructed a *Bom<sup>455C</sup>; GNBP-like 3* KO recombinant double mutant. With the infection of *Candida glabrata*, the double mutant died faster than *GNBP-like 3* KO single mutant; it however died at the same rate as *Bom<sup>455C</sup>* single mutant, which are almost as sensitive as *MyD88* mutants (Fig. 4D). Thus, the high sensitivity of *Bom<sup>455C</sup>* precludes us from detecting a potential synergy between BomS and GNBP-like 3. Whereas the injection of GNBP-like 3 recombinant protein did rescue the *GNBP-like 3* *C.*



*glabrata* susceptibility phenotype, it could not rescue the *Bom*<sup>A55C</sup>, the *MyD88* nor the *GNBP-like 3*; *Bom*<sup>A55C</sup> phenotypes (Fig. 4E). Unexpectedly, the double-mutant *GNBP-like 3*; *Bom*<sup>A55C</sup> was partially rescued, albeit weakly, by the injection of GNBP-like3 recombinant protein together with synthetic BomS3 whereas the rescue was full with respect to *GNBP-like 3*, as expected (Fig. 4F). In contrast, synthetic BomS3 peptide could not rescue the *Bom*<sup>A55C</sup>, the *GNBP-like 3* mutants, nor the double-mutant (Fig. 4G). Thus, synthetic BomS3 is sufficient to allow a rescue of the double mutant by recombinant GNBP-like 3 whereas the combination of the two peptides is not able to rescue the *Bom*<sup>A55C</sup> mutant. Taken together, these data suggest that the rescuing function of recombinant GNBP-like3 may marginally function together with BomS3, or possibly a BomS peptide. The comparison of the microbial burden in single *vs.* double-mutant did not reveal any additivity of the two mutations as the same global burden was measured in all three conditions (Fig. 4H). Of note, these data establish for the first time that the 55C locus may be involved overall in the resistance against *C. glabrata* infection, although more experiments would be required to determine whether the bimodal distribution observed in the two independent experiments is reproducible.

We also tested *in vitro* a putative interaction between recombinant GNBP-like3 and synthetic BomS3 using a checkerboard method in which we vary the concentration of recombinant GNBP-like 3 and of synthetic BomS3 and then monitor the growth of *C. glabrata*. In one experiment (Fig. S8A), there was some evidence for an interaction between the two peptides in the high concentration range. Of note, no action of BomS3 on its own was detected. These promising results were however not confirmed in a second experiment (Fig. S8B) and further experiments are definitely required to assess a potential additive or synergistic effects *in vitro* of these two Toll pathway effectors.

## Discussion

In this study, we document a strong induction of GNBP-like 3 in the hemolymph upon a variety of immune challenges and not solely fungal ones as previously reported [118]. The protein expression mirrors the induction of transcripts by the Toll and IMD pathways. Unexpectedly, GNBP-like 3 ectopic expression inhibits somewhat the activation of the

melanization cascade. Our data taken together indicate a role for GGBP-like 3 in resistance against *C. glabrata* and not the other tested pathogens.

The use of Western blot analysis allowed us to test multiple infections and time points that would have been too difficult to investigate through proteomics analysis at the time. Thus, the induction by bacteria was missed in the original study. From our phenotypic analysis, it remains unclear why *GGBP-like 3* is also regulated by the IMD pathway, as we did not detect a susceptibility phenotype with *Ecc15*. While it can be argued that we have tested only one Gram-negative bacterial species, the fact that GGBP-like 3 binds specifically to  $\beta$ -glucans suggests this finding may be generalized since this structural compound is not synthesized by this class of bacteria. An alternative possibility would be that the indirect inhibitory role that GGBP-like 3 plays on melanization in a feedback loop might be physiologically relevant to the host to limit potential damages elicited by PO activation. This however was not apparent in survival studies with several pathogens. We found that *GGBP-like 3* mutants are susceptible to only *C. glabrata* among the tested pathogens. It is not certain that *Drosophila* encounters this yeast frequently in its original natural environment as it is mostly a human commensal and also found in pet birds [237]. We note however that *Drosophila* has become a likely human commensal for a million years as both flies and humans are able to withstand moderate doses of ethanol. It remains an open possibility that GGBP-like 3 may participate in the host defense against dimorphic fungi that present a yeast-form in the hemolymph, as is the case for *M. robertsii* in a septic injury model. Indeed, we did find a sensitivity to this pathogen prior to isogenization of the *GGBP-like 3* KO line.

The monitoring of the *C. glabrata* titer during infection revealed a function in resistance against the fungus. Interestingly, a fungistatic activity was detected *in vitro* for concentrations in the 25-50  $\mu$ M range. It is not known whether these concentrations can be reached *in vivo*, which is the case for several *Drosophila* AMPs. While its concentration is likely high, its high molecular weight would represent a much higher metabolic Toll than the synthesis of shorter AMPs. It would thus be valuable to estimate the concentration from collected hemolymph by comparing it to a standard curve made with the recombinant protein.

The FLUD data should allow us to infer whether GGBP-like 3 is also involved in the resilience against *C. glabrata* [181]. However, the FLUD concept may not be appropriate

for pathogens that are unable to kill wild-type flies. It is likely that the few wild-type flies that succumb to this challenge are not killed by the proliferation of the fungus but rather are a consequence of the wound [222]. It would nevertheless be interesting to test whether *GNBP-like 3* mutants are sensitive to injected microbial toxins, although few secreted virulence factors are known for *C. glabrata*. In addition, its concentrated supernatant did not kill *MyD88* mutants. Nevertheless, *C. glabrata* has been reported to be able to cross the blood brain barrier [238] and might interact with the host through unidentified virulence factors at this level.

As mentioned earlier, no AMP with a documented activity against *C. glabrata* has been identified so far. The finding that *Bom<sup>455C</sup>* deletion mutant is almost as susceptible as *MyD88* to this pathogenic yeast opened the possibility that some *Bomanin* genes might encode effectors active against *C. glabrata*. Our data on the fungal burden of *Bom<sup>455C</sup>* mutants supports a potential role in resistance, as is the case for *GNBP-like 3*. Lindsay *et al.* have investigated in some detail the role of 55C Bomanins and found that the susceptibility phenotype of *Bom<sup>455C</sup>* mutants can be rescued by the strong overexpression of short Bomanins [133]. They proposed that the important parameter is the quantity of the short Bomanin and not so much its identity, in keeping with a strong sequence similarity of the short Bomanins that basically are formed only by the conserved Bomanin domain [133]. Interestingly, as was the case for experiment performed in Strasbourg 20 years ago, they failed to detect an antifungal action of short Bomanins *in vitro*. However, collected hemolymph displayed a candidicidal activity that was dependent on the 55C *Bomanin* locus. Thus, it is likely that other effectors regulated by the Toll pathway are acting in concert with short Bomanins to kill *C. glabrata*. We therefore tested whether *GNBP-like 3* might be the missing link. First, the *GNBP-like 3* susceptibility phenotype is much weaker than that of *Bom<sup>455C</sup>* mutant, making it unlikely that *GNBP-like 3* would be an obligate partner. Our genetic interaction data were not resolute enough because of the strength of the *Bom<sup>455C</sup>* phenotype alone in terms of survival, which precludes the reliable observation of a stronger phenotype in the double mutant. Intriguingly, the fungal burden in *GNBP-like 3* mutants was in the same range as that of *MyD88* or *Bom<sup>455C</sup>* mutants. Thus, one may wonder whether 55C Bomanins may not have an additional function in host defense besides resistance. Unfortunately, FLUD data do not appear to be that informative in the case of *C. glabrata* infections and a more

detailed analysis, especially as regards a potential targeting of the brain by the pathogen needs to be implemented. One should therefore keep in mind that the difference in phenotypic strength between *GNBP-like 3* and *Bom<sup>455C</sup>* mutants may actually result from an additional function from Bomanins in host defense, leaving open a possible direct interaction between short Bomanins and *GNBP-like 3*. Thus, further genetic and biochemistry approaches are warranted. It would be highly useful to dissect the 55C locus and to generate mutants for each single *Bomanin* gene and possibly generate compound mutants deleting several Bomanins, possibly by focusing on the four distal genes of the locus, which together can rescue the *Bom<sup>455C</sup>* mutant phenotype [133]. Should we confirm an interaction *in vitro*, then it would be worth testing physically a direct interaction, for instance using Microscale Thermophoresis.

## Materials and Methods

### Fly strains

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.

*w<sup>45001</sup>* flies were served as wild type controls. The positive controls for infection assays for Gram-positive/fungal infections and Gram-negative infections were respectively *MyD88* and *key* in the *w<sup>45001</sup>* background. *GNBP-like 3* KO mutant flies were isogenized in the *w<sup>45001</sup>* background.

*GNBP3<sup>hades</sup>*, *psh<sup>4</sup>-GNBP3<sup>hades</sup>*, *psh<sup>4</sup>*, *GNBP<sup>1osi</sup>*, *Dif<sup>l</sup>*, *spz*, *MyD88*, *key<sup>l</sup>*, *UASGNBP1-PGRP-SA*, *UAS-psh*, *UAS-GNBP3*, *UAS-serpin 27 A* and *hspGal4* stocks have been described previously [64, 66, 81, 239, 240]. *imd<sup>BWIV9</sup>* flies were generated by Dominique Ferrandon.

Flies were used in this study were females, except lifespan experiments that both females and males were used.

### Generation of CRISPR/Cas9-mediated null mutants

The *GNBP-like 3* (*CG13422*) 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.

