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***Vers une meilleure compréhension des infections intestinales: études des relations hôte-pathogène chez l'organisme modèle* Drosophila melanogaster**

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RÉSUMÉ

L'intestin représente la barrière épithéliale la plus exposée au monde microbien, en particulier chez les vertébrés où le microbiote contient environ dix fois plus de cellules que l'organisme-hôte. Par ailleurs, de nombreux microbes potentiellement pathogènes sont ingérés avec les aliments ou la boisson. Effectivement, certains microbes sont capables de traverser la paroi intestinale et de provoquer des infections graves, surtout lorsque l'hôte est immunodéprimé. Cette paroi consiste en fait en un simple épithélium monocouche. Cette thématique fait l'objet de nombreuses études à l'heure actuelle. Le problème est difficile à étudier chez les vertébrés en raison de la complexité du microbiota et des deux niveaux imbriqués de la défense de l'hôte que sont l'immunité innée et l'immunité adaptative. Des études génétiques récentes chez un organisme modèle, *Drosophila melanogaster*, ont souligné que la défense de l'hôte contre les infections intestinales ne se résume pas à la seule réponse immunitaire dont le rôle est d'attaquer les pathogènes et de contenir le microbiota. Il est apparu qu'il est également important d'être capable de subir et de réparer les dommages entrainés par l'infection, soit directement par l'intermédiaire des facteurs de virulence microbiens, soit par la propre réponse immunitaire de l'hôte contre le pathogène. Par exemple, une infection orale de drosophiles par *Serratia marcescens* entraîne une mort cellulaire importante des entérocytes. La disparition des entérocytes est compensée par la prolifération des cellules-souches intestinales qui est elle-même déclenchée par l'activation de la voie JAK-STAT.

S. marcescens est une enterobacteriacae Gram-négative qui est ubiquitaire dans l'environnement. C'est un pathogène opportuniste capable d'infecter aussi bien les plantes que les animaux. Elle se retrouve aussi dans les services de soin intensif et de néonatalité et peut poser problème en raison de ses multiples gènes de résistance aux antibiotiques. *S. marcescens* est une bactérie entomopathogène très virulente chez la drosophile: quelques bactéries injectées dans la cavité interne, l'hémocoele, tuent la mouche en moins de 24 heures. Cependant, les mouches ne succombent qu'au bout de six jours dans un modèle d'infection orale. Une hypothèse était que *S. marcescens* resta confinée au tractus digestif, ce qui expliquerait ce phénotype. Cependant, bien que la majorité des bactéries se retrouve effectivement dans le lumen du tube digestif, quelques bactéries sont capables de traverser l'épithélium intestinal ainsi que la matrice péritrophique, une membrane chitinoprotéique qui borde intégralement l'épithélium de l'intestin moyen. Les bactéries traversent la paroi intestinale continuellement au cours de l'infection; cependant, elles ne semblent pas être en

mesure de proliférer dans l'hémocoele. De plus, elles sont phagocytosées efficacement par les hémocytes. De manière intéressante, bien que présentes dans l'hémocoele en quantités suffisantes pour déclencher une réponse immunitaire humorale systémique dans le modèle d'injection, une telle induction de cette réponse ne prend pas place après ingestion. Ainsi, selon le mécanisme d'introduction de *S. marcescens* dans l'hémolymphe, le résultat de l'infection peut varier considérablement, résultant en une bactérie virulente (injection) ou peu ou prou pathogène (ingestion). Il est vraisemblable que *S. marcescens* atténue son programme de virulence suite à son passage à travers la paroi intestinale, ce qui pourrait s'interpréter comme la conséquence d'un dialogue entre hôte et pathogène.