### **Pathogen infections**

The bacterial strains used in this study include the Gram-negative bacterium *Erwinia carotovora carotovora 15* (strain *Ecc15*, OD<sub>600</sub>=50) and the Gram-positive strains *Enterococcus faecalis* (*ATCC 19433*) (OD=0.1), *Micrococcus luteus* (OD=200). Yeasts we used in this study, *Candida albicans* (Caf 2.1 strain) and *Candida glabrata* (BG2 strain). Fungi used in this study, *Beauveria bassiana* and *Metarhizium robertsii*. The following media were used to grow the strains: Yeast extract- Peptone-Glucose Broth Agar (YPDA, *C. albicans* and *C. glabrata*), Luria Broth (LB, Gram-positive and -negative bacteria), or Potato Dextrose Agar (PDA, *Beauveria bassiana* and *Metarhizium robertsii*). For natural fungal infections, flies were shaken on a lawn of sporulating *Beauveria bassiana*. They were then put back into vials and processed as described above. Results are expressed as percentage of surviving flies at different time points after infection.

### **Pathogens Load Quantification**

To characterize the dynamics of within-host microbial loads FLUDs, flies were monitored every 30 minutes for newly dead flies. These flies were then individually homogenized with a bead in 100 µl PBS with 0.01% tween20 (PBST). Homogenates were diluted serially in PBST and spread on YPDA plates for incubation at 29°C overnight. Colonies were counted manually. Data were obtained from at least three independent experiments and pooled.

### **Gene Expression Quantitation**

Total RNA was extracted from 5 adult flies collected at different time points after Toll activation with TRIzol (Invitrogen). cDNA was synthesized from 1000 ng total RNA using the SuperScript II Reverse Transcriptase kit (Transgene). Quantitative RT-PCR was performed on an iQ5 cycler (BioRad) using SYBR Green Supermix (Vazyme). Quantification of mRNA levels was calculated relative to levels of the ribosomal protein gene *rpl32*. Primers for GGBP-like 3 sequences are as follow: forward 5'-GCTCCAGCCTGTCCTACG-3' reverse: 5'-AATCCCTTCGGTGAGTTGA-3'.

### **Pull-down binding assays**

Overnight cultures of microorganisms were collected by centrifugation, washed three times with PBS and resuspended in PBS to an OD=1. Microbes were then fixed with 4% paraformaldehyde (PFA) o/n at 4°C and washed with PBS. 1 ml of OD=1 killed microbes was added to 5 µg of purified GGBP-like3 and incubated in 200 µl of binding buffer (10 mM Tris-HCl (pH 7.5), 500 mM NaCl) at room temperature with mild agitation for 1 hr. The solution containing both recombinant protein and microorganisms was centrifuged (14,000g for 5 min), and the pellet was washed three times with 0.5 ml of washing buffer (10 mM Tris (pH 7.5), 500 mM NaCl, 0.02% Tween-20). The proteins bound to microbial cells were detached by adding SDS-PAGE sample buffer and analyzed.

### **Antibody production**

The purified recombinant GGBP-like 3 protein from *E. coli* expression was used to produce polyclonal mouse antisera. The anti-GGBP-like3 antisera were screened for specific staining of GGBP-like 3 and *Drosophila* endogenous GGBP-like 3 by western blot analysis. The specificity of the antibody was assessed by comparing extracts of wild-type flies to those of a UAS-GGBP-like 3 and RNAi-GGBP-like 3 strains. PO antibody has been originally derived against *Anopheles gambiae* PO. The conditions for its use in *Drosophila* are described in Leclerc *et al* [241]. The specificity of anti-PO antibody was confirmed by MALDI-TOF analysis.

### **Western blot**

All hemolymph extracts, containing 10 µg of protein were equilibrated in Laemmli solution and denatured at 95°C for 5 min prior to loading on a 12% or 8% SDS-polyacrylamide gel. Following SDS-PAGE, proteins were blotted to hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham) and proteins loading were controlled by PonceauS staining. Membranes were then blocked for 2h at Room Temperature in 5% fat dry milk (Bio-Rad) in TBS-T (0.1% Tris buffer saline-Tween). Blots were incubated overnight at 4°C with the anti-GNBP-like 3 antibodies or the anti-PO antibodies [241] in 0.5% fat dry milk in TBS-T. After washing with TBS-T, the blots were incubated for 2 h at room temperature with the horseradish peroxidase (HRP)-conjugated donkey anti-mouse secondary antibody (Amersham) or the horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (Amersham). After washing with TBS-T, blots were detected by enhanced chemiluminescence according to the manufacturer's instructions.

### **Synergy checkerboard experiments**

This experiment was performed in the 96 wells plate. Columns 2 to 10 contain 2-fold serial dilutions of BomS3 synthetic peptide, and rows B to H contain 2-fold serial dilutions of GNBP-like 3 recombinant protein. Column 10 contains a serial dilution of GNBPlike 3 recombinant protein alone, while row H contains a serial dilution of BomS3 synthetic peptide alone. About 1000 cells of *C. glabrata* in each well (except blank control), and co-incubated at 29°C with 100 rpm/min shaking. OD was checked with Virioshan machine at the indicated time points with 630nm wavelength.

### **Survival tests**

Survival tests were performed using 20-25 flies per vial in biological triplicates. Adult flies used for survival tests were 5–7-day old. Infected flies were incubated in 25°C (bacterial infections) or 29°C (fungal infections). Statistical analysis were performed with Log-rank test.



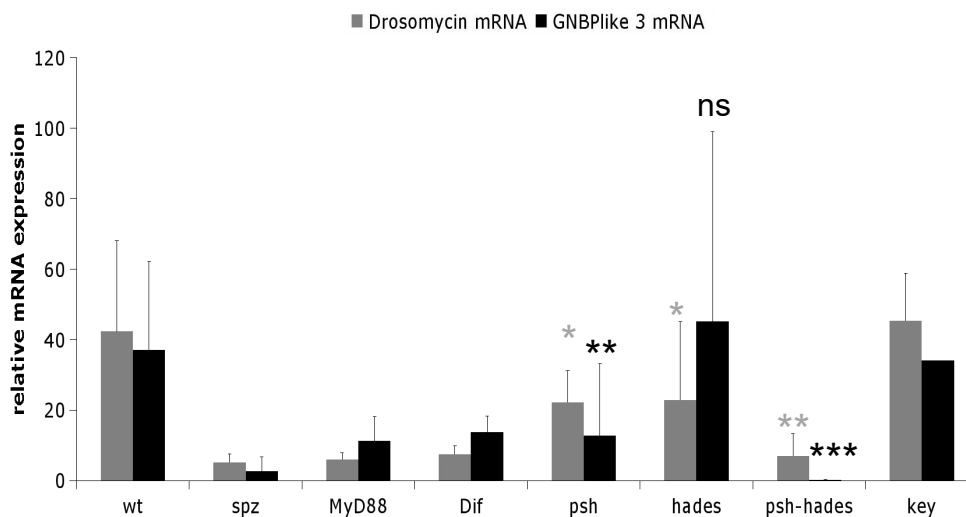


**Fig. 2**

**A**

GNBP-like 3 mRNA	<i>B. b</i> NI 48h	<i>M. l</i> 24h	<i>E. coli</i> 6h	<i>C. a</i> 24h	<i>C. g</i> 24h
wt	+	+	+	+	+
<i>spz</i>	-	-	ND	-	-
<i>key</i>	ND	ND	-	ND	+

**B** *C. glabrata*

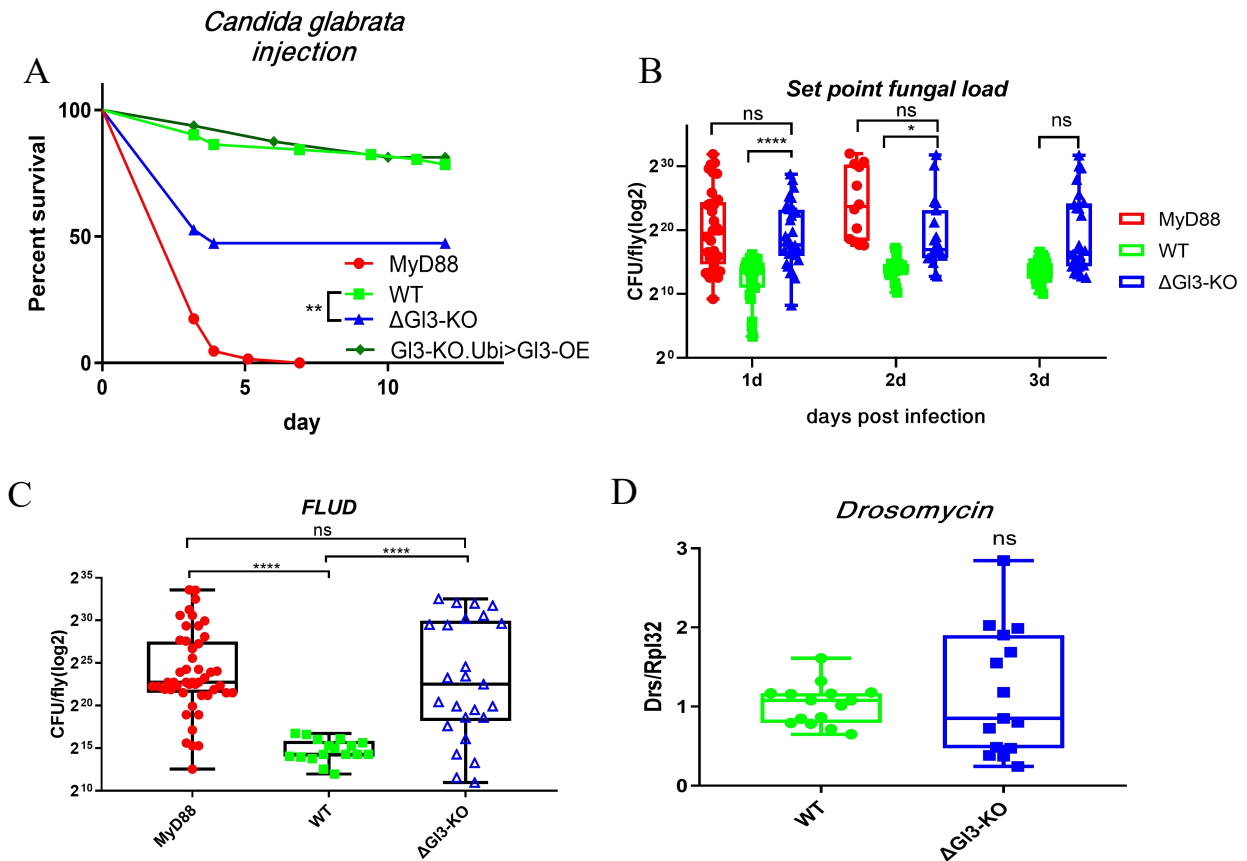


**Figure 2. *Drosomycin* and *GNBP-like 3* mRNA expression after septic injury with *C. glabrata* 24 hours post-infection. (data from Jessica Quintin)**

(A) *GNBP-like 3* mRNA expression summary after Natural infection (NI) with *B. bassiana* or septic injury challenges with bacteria or yeasts. Hours indicate the time post infection. -: not significant ( $p > 0.05$ ); +: significant ( $p < 0.05$ ) for a comparison with corresponding challenge wild-type (wt). ND: Not Determined. (B) Histograms showing the mRNA induction analyzed by qRT-PCR. ns: not significant ( $p > 0.05$ ) for a comparison to the corresponding challenge wild-type (wt), \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . *spz* : spätzle, *psh* : persephone. ND: Not Determined.

*M. l*: *M. luteus*; *B. b* NI: *B. bassiana* Natural infection; *C. g*: *C. glabrata*; *C. a*: *C. albicans*. *osiris*: *GNBP1* mutant; *hades*: *GNBP3* mutant.

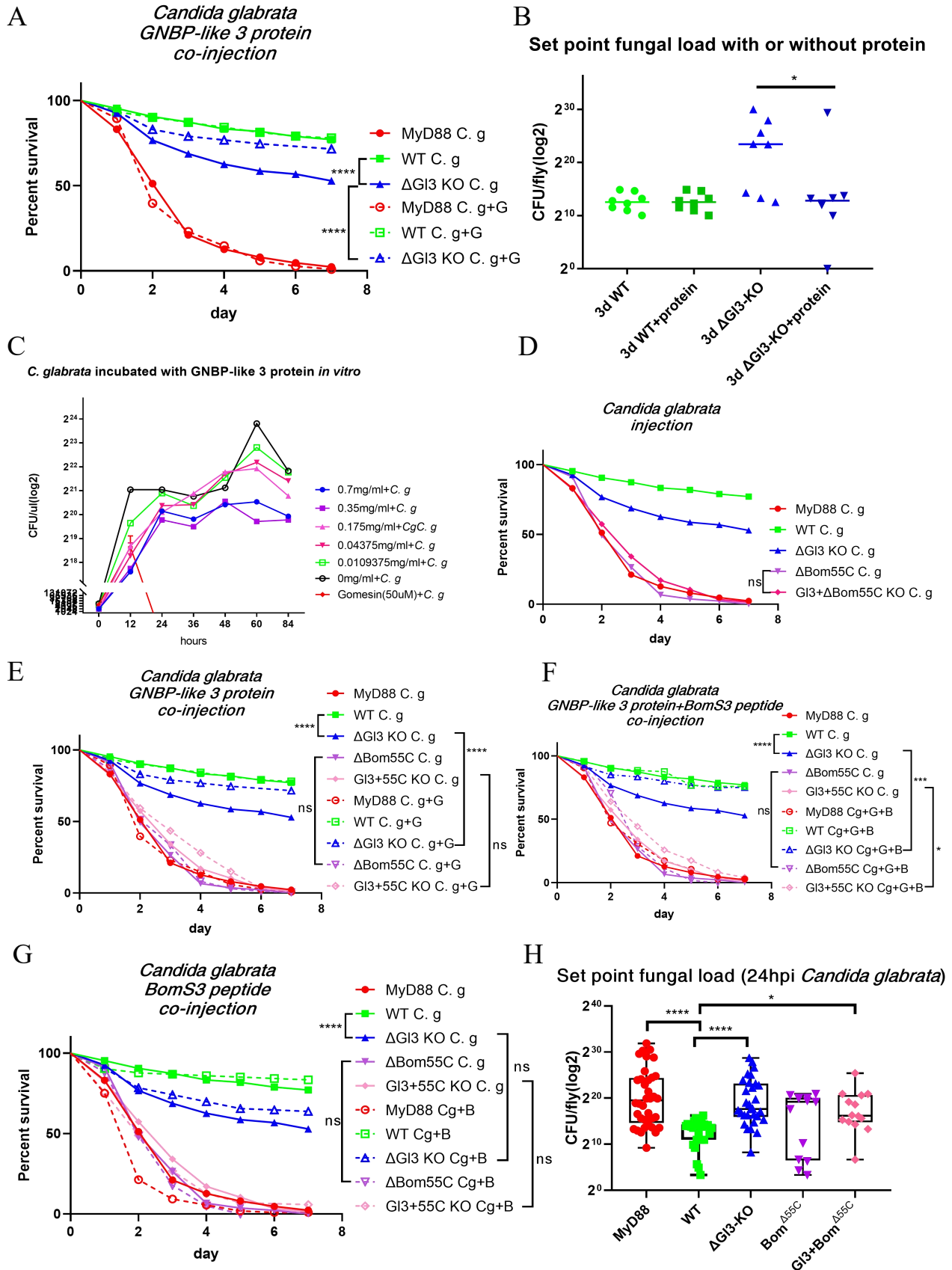
**Fig. 3**



**Figure 3. The *GGBP-like 3* KO mutant is susceptible to *Candida glabrata* infection**

(A)  $10^9$ , 4.6nl of *C. glabrata* was injected. *GGBP-like 3* KO mutant displayed an intermediate phenotype. Overexpressing *GGBP-like 3* in the background of *GGBP-like 3* mutant (driven by Ubi driver) can rescue the sensitive phenotype. Three independent experiments have been performed. (B) Monitored fungal load during infection. After challenge from day 1 to day 2 exhibited increasing load in *GGBP-like 3* KO mutant flies. Pooled data of at least three independent experiments. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ . (C) Quantified the fungal load upon death (FLUD) by individual fly within 30min after their death. Both *MyD88* mutant and *GGBP-like 3* mutant flies showed an increasing burden compared to wild type flies. Pooled data of at least three independent experiments. \*\*\*\*  $p < 0.0001$ . (D) Loss-of-function of *GGBP-like 3* had no effect on Drosomycin induction by *C. glabrata* septic injury. No significant difference compared WT to *GGBP-like 3* mutant flies. Pooled data from five independent experiments.

**Fig. 4**

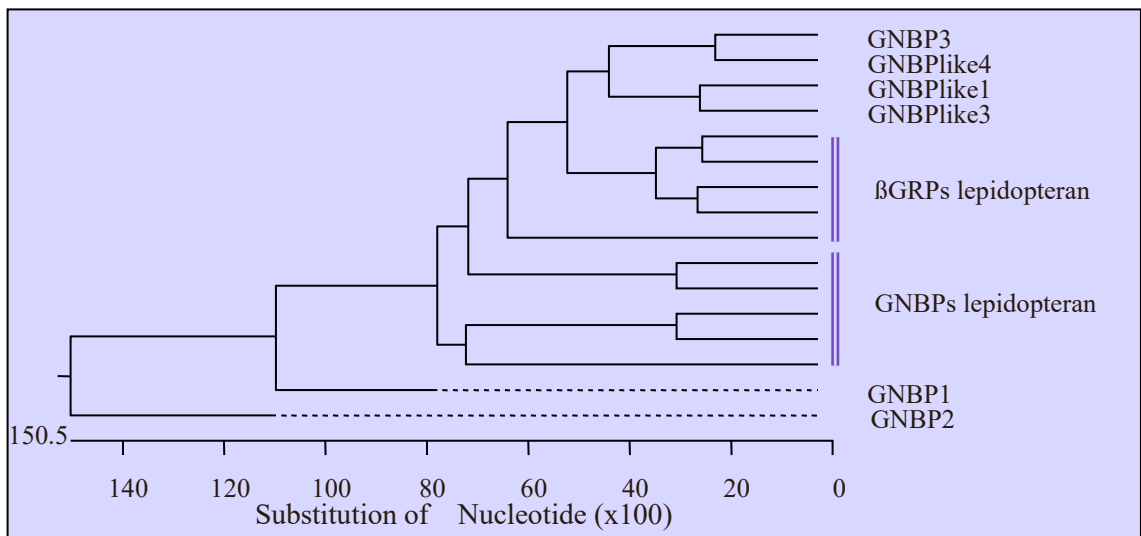


**Figure 4. Sensitivity phenotype upon *C. glabrata* can be rescued by GGBP-like3 recombinant protein**

(A) 0.5mg/ml GGBP-like 3 recombinant protein and  $10^9$  *C. glabrata* were co-injected. Sensitivity phenotype from *GGBP-like 3* mutant can be rescued by the recombinant protein. Pooled data of seven independent experiments. \*\*\*\*  $p < 0.0001$ . (B) Monitored the fungal burden of day 3 in (A). Fungal load decreased in co-injected group of *GGBP-like 3* mutant flies, \*  $p < 0.05$ . Pooled data of two independent experiments. (C) Different concentrations of GGBP-like 3 recombinant protein have been co-incubated with  $2.5 \times 10^6$ /ml *C. glabrata* in 10ul, incubating in microplate with gentle shaking. Gomesin was as a positive control. 0.5ul of the incubated solution was taken to plate to monitor the CFU. When the protein concentration reaches to 0.35mg/ml, GGBP-like 3 recombinant protein shows killing activity to *C. glabrata in vitro*. Three independent experiments have been performed. (D) *Bom<sup>A55C</sup>* mutant flies did not show significance as compared to *Bom<sup>A55C</sup>*; *GGBP-like 3* double mutant upon *C. glabrata* infection. Pooled data of seven independent experiments. (E) GGBP-like 3 recombinant protein can rescue the sensitivity phenotype of *GGBP-like 3* mutant flies upon *C. glabrata* infection. Pooled data of seven independent experiments. \*\*\*\*  $p < 0.0001$ . (F) GGBP-like 3 recombinant protein and BomS3 synthetic peptide together can rescue the sensitivity phenotype of *GGBP-like 3* mutant flies and slightly rescued *Bom<sup>A55C</sup>*; *GGBP-like 3* double mutant upon *C. glabrata* infection. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . (G) 100 $\mu$ M, 4.6nl of BomS3 synthetic peptide was injected. BomS3 peptide could not rescue the sensitivity phenotype of MyD88, *GGBP-like 3* KO nor *Bom<sup>A55C</sup>*; *GGBP-like 3* double mutant. (F) and (G) were relatively experiments. (H) Set point fungal load of mutants after 24 hours post infection upon *C. glabrata* infection. *C. glabrata* burden increased in mutants flies as compared to wild type flies. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ .

**Fig. S1**

**A**



**B**

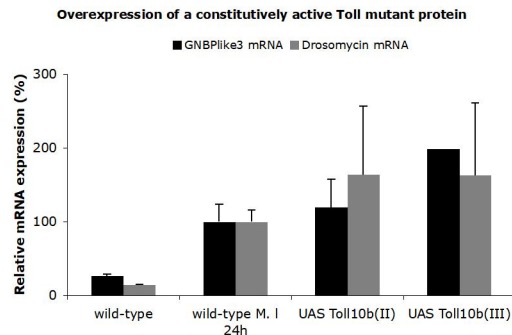
GNBP-like 3 protein	<i>Non challenged</i>	<i>B. bassiana</i>	<i>M. luteus</i>	<i>E. coli</i>
<i>wt</i>	-	+	+	+
<i>spz</i>	-	-	-	-
<i>Dif<sup>1</sup></i>	-	+/-	+/-	+
<i>key<sup>1</sup></i>	-	+	+	-
<i>imd<sup>BWIV9</sup></i>	-	+	+	-

**Figure S1. Phylogenetic tree and activated expression of GNBPs-like 3 (data from Jessica Quintin)**

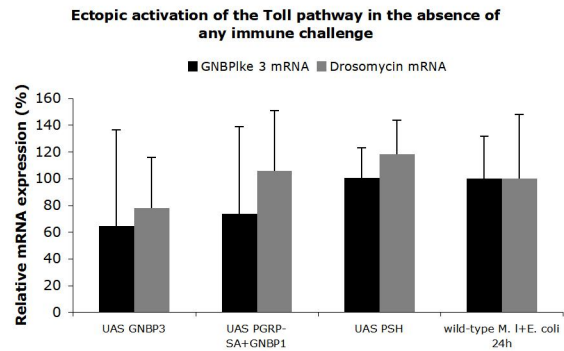
**(A)** Phylogenetic tree of the GNBPs/ βGRPs of *Drosophila* and lepidoptera insects. **(B)** Expression of GNBPs-like 3 in adult *Drosophila* hemolymph analysed by Western Blot. The hemolymph of unchallenged or infected flies was collected. (*M. luteus* 24h post infection, *E. coli* 10 to 16h post-infection and *B. bassiana* 48 and 72h post infections). +/-: reduced amount but not completely absent.

**Fig. S2**

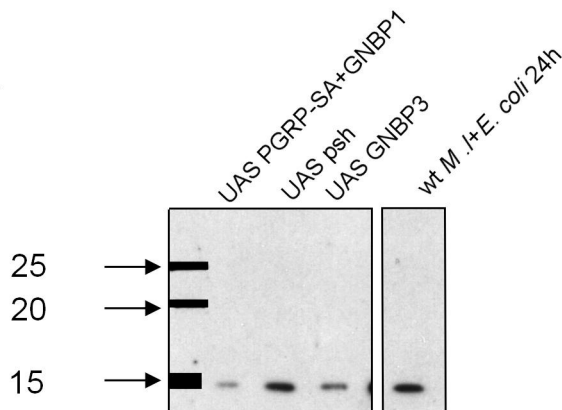
**A**



**B**



**C**

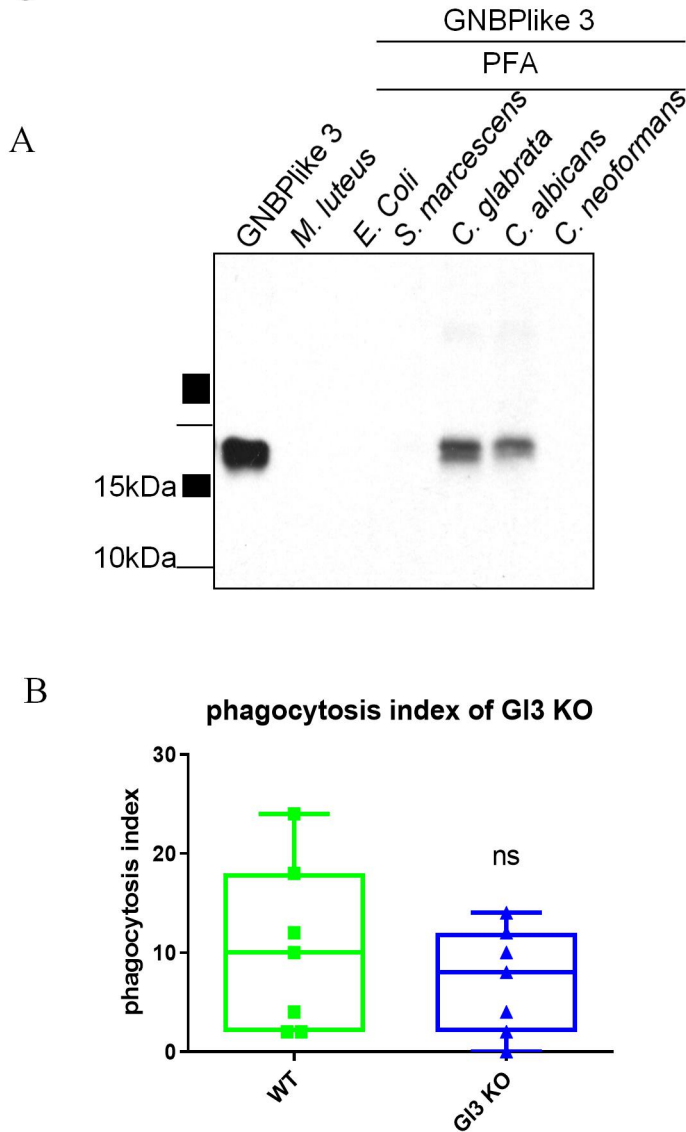


**Figure S2. Ectopic activation of the Toll pathway induces GNBPlike 3 expression (data from Jessica Quintin)**

(A, B) *GNBPlike 3* and *Drosomycin* mRNA expression after artificial activation of the receptor Toll (A) or of the circulating receptors (B) using the ubiquitous driver *hspGal4* and in absence of any challenge. (C) GNBPlike 3 protein detected by western-blot analysis on adult hemolymph. Forced Toll activation induces *GNBPlike 3* mRNA (A) and protein (C) to a level of a *M. luteus* or a mix (*M. luteus* and *E. coli*) infection (differences between expression upon overexpression and challenge are not significant).

*M. l.*: *M. luteus*; UAS Toll10b: UAS construct driven by *hspGal4* which overexpresses a constitutively active form of Toll the receptor; UAS GNB3: UAS construct driven by *hspGal4* which overexpresses a wild-type form of the GNB3 PRR; UAS PSH: UAS construct driven by *hspGal4* which overexpresses a wild-type form of the Persephone protease; UAS PGRP-SA+GNBP1: UAS constructs driven by *hspGal4* which concomitantly overexpress wild-type forms of the GNBP1 and PGRP-SA PRRs.