Une partie conséquente de mon travail a été d'effectuer un crible génétique en utilisant une bibliothèque de mutants générés par insertion aléatoire de Tn5-Sm, un minitransposon bactérien. Le crible a été réalisé dans un contexte défini: celui de mouches-hôtes auxquelles manquait le gène *Eater*, lequel code un récepteur de phagocytose. Dans ces mouches, l'infection n'est plus contrôlée dans l'hémocoele par les hémocytes et les drosophiles mutantes succombent rapidement à une bactériémie. Plusieurs phénotypes bactériens étaient attendus à l'issue de ce crible. Une première catégorie de phénotype prévisible était une virulence accrue, par exemple si les bactéries mutantes devenaient capables de traverser plus rapidement ou efficacement la barrière intestinale conséquemment à la perte d'un régulateur négatif. Un deuxième type de phénotype attendu était une virulence atténuée pouvant s'expliquer de plusieurs manières: 1- perte de résistance à l'environnement existant dans le lumen intestinal (enzymes digestives et lysozyme, radicaux libres et peptides antimicrobiens induits au niveau de l'épithélium intestinal dans le cadre d'une réponse immunitaire locale de l'hôte); 2- incapacité à traverser la matrice péritrophique; 3-incapacité à envahir les cellules épithéliales (adhésion, pénétration); 4- incapacité à résister aux défenses intracellulaires potentielles; 5- incapacité à sortir du côté basal des entérocytes 6- incapacité à proliférer dans l'hémolymphe ou perte de la résistance à l'action de la réponse immunitaire systémique qui est, quant à elle, fortement induite en l'absence de phagocytose, laquelle empêche chez les mouches sauvages la prolifération des bactéries ayant traversé la paroi intestinale.

J'ai ainsi isolé 58 lignées candidates après avoir criblé 1348 mutants bactériens. Je me suis par la suite plus particulièrement intéressé à un mutant affectant *FliR*, un gène de l'opéron impliqué dans la formation des flagelles. Le mutant *FliR* a effectivement une motilité atténuée et un moindre virulence *in vivo* qui corrèle avec un titre bactérien atténué dans l'hémolymphe. Ce phénotype suggère une capacité diminuée des mutants *FliR* à traverser la paroi intestinale. Ce gène pouvait soit être requis pour la sécrétion d'un facteur de virulence via l'appareil de

construction du flagelle, soit plus vraisemblablement impliquer directement le flagelle comme facteur de virulence, par exemple en raison de son rôle dans la motilité bactérienne.

Mes travaux ont établi que le mutant *FliR* est capable de traverser la matrice péritrophique. Dans un modèle de culture de cellules, ce mutant semble capable de s'attacher aux cellules avec la même efficacité que la souche sauvage de *S. marcescens*. Cependant, il ne semble pas capable d'entrer efficacement dans les cellules en culture ou les entérocytes. Une interprétation alternative serait qu'il pénètre normalement dans les cellules mais soit incapable d'affronter les éventuelles défenses intracellulaires de l'hôte. Ainsi, un blocage au niveau de l'épithélium expliquerait le phénotype de moindre virulence du mutant FliR dans des mouches mutantes pour *Eater*. En conclusion, l'appareil de synthèse du flagelle est important pour la traversée de l'épithélium intestinal et constitue donc un facteur de virulence

La plupart des modèles d'infection intestinale utilisent des bactéries à Gram-négatif ou des champignons. Il semblait donc intéressant d'établir un tel modèle avec des bactéries à Gram-positif. Notre choix s'est porté sur *S. xylosus* car cette bactérie avait été retrouvée dans l'hémolymphe de certaines de nos cultures de drosophile affectées par une infection à microsporidies.

Une première étape a été la caractérisation de *S. xylosus* dans le modèle d'infection systémique après piqûre septique. La bactérie se comporte comme la plupart des bactéries à Gram-positif ayant un peptidoglycane de type Lysine, c'est-à-dire incapacité à tuer rapidement des mouches sauvages, mais virulence accrue dans les mutants affectant soit la voie Toll, soit la réponse cellulaire. Les mouches mutantes pour la deuxième voie de régulation de la réponse humorale systémique (la voie Immune deficiency [IMD]) se comportaient comme les mouches sauvages.