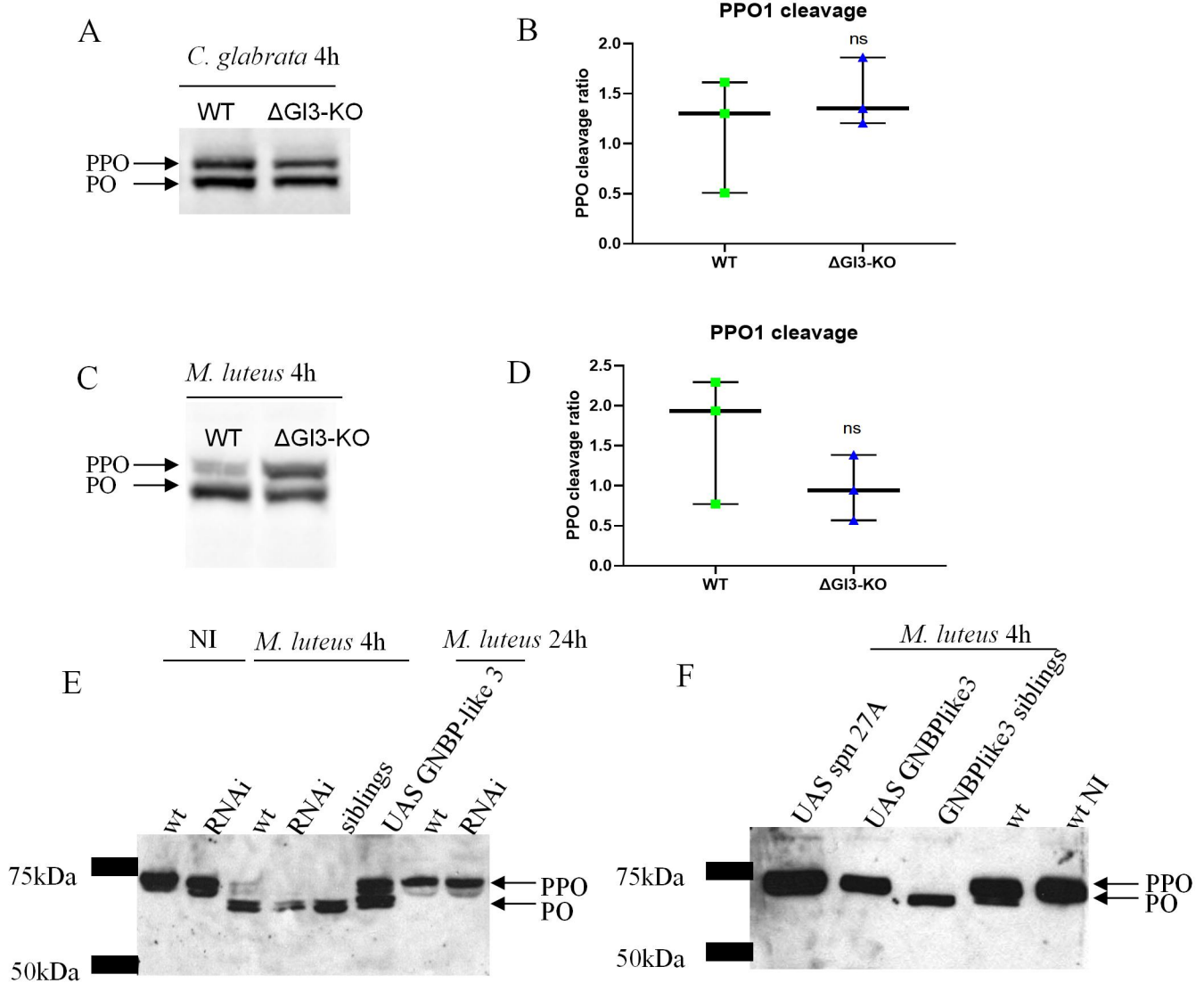
**Fig. S3**



**Figure S3. GGBP-like 3 binds to yeasts, but is not essential to act as an opsonin.**

(A) Pull-down experiments with GGBP-like 3 and different microbes. GGBP-like 3 recombinant protein binds to dead *C. albicans* and *C. glabrata* (PFA killed) in vitro. Interestingly, neither the loss of function nor the overexpression of the protein has an effect on fly survival after *Candida albicans* infection. (data from Jessica Quintin) (B) Injected *C. glabrata* into third instar larvae, with in/ out differential immune staining and one dot represents the phagocytosis index that 50 cells were counted. No significant difference was observed. Pooled data of two independent experiments.

**Fig. S4**

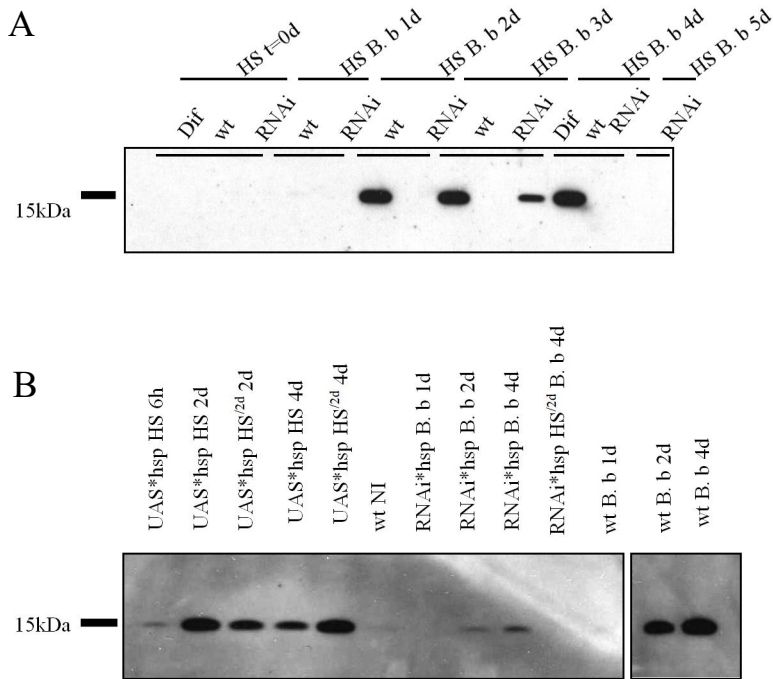


**Figure S4. Loss of function of *GNBP-like 3* does not activate constitutive cleavage of PPO, whereas overexpression of *GNBP-like 3* causes inhibiting cleavage of PPO.**

(A, C) Collected hemolymph from flies after *C. glabrata* (A) or *M. luteus* (C) challenge 4h post infection. Western blot results revealed that no difference between wild type flies (WT) and *GNBP-like 3* mutant flies in the cleavage rate of PPO. Three independent experiments have been performed. (B, D) Grey analysis with ImageJ of (A) or (C). (E) Knock-down of *GNBP-like 3* expression alone has no effect on PPO cleavage neither after *M. luteus* challenge nor on the kinetics of the activation. (F) Overexpression of *GNBP-like 3* inhibits PPO cleavage after an *M. luteus* (*M.l*) challenge. (E, F data from Jessica Quintin).



**Fig. S5**



**Figure S5. Validation of UAS-RNAi GGBP-like 3 (data from Jessica Quintin)**

**(A)** The UAS-RNAi GGBP-like 3 B6R11 construct driven by hspGAL4 completely blocks GGBP-like 3 expression in the hemolymph of adult flies after *B. bassiana* natural infection. Heat shocks (HS) were performed every two days. **(B)** The UAS-GGBP-like3 O17A5(3) construct driven by hspGAL4(UAS\*hsp) induces more GGBP-like 3 expression in the hemolymph of adult flies at 4 days when a Heat shock is performed every two days (UAS\*hsp HS/2d 4d compared to UAS\*hsp HS 4d). No differences are visible 2 days post Heat-shock.

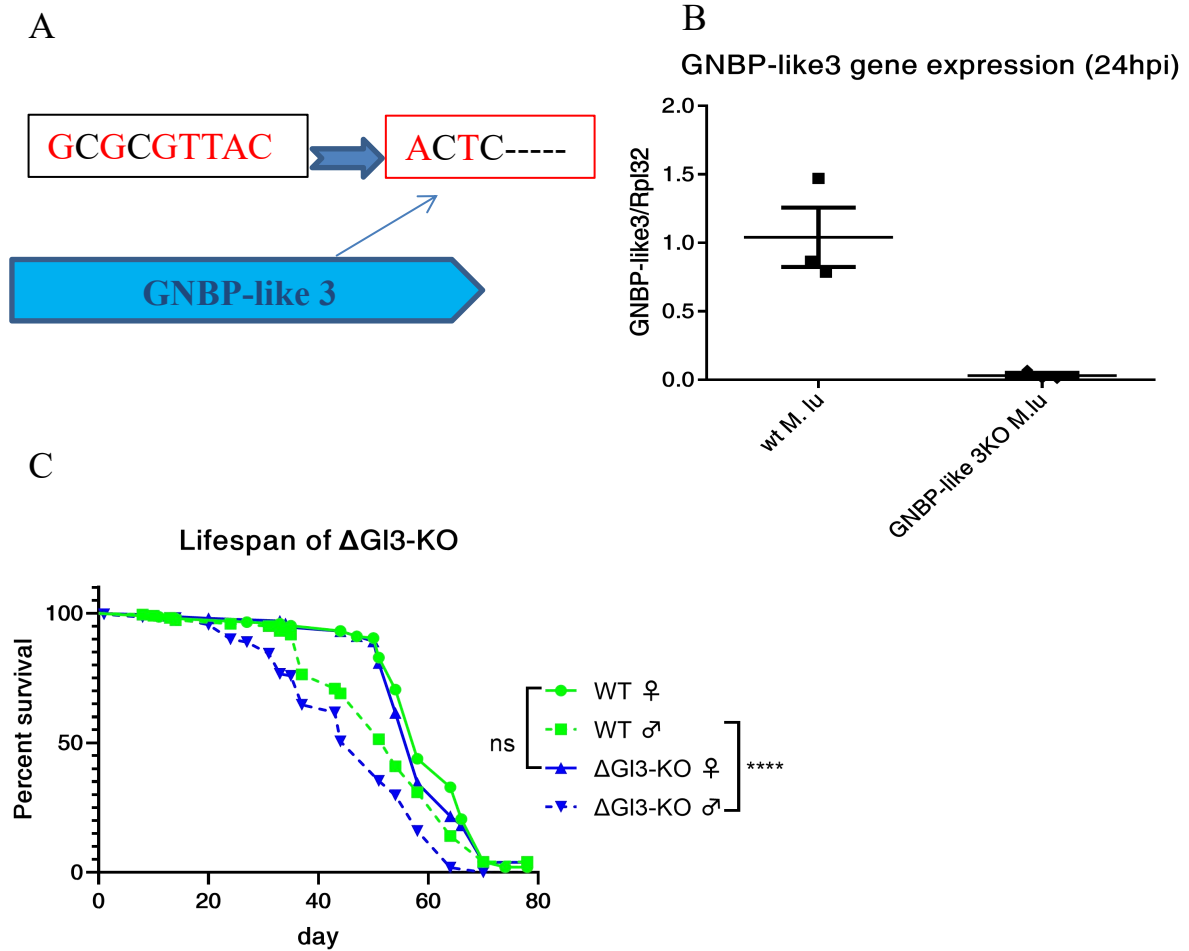
The UAS-RNAi GGBP-like 3 B6R11 construct driven by hspGAL4 (RNAi) efficiently blocks GGBP-like 3 expression in the hemolymph of adult flies after *B. bassiana* natural infection when a Heat shock is performed every two days (RNAi\*hsp HS/2d *B. b* 4d compared to RNAi\*hsp *B. b* 4d).