Dans le cadre d'une infection intestinale, les mouches sauvages (et *imd*) succombaient en six jours alors que, de manière surprenante, les mouches mutantes de la voie Toll périssaient plus lentement, une situation opposée à celle du modèle de la piqûre septique. Quelques bactéries sont capables de traverser la paroi intestinale mais sont incapables de proliférer à moins que la réponse cellulaire ait été préalablement bloquée. L'épithélium intestinal apparaissait normal à la dissection et la presque totalité des bactéries ingérées étaient tuées dans l'intestin. Après avoir exclu l'hypothèse d'une toxine sécrétée dans le surnageant des bactéries adsorbées sur le filtre sur lequel viennent se nourrir les mouches, nous avons testé l'hypothèse qu'une suractivation de la réponse immunitaire était à l'origine du décès des mouches. La génétique mettant hors de cause les peptides antimicrobiens, la voie Toll n'étant apparemment pas activée dans l'épithélium intestinal, nous avons alors étudié la

réponse oxydative induite par l'ingestion de bactéries, laquelle est capable de tuer les mouches lorsqu'elle n'est pas régulée correctement. Là-aussi, le résultat s'est avéré négatif. En fin de compte, j'ai pu établir que la mort des mouches était due à un état de famine, confirmé par des mesures des réserves métaboliques. Mes travaux ont permis d'établir un nouveau rôle de la voie Toll dans la résistance à la famine, en présence ou absence d'infection, qui sera peut-être à mettre en relation avec un rôle métabolique de la voie Toll consistant à bloquer la voie de réponse à l'insuline lors d'une infection. En conclusion, mes travaux permettent de mieux comprendre les relations hôte-pathogène qui s'établissent lors d'une infection intestinale.

SUMMARY

The intestine is the organ most exposed to the microbial communities, especially in vertebrates where the number of individual microbes is ten times more as compared to the number of cells in the host organism. The epithelial barrier restricts these microbes into the lumen thus preventing them from causing local or systemic infections. Yet, some pathogenic bacteria cross the epithelial barrier, especially in immunocompromised people, and cause sever pathologies. Due to the complexity among the microbiota communities and the immune response of the host, consisting of innate and adaptive defenses, it is difficult to study specific interactions between individual microbial species and the host response. *Drosophila melanogaster* contains simple microbiota and lacks adaptive immune response. Also the availability of powerful genetic tools makes *D. melanogaster* a suitable genetic model to study host-pathogen interactions in the intestine. Some potential pathogens, like *Serratia marcescens*, can cross the intestinal epithelial barrier of *D. melanogaster* as well as damage the enterocytes, the dominant cell population in the intestinal epithelium. Consequently the intestinal stem cells undergo compensatory proliferation to maintain intestinal homeostasis.

The entomopathogenic Gram-negative bacterium *S. marcescens* infects a wide range of hosts. It opportunistically infects humans, especially immunocompromised people and neonates. It is posing a growing health hazard due to its resistance to multiple antibiotics. It behaves differently in distinct infection models. When directly injected into *Drosophila* hemolymph, *S. marcescens* kills the flies within one day but upon oral feeding the flies succumb only after six days of the start of infection. We reasoned that the bacteria exhibit differential virulence program depending on the mode of penetration in the flies. In the latter case there is an early degradation of the gut epithelium as early as a few hours after infection that is caused by the *S. marcescens* hemolysin. Following the initial regeneration of the gut epithelium from early damage prolonged exposure to *S. marcescens* leads to the gut damage possibly due to bacterial proteases. Interestingly, the bacteria cross the gut of the flies just after a few hours of infection but are not able to trigger the systemic immune response unless the cellular immune system is blocked. Bacteria that have crossed the gut barrier appear to have down regulated their virulence programs. As a result they are easily controlled by phagocytosis. I have performed a genetic screen to identify the bacterial virulence factors and genes responsible for the crossing midgut barrier. In this screen a bank of bacterial mutants generated by transposon insertions randomly into the genome of *S. marcescens* were tested in survival experiments using phagocytosis-deficient flies.