*M. l*: *M. luteus*; *B. b*: *B. bassiana* natural infection; NC: Non Challenged; wt: wild-type flies; HS: Heat shocked flies; HS/2d: Heat shock performed every two days at 0h, 48h, 96h. Hemolymph collected after Heat-shock when performed the same day.

Hours (h) and days (d) indicate the time post infection.

First Heat shock is performed 18hours before any infection.

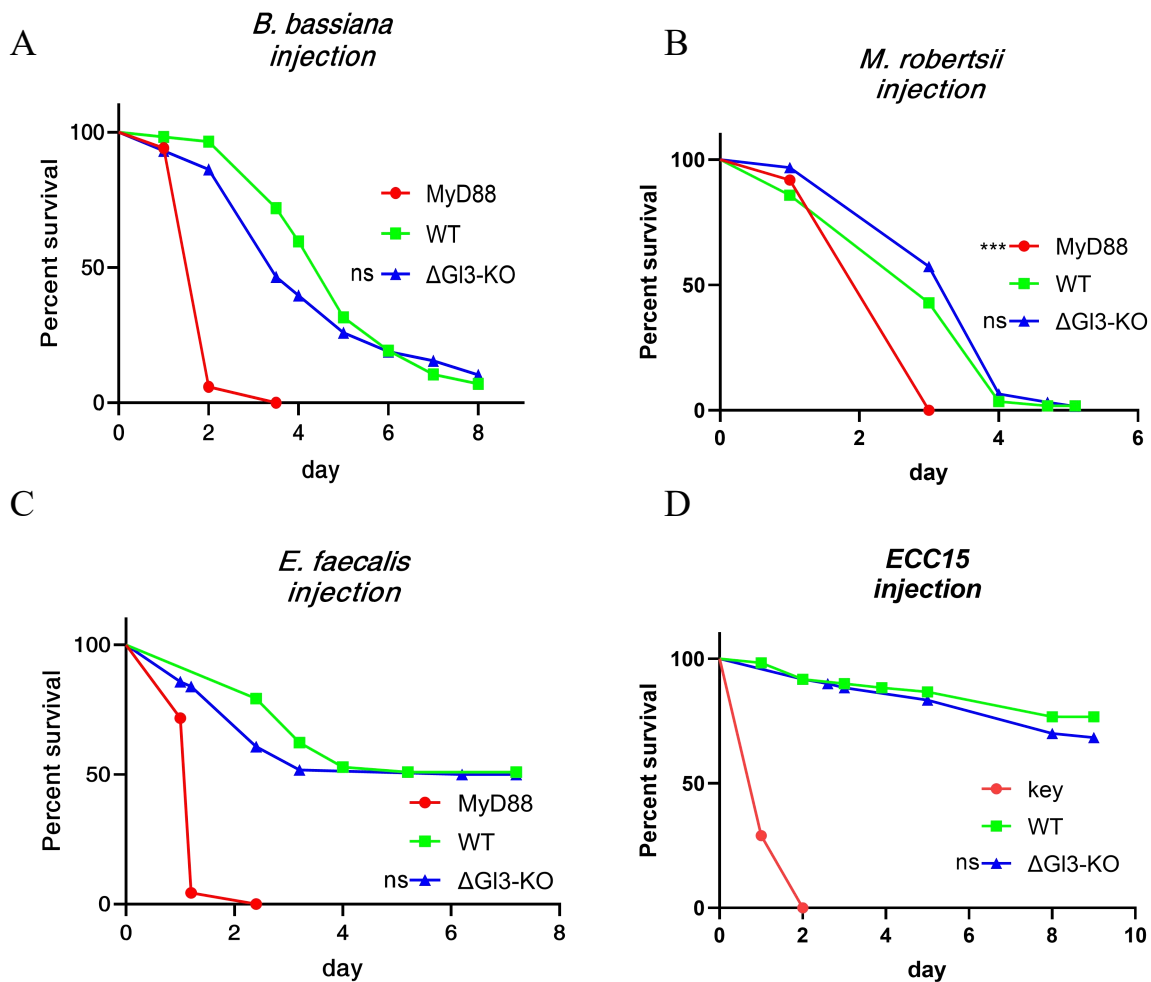
**Fig. S6**



**Fig. S6** *GNBP-like 3* KO mutant flies are viable but displayed a slightly-impaired fitness.

(A) *GNBP-like 3* CRISPR/Cas 9 mutant is five nt deletion and two nt mutations (GCGC→ACTC, as marked in red). (B) No *GNBP-like 3* mRNA expression in *GNBP-like 3* KO mutant after a *M. luteus* challenge. (C) Lifespan of *GNBP-like 3* KO mutant flies. *GNBP-like 3* KO male mutants showed significant shorter lifespan as compared to wild type males, while no significant difference between *GNBP-like 3* KO mutant females and wild type females. Three independent experiments have been performed.

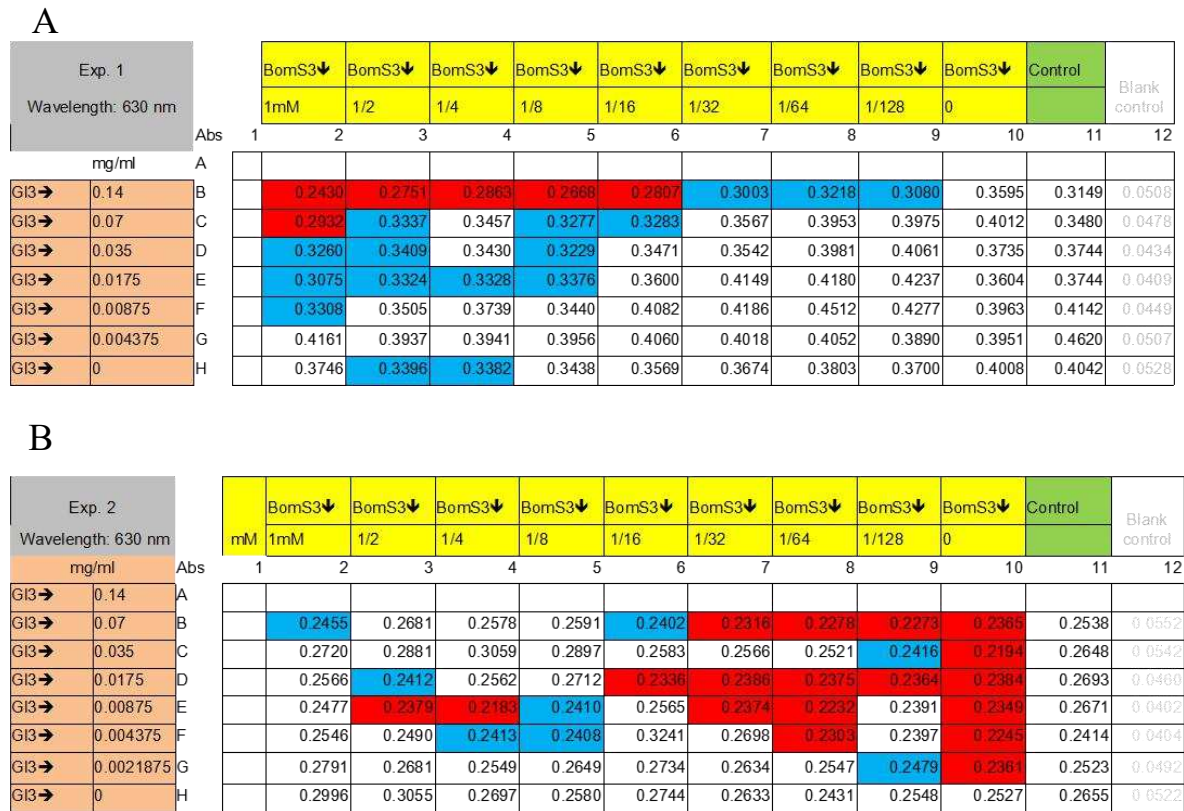
Fig. S7



**Fig. S7 *GNBP-like 3* KO mutant flies do not show sensitivity phenotype to other kinds of pathogens.**

(A) About 250 *B. bassiana* spores were injected. *GNBP-like 3* KO mutant flies did not show significant difference compared to wild type flies. Two independent experiments have been performed. (B) About 50 *M. robertsii* spores were injected. *GNBP-like 3* KO mutant flies behaved like wild type flies. Four independent experiments have been performed. (C) The injected concentration of *E. faecalis* was 0.1 OD, 4.6nl. There was no statistic difference between wild type and *GNBP-like 3* KO mutant flies. Two experiments have been performed. (D) 50OD, 4.6nl of *ECC15* were injected. *GNBP-like 3* KO mutant flies showed the same survival rate as wild type flies. One experiment has been performed

**Fig. S8**



**Figure S8. Checkerboard experiments with GGBP-like3 recombinant protein and synthetic peptide BomS3 co-incubation with *C. glabrata* in vitro.**

Two independent experiments of synergy checkerboard assay with GGBP-like 3 recombinant protein and BomS3 synthetic peptide. As marked in red represents OD value lower than two standard deviation of control, blue means OD value lower than one standard deviation of control. Table shows here was at 16h, continuous time points still had been observed.

## General discussion

This work has focused on the elucidation of the biological function in host defense against microbial infections of two secreted effectors of the Toll pathway, BaramicinA and GGBP-like 3. The former has initially been identified through its differential immune-dependent expression in the hemolymph of single flies by MALDI-TOF mass-spectrometry some 25 years ago [136]. The latter had been identified based on its sequence homology following the publication of the *D. melanogaster* genome. Yet, a proteomics analysis relying on 2D-gel electrophoresis revealed it was one of the most induced protein upon a fungal challenge, in keeping with a potential effector function [118]. The genetic analysis of *BaraA* presented here reveals a role for BaraA in resilience against two specific infections, the Gram-positive bacterium *E. faecalis* and the entomopathogenic fungus *M. robertsii*. An important feature of the mutant phenotype is that we did not detect an enhanced microbial load at our level of resolution, which excludes a role in resistance [181]. According to a couple of studies that introduced the concept of Microbial Load Upon Death (MLUD), mutants of genes involved in resistance display an unaltered MLUD (they just reach it faster) whereas genes involved in resilience are expected to show a decreased MLUD (it takes fewer microorganisms to kill the host as it is unable to cope with the deleterious consequences of infection) [181]. However, the hidden assumption of this type of analysis is that damages are directly correlated to the microbial burden. This assumption is not always true, as exemplified by the case of *A. fumigatus* that kills its host without invading it through the secretion of mycotoxins (Xu *et al.*, submitted). Here, we have demonstrated a protective function of BaraA against two toxins of bacterial and eukaryotic origins. It would be interesting to determine whether these toxins are constantly secreted during infection or whether just an initial dose is sufficient to reach a threshold level of damages in *BaraA* mutants. To account for the unmodified MLUD, an open possibility is that the toxins are no longer produced at high microbial concentrations and that their expression would be down-regulated in a quorum-sensing-dependent manner. In contrast, *GGBP-like 3* mutants exhibit a clearly increased *C. glabrata* load during the infection. However, whether GGBP-like 3 functions as a genuine AMP needs to be discussed. Taken together, this

work opens novel research directions and some of the outstanding issues are discussed further below.

The analysis of *BaraA* function in host defense was initially puzzling: there was a clear-cut sensitivity phenotype, not to a broad category of microbes but to two distinct species that are not even originating from the same kingdom. When we set out to check whether the different facets of the host defense were affected in the *BaraA* mutant, we found that the cleavage of PPO into active PO was altered. As melanization has been documented to play a role in host defense against fungi and against Gram-positive bacteria [70, 242], it might account for the susceptibility to these two pathogens. However, one then does not understand why *BaraA* mutants did not display any heightened sensitivity to *S. albus* or to *C. albicans*. Also, the microbial titer is not increasing even though melanization has been shown to limit the proliferation and dissemination of at least *A. fumigatus* (Xu *et al.*, submitted). Thus, we conclude that melanization is likely not affected severely enough in the *BaraA* mutant to contribute to its phenotype. How *BaraA* affects melanization remains to be determined.

The silencing of *BaraA* expression in hemocytes led to an increased sensitivity to *E. faecalis* that unexpectedly was as pronounced as when *BaraA* expression was silenced ubiquitously. As we did not detect a role for *BaraA* in the uptake of killed *E. faecalis*, its function in cellular host defense remains uncharacterized. Again, a role in phagocytosis would have been expected to lead to an increased microbial titer in the *BaraA* mutant. Besides phagocytosis, hemocytes represent also a signaling platform, *e.g.*, for emitting cytokines such as Eiger (*Drosophila* TNF $\alpha$ ) or Unpaired3 (IL-6 -like cytokine) [243, 244]. Thus, more work is required to understand its function. A stimulating possibility would be that some *BaraA*-derived peptides function themselves as cytokines. We note in this respect that *BaraA* expression in hemocytes is required to protect the fly from the action of DestruxinA or EntV.

We have also found that the uptake of injected *M. robertsii* conidia by hemocytes was affected in the *BaraA* mutants. Its function in the host defense against *M. robertsii* was not required in the hemocytes, but in the fat body, in keeping with a possible role of some *BaraA*-derived peptides as opsonins. We note however that the results of the opsonization experiments were not as clear-cut as those reported for TEP4 [245] in that we did not observe any rescue from *M. robertsii* conidia coated with *BaraA*-expressing donor

hemolymph in *BaraA* recipient larvae (only in wild-type recipient larvae). As for melanization, the absence of an altered microbial titer in the *BaraA* mutant suggests that phagocytosis is not a major contributor to the host defense against this fungus. This result however is in conflict with other studies in which hemocytes were blocked by the injection of latex beads or genetically ablated and led to an impaired survival to *M. robertsii* injected conidia (Wang *et al.* in preparation).

Of note, silencing *BaraA* in enterocytes did not lead to a heightened susceptibility to either the bacterium or the fungus. These results are negative controls that underline the restriction of *BaraA* to a limited set of tissues that correspond to the expression of the knock-in reporter *mCherry* gene.

Given the roles of *BaraA* in protecting the host from specific secreted toxins, we also investigated the tissue/cell type-specific requirements for these *BaraA* functions. We have already referred to the necessity of *BaraA* expression in hemocytes as regards protection from Destruxins and EntV. A similar requirement was found as regards the fat body. These results are somewhat unexpected for a gene the products of which are secreted. One would have expected that *BaraA*-derived peptides produced by the fat body, with its high biosynthesis capacities, would be able to maintain the concentration of these peptide in the hemolymph when also not released by hemocytes. This may suggest a short range, temporal or spatial, for the action of the secreted peptides that might protect the tissues targeted by the toxins only locally.

The brain represents a special organ, as it is insulated by the blood brain barrier (BBB). In addition, Destruxins may target partially the nervous system as the injection of DtxA leads to a paralysis that is reversible in wild-type flies but not in *BaraA* or *MyD88* mutants. Interestingly, silencing *BaraA* expression with an *ELAV*-Gal4 driver did not yield any enhanced sensitivity phenotype to injected toxins. Of note, it might be worth using a stronger neuron-specific driver such as *nSyb*-Gal4. The knock-down of *BaraA* expression in all glial cells or specific subsets of glial cells such as cortex glia, and cells that form the BBB such as perineurial and subperineurial populations (with a strong dependence on the driver used as regards subperineurial glial cells) yielded also a susceptibility to both toxins. As regards EntV challenge, the phenotype was somewhat variable. In contrast, the sensitivity to DtxA was consistent when using a *repo*-Gal4 pan-glial driver; Of interest is the observation of some sensitivity to only injected DtxA upon

using two subperineurial drivers that form the tight epithelium that delimits the brain. This suggests that BaraA peptides might contribute to the establishment of chemical barrier to toxins that complements the physical BBB.

We have so far been unable to ascribe a function to specific BaraA-derived peptides. It is likely that the short BaraA peptides function somewhat redundantly given their high sequence similarity. Our approaches based on genetic overexpression of specific peptides have not been conclusive so far. We cannot exclude that a pyroglutamic acid modification present in most cleaved BaraA peptides would not be produced when expressing only the selected peptide from a transgene. Of note, this modification was present in the synthetic peptides used in this study. We have so far limited ourselves to the study of survival phenotypes and did not investigate the toxins as they are not active on wild-type flies. A preliminary experiment on rescued *BaraA* flies by the peptide transgenes failed to reveal any protection against injected DtxA in survival experiments. One last attempt will consist in overexpressing the single peptides or pre-injecting the synthetic peptides in a wild-type background and analyze whether all flies undergo paralysis. Of note the overexpression of a constitutively-active allele of Toll protects 50% of the flies from a transient paralysis caused by the injection of verruculogen. Finally, it is puzzling that we revealed a brain-specific requirement for *BaraA* function as we did not observe any expression in the nervous system with the knock-in *mCherry* reporter. Another study from the Lemaitre laboratory documented an expression for *BaraA* in the brain using a reporter transgene, which is in keeping with our functional analysis [137]. In another study devoted to the evolution of the BaraA locus and of two paralogs, Hanson *et al.* proposed that the DIM24 peptide might represent a defining feature of the Bara family [246]. He found that BaraB functions during development and not in immunity and that this developmental function requires its expression in glial cells. Another paralog, BaraC, is also expressed in glial cells. Thus, it will be interesting to experimentally test the possibility that DIM24 is the peptide conferring protection against toxins in glial cells. One approach would be to perform a genetic complementation of the *BaraA* null mutant by a DIM24 transgene using glial-specific drivers. This may not work as the rescue using a strong ubiquitous driver failed. Finally, Mark Hanson *et al.* reported that *BaraB* null mutants are lethal when isogenized in the *w* [DrosDel] background. We had also generated such a null mutant using the SFHI CRISPR-Cas9 platform and did not notice



any viability problem of homozygous flies. We also failed to find an immune-related phenotype for *BaraB* [247].

A major challenge will be to understand how the innate immune system protects the host from the action of secreted microbial toxins via the Toll pathway.

A first point of discussion is the relationship between AMP function and antitoxin peptide (ATP) role of secreted effectors of the innate immune response. Actually, it might also be the case for some peptides to mediate both functions as has been reported for some human alpha-defensins [248]. Mark Hanson and colleagues published a concurrent study on *BaraA* and proposed that it functions as an antifungal peptide. This was based on two arguments: they detected an increased fungal load in *BaraA* mutants naturally infected by *B. bassiana*; in *in vitro* assays, a combination of short *BaraA*-derived peptides enhanced the fungicidal activity of the pimaricin commercial antifungal compound against *C. albicans*. We have ourselves reported an antibacterial activity of DIM12 and Dim13 against *E. faecalis*, which was significant only at millimolar concentrations that are likely biologically irrelevant unless reached in a very limited spatial environment. We have also found that *BaraA* mutants are susceptible to *B. bassiana* injected conidia but did not find an increased fungal load at 24 hours by monitoring colony-forming units. In contrast, Mark Hanson used a natural infection model and monitored the fungal burden by RTqPCR. Whereas CFU may not be that reliable to assess the fungal burden of a filamenting fungus RTqPCR may also “count” signals emanating from killed fungi. Thus, we cannot formally exclude an AMP function for some of the *BaraA*-derived peptides.

Interestingly, we also tested whether *BaraA* was required to protect the host from the action of Beauvericin, a major *B. bassiana* mycotoxin and did not find it. Of note, we had initially unsuccessfully tested a role for *BaraA* against the major *E. faecalis* virulence factor, a secreted cytolysin, prior to investigating EntV. Thus, *BaraA* might counteract the effects of other *B. bassiana* secondary metabolites.