A screen was performed to identify bacterial virulence factors. A mutant strain with the transposon inserted into the *fliR* gene, a component of the type III flagellar protein export system, exhibited attenuation of virulence in oral infection assays in *D. melanogaster*. The plasmid insertion mutant strain generated to interrupt the gene *fliR* reproduced the fly survival phenotype, indicating that the *fliR* gene is important for the virulence of *S. marcescens*.

The *fliR* mutants are able to cross the peritrophic matrix, functionally similar to the human mucus. The bacteria were found in the vicinity of the epithelial cells but were not able to efficiently invade the intestinal epithelium as compared to the wild-type strain. Consequently lower titer of *FliR* mutants was found in the hemolymph. The inefficiency of the *FliR* mutants to invade cells was also confirmed in *ex-vivo* assay using insect cells.

I thus demonstrated that the *fliR* gene which is important in the motility apparatus is also required by *S. marcescens* for the crossing of the epithelial barrier of *D. melanogaster*.

Most of the intestinal infections utilize Gram-negative bacteria or fungi. It is therefore interesting to develop an infection model with a Gram-positive bacterium. The *Staphylococcus xylosus* strain Argentoratum was the microorganism of choice because it was isolated from the hemolymph of moribund flies coinfected with microsporidia.

First the *S. xylosus* was characterized in septic injury model. Like other Gram-positive bacteria, *S. xylosus* did not kill the wild-type flies but Toll pathway mutants, the immunity pathway responsible to sense Gram-positive bacteria through their structure Lys-type peptidoglycans as well as phagocytosus-deficient flies succumbed in a concentration dependent manner. The mutants for the other immunity-, Immune deficiency (IMD)-, pathway died like wild-type controls.

When the wild-type flies were orally infected with *S. xylosus* A. they succumbed to the infection within six days. The midgut structure was intact when visualized a moment before the flies die. No protection was provided by IMD pathway as *kenny* mutant flies were dying at the same rate as wild-type flies. The bacteria were able to cross the gut barrier but were not able to proliferate in the hemolymph and their numbers remained very low even when phagocytosis was blocked.

A strong oxidative response is triggered by *D. melanogaster* in the midgut against commensals and pathogens. In order to check whether the strong oxidative immune response is eventually killing the flies themselves, hydrogen peroxide was chemically neutralized in the midgut during the *S. xylosus* A. oral infection. No difference in the fly survivals was observed with or without neutralization of the oxidative response indicating that over-production of reactive oxygen species (ROS) does not seem to be responsible for the fly death caused by a

very low number of bacteria. Flies could efficiently survive to killed bacteria and filtered supernatant solution from overnight bacterial culture indicating that they do not die to the toxins released by the bacteria. Most surprisingly *MyD88*-, the Toll pathway-, mutant flies were surviving better to *S. xylosus* A. oral infection. A series of experiments lead us to the finding that the flies actually succumbed to starvation when orally infected with *S. xylosus* and that the MyD88 is required for the starvation susceptibility in microbiota-mediated manner. In conclusion my work has lead us to the better understanding of the host-bacterial interactions in the intestine.

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Abbreviations:

AMP	: Antimicrobial peptides
CGD	: Chronic granulomatous
DAP	: Diamiopimelic acid
DIF	: Dorsal-related immunity factor
DTT	: Dithiothreitol
FOXO	: Forkhead box O
GlcNAc	: N-acetylglucosamine
GNBP3	: Gram negative protein 3
IMD	: Immune deficiency
ISC	: Intestinal stem cell
IRC	: Immune response catalase
JAK/STAT	: Janus kinase/signal transducers and activators of transcription
LPS	: Lipoplysaccharide
MAMP	: Microbe-associated molecular patterns
MurNAc	: N-acetylmuramic
NADPH	: Nicotinamide adenine dinucleotide phosphate-oxidase
PGN	: Peptidoglycan
PGRP	: Peptidoglycan recognition protein
PO	: Phenoloxidase
ROS	: Reactive oxygen species
PRR	: Pattern recognition receptor
SPZ	: Spätzle
TCT	: Tracheal cytotoxin
TLR	: Toll like receptor

1. Introduction

1.1. Preface

Animals and microorganisms have evolved together. Regular contacts between these living entities throughout the process of evolution have lead to the development of versatile molecular interactions. The animal hosts have acquired the ability to recognise various categories of microbes and induce appropriate defense response that allows them to eradicate the hazardous microbes and tolerate the commensals. The innate immune response is the first lines of defense. It is composed of germ-line encoded receptors that recognize the structural components and danger signals from the pathogen. The adaptive immune response, first appeared in cartilaginous fish, is the second line of defense that is only present in higher animals. During the adaptive defense, a complex repertoire of the immune receptors is produced as a result of somatic gene rearrangements. These immune responses, however, utilize available energy resources of the host. Thus, the processes that interconnect immunity and metabolism are of immense importance for an efficient and cost-effective management of interacting microbes. Moreover, pathogens have also acquired strategies to overcome the host immune response. A large variety of virulence factors are secreted by various microbes that encounter the immune response and damage the tissues of their hosts at various stages of infection. The hosts, therefore, must repair the damages to maintain cellular and metabolic homeostasis essential for their survival.

The human intestine hosts a large variety of microorganisms. Abnormalities in the interactions with resident microbiota in the intestine result in sever pathologies. Many diseases like chronic and acute inflammation, cancer and metabolic disorders are associated with the interacting microbes. Not all mechanisms that ensure a healthy life by providing successful defense against microbes are known to date. In addition, there is a need to uncover the virulence factors of many life-threatening microbes.

Thus, the objective of my study was to use the powerful genetics of the model organism *Drosophila melanogaster* for the better understanding of intestinal infections, using a Gram-negative bacterium *Serratia marcescens* and a Gram-positive bacterium *Staphylococcus xylosus*. My work also led to the study of the mechanisms that connect immunity and metabolism.

1.2. *Drosophila melanogaster*

The fruit fly is widely used as a model organism because of numerous advantages. This small insect is cheap to maintain and easy to handle. As compared to other insects, *Drosophila* has a fast life cycle producing large number of offspring, allowing medium-throughput studies. Moreover, powerful genetic tools have been developed after a work spread over a century. The *Drosophila* genome has been sequenced (Adams et al., 2000) and large number of genetically modified strains are easily available. Induction of various transgene expressions can be achieved in a spatio-temporal manner using yeast UAS-GAL4 system (Brand and Perrimon, 1993; Zhong and Yedvobnick, 2009). Downregulation of gene expression can be achieved by combining UAS-GAL4 system with RNA interference (RNAi) where genes of interest are targeted by constructing hairpin dsRNA induced under the control of GAL4 promoter (Kennerdell and Carthew, 2000). Mutants can also be generated using transposon mutagenesis (Rubin and Spradling, 1982; Searles et al., 1982; Swaroop et al., 1985). Whole genome based *Drosophila* mutant libraries can be generated by feeding flies on ethyl methanesulfonate (EMS) (Jenkins, 1967a, b; Williamson, 1970). Fate of proteins or individual cells can easily be traced *in vivo* using genetically marked fluorescent clones (Luo, 2007; Shrestha and Grueber, 2011; Wu and Luo, 2006). Finally, additional genetic tools like balancer chromosomes make *Drosophila* a power genetic model organism.

1.2.1. Immunity

Drosophila larvae feed on decaying fruits and vegetables and thus come across a large number of microbial communities. The microbes interact with *Drosophila* in the digestive tract, trachea, genitalia and other epithelia as well as by systemic dissemination through physical injuries. Although *Drosophila* lacks an adaptive immune response to microorganisms, the innate immune system provides an efficient protection to the flies against a majority of microbes. This makes *Drosophila* a suitable model to study innate immunity. Innate immune responses in *Drosophila* are well characterized and have lead to the discovery of various homologous innate systems in mammals (Tukhvatulin et al., 2010).