A surprising result was the finding that the  $\Delta$ AMP mutant was sensitive to both DtxA and EntV, a feature in common with *BaraA* mutants. Preliminary experiments failed to reveal a protective function for Group B (most IMD-dependent AMP genes) and Group C (Drosomycin and Metchnikowin) mutants, implying that Defensin may actually mediate this function. Of note, *Defensin* expression is abolished in *imd* mutants, which are not

susceptible to fungal and Gram-positive bacterial infections. It will nevertheless be important to test *imd* pathway and the *Defensin* mutant for a sensitivity to DtxA or EntV. When considering the results from different lines of investigations of the Toll-mediated protection against secreted microbial toxins, several categories can be delineated. A first one regroups toxins such as Beauvericin and ergot alkaloids that kill Toll pathway mutants but not the *Bom*<sup>A55C</sup> nor the *BaraA* mutants. It will be worth investigating whether the two *Bomanin* genes that are outside of the 55C locus mediate the Toll-dependent protection. A second category regroups toxins that are active on *Bom*<sup>A55C</sup> deletion mutants such as restrictocin or verruculogen/fumitremorgins from *A. fumigatus*. Of special interest is the observation that the *Bom*<sup>A55C</sup> were also susceptible to DtxA and to the *E. faecalis* supernatant. A third category regroups toxins that specifically kill *BaraA* mutants, including DtxA and EntV, but surprisingly also OMVs/bacterial metalloproteases such as PrtA. Interestingly, we have identified independently in the laboratory two further peptides that share this phenotype with *BaraA*. Thus, the mechanism of action of the Toll-dependent secreted peptides against diverse categories are likely to be complex on two levels: the variety of toxins counteracted by specific host peptides and the diversity of the host secreted peptides involved in the defense against a given toxin. For instance, we have mentioned the common properties of DtxA, EntV, and OMVs/metalloproteases on the one hand, and then up to five peptides required in the host defense against DtxA and EntV.

A previous study has found a common mechanism of action for the neutrophil  $\alpha$ -defensin HNP1 against a variety of cholesterol-binding pore-forming toxins and extracellularly-secreted bacterial enzymes that act inside the host cells [248]. The common property of these microbial virulence factors is a thermodynamic degree of instability required for forming pores within host membranes or for entering the host cells through channels. HNP1 is forming amphipathic helices that allow to partially denature the toxins by hydrophobic interactions, which make them susceptible to host proteases or undergo full denaturation. This elegant mechanism is however unlikely to underlie the mechanism of *Drosophila* ATPs since several toxins are actually short circular hexadepsipeptides with a likely constrained structure.

An important consideration for the thorough understanding of the actions of ATPs will be to determine how each toxin mediates its noxious action on the host and especially whether there are specific cell types or tissues targeted by a given toxin, such as the brain for verruculogen, and possibly also for DtxA.

An additional feature of the Toll response against toxins is that it appears to often involve a noncanonical Toll pathway that acts through unidentified transcription factors likely activated by dedicated intracellular adapters. A puzzling observation is that this noncanonical pathway does not appear to regulate *Drosomycin* expression, a classical read-out of the Toll pathway but ought to regulate at least the *Bomanin* genes that can rescue the sensitivity phenotype of *Bom*<sup>A55C</sup> to restrictocin or verruculogen (Xu *et al*, submitted). There is a frontier in determining how the Toll pathway gets activated by a proteolytic cascade that involves at least one protease of the developmental Toll pathway, which is itself intricately and complex. In this respect, we note that Toll has been shown to be required in a specific neuronal network regulating sleep homeostasis, with Spätzle being provided by astrocytes [249]. Thus, much exciting work needs to be done to understand how innate immunity protects against the actions of secreted microbial toxins. Whether this protection mode has been conserved during evolution remains an open question as most of the effectors appear to be restricted to *Drosophila* species. It is conceivable that the pressure applied by the pathogens that employ diverse type of toxins led to the selection of other strategies in different organisms, such as defensins in vertebrates, which does not however exclude the possibility of other secreted peptides to act against other sets of toxins.

Given the similarity of susceptibility phenotypes of several ATP genes against the same set of specific but different virulence factors, it is likely that a biochemical approach should be implemented to assess whether these peptides function together in a complex. This question is also open for GGBP-like3 and whereas we have not yet been able to demonstrate an interaction with BomS3, we think that this possibility should be further investigated. One has to remember that GGBP3 itself was found to form a large complex in the hemolymph that also included PPOs [117]. It may be also the case for GGBP-like 3 and such a complex might trap POs thus explaining why the ectopic overexpression of *GGBP-like 3* may inhibit melanization to some degree.

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## Acknowledgments

I would like to give my deepest gratitude to my supervisor Prof. Dominique FERRANDON, for his patient guidance and giving me encouragement and motivation during these six years. He is an excellent scientist and always shares his great knowledge and ideas with me. I will always remember in my whole life for the rigorous scientific attitude I learned from him, and “good science, good control”. And thanks to him for taking me in the joint PhD program. It is an amazing year in Strasbourg.

I am sincerely thankful to my jury members, Prof. Bruno LEMAITRE, Prof. Robert UNCKLESS, Prof. Gilles PREVOST, Prof. Renjie JIAO, Prof. Zhongfang WANG, and Prof. Zi LI, for accepting to read and evaluate my work.

I would like to thank the director of SFHI, Prof. Renjie JIAO, and the director of UPR\_9022 Prof. Jean-Luc IMLER, for accepting me to work in the unit, and giving me the opportunity to attend the joint PhD project.

I would like to appreciate Professor Samuel LIEGEOIS, who is always patient and very kind to my questions. And Prof. Zi LI for her kindness help during my study in SFHI.

I also extend my appreciation to members in SFHI: Jing CHEN, Marion DRAHEIM, Chuqin HUANG, Aichun CHEN, Rui XU, Wenhui WANG, Yanyan LOU, Chuping CAI, Rong YAN, Jianwen YANG, Qinglin QU, Guiying LIN, Yaotang WU, Bo ZHANG, Kaiyu MA, Lu LI, Xiaomin CHANG; members in UPR\_9022: Gaëtan CARAVELLO, Ines PAIS, Wennida Miriam Yamba, Jenny NGUYEN, Rachel WALTHER, Claire, Matthieu, Nathalie, and Anais for their warmhearted help to my research. And also, thanks to Valerie WOLF for all the helps in administrative works.

Last but not least, I would like to express my special thanks to my beloved family and friends, for their support and care. I also would like to thanks to my cheer leader Xulin ZENG, for your unconditional and endless support. You will always be my spiritual and motivation to keep going.



## Résumé en français suivi des mots-clés en français

La réponse immunitaire systémique de la drosophile contre de nombreuses bactéries et champignons à Gram-positif est assurée par la voie Toll. La façon dont les effecteurs régulés par la voie Toll remplissent réellement ce rôle reste mal connue car les gènes des peptides antimicrobiens régulés par cette voie ne sont actifs que contre les champignons filamenteux et non contre les bactéries à Gram-positif ou les levures. Ce travail a porté sur l'élucidation de la fonction biologique dans la défense de l'hôte contre les infections microbiennes de deux effecteurs sécrétés de la voie Toll, BaramicinA et GNBP-like3. L'analyse génétique de *BaraA* présentée ici révèle un rôle pour BaraA dans la résilience contre deux infections spécifiques, la bactérie à Gram-positif *Enterococcus faecalis* et le champignon entomopathogène *Metarhizium robertsii*. Pourtant, une analyse protéomique basée sur l'électrophorèse sur gel bidimensionnel a révélé que GNBP-like 3 était l'une des protéines les plus induites par une infection fongique, en accord avec une fonction effectrice potentielle. Ici, nous avons démontré une fonction protectrice de BaraA contre deux toxines l'EnterocinV de *E. faecalis* et la Destruxin A de *M. robertsii*. Il serait intéressant de déterminer si ces toxines sont constamment sécrétées au cours de l'infection ou si une dose initiale est suffisante pour atteindre un seuil de dommages chez les mutants de *BaraA*. En revanche, les mutants de *GNBP-like3* présentent une charge en *Candida glabrata* nettement accrue au cours de l'infection. Cependant, il faudra déterminer si GNBP-like 3 fonctionne comme un véritable peptide antimicrobien. Ce travail ouvre de nouvelles directions de recherche et certaines des questions en suspens sont évoquées dans la Discussion finale.

**Mots clés :** BaramicinA, toxines microbiennes, GNBP-like 3, résilience/tolérance aux maladies

## Résumé en anglais suivi des mots-clés en anglais

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 Gram-positive bacteria or yeasts. This work has focused on the elucidation of the biological function in host defense against microbial infections of two secreted effectors of the Toll pathway, BaramicinA and GNBP-like 3. The genetic analysis of *BaraA* presented here reveals a role for BaraA in resilience against two specific infections, the Gram-positive bacterium *Enterococcus faecalis* and the entomopathogenic fungus *Metarhizium robertsii*. A proteomics analysis relying on 2D-gel electrophoresis revealed that GNBP-like 3 was one of the most induced protein upon a fungal challenge, in keeping with a potential effector function. Here, we have demonstrated a protective function of BaraA against two toxins EnterocinV from *E. faecalis* and Destruxin A from *M. robertsii*. It would be interesting to determine whether these toxins are constantly secreted during infection or whether just an initial dose is sufficient to reach a threshold level of damages in *BaraA* mutants. In contrast, *GNBP-like 3* mutants exhibit a clearly increased *C. glabrata* load during the infection. However, whether GNBP-like 3 functions as a genuine AMP needs to be assessed. This work opens novel research directions and some of the outstanding issues are outlined in the concluding Discussion.

**Keywords :** BaramicinA, microbial toxins, GNBP-like 3, resilience/disease tolerance