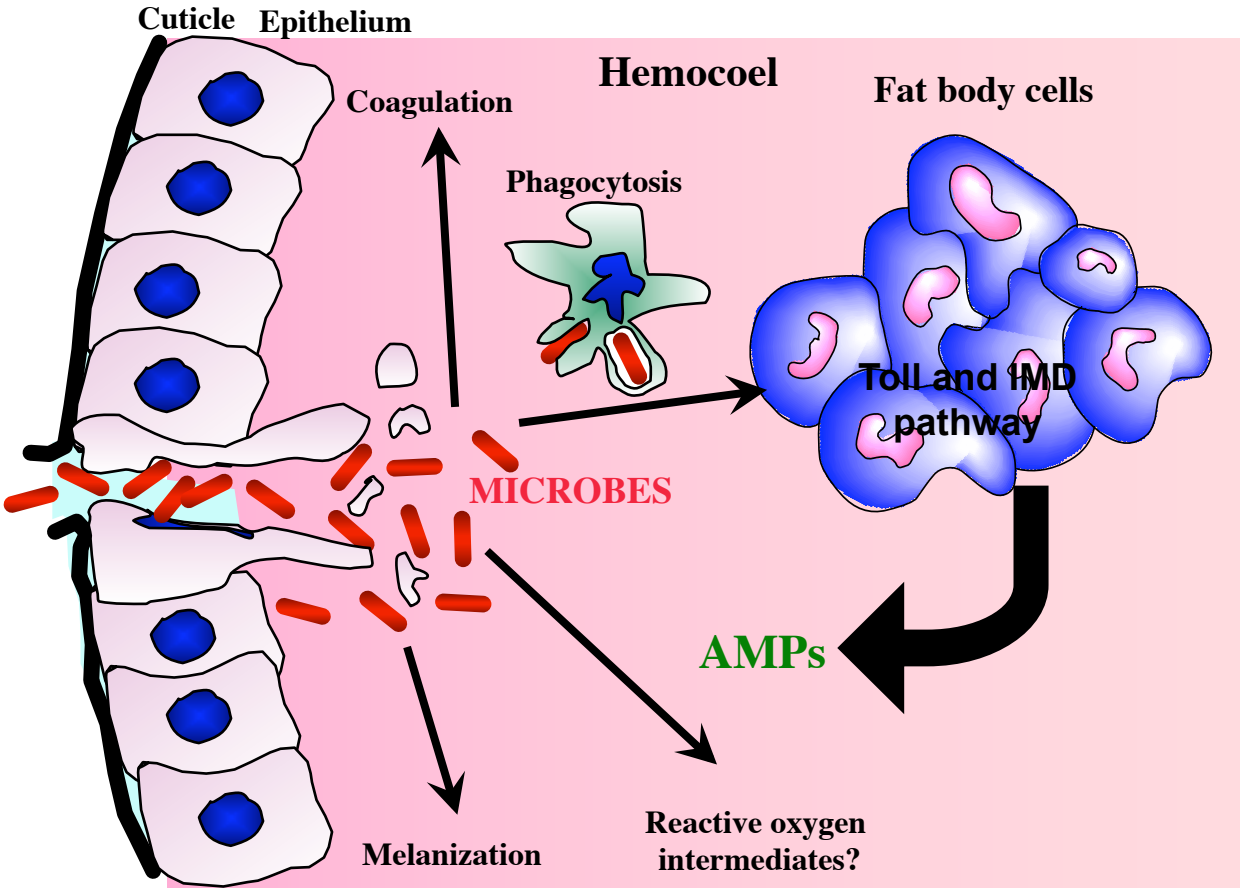

Figure 1.1: **The *Drosophila* systemic immune response**

The systemic immune response is triggered upon a septic injury. Coagulation and melanization are triggered and close the wound and trap the pathogen. Hemocytes engulf the invading microbes and phagocytose them. Reactive oxygen intermediates might be produced as by products of melanization reactions catalysed by the phenol oxidase enzyme. Toll and IMD pathway are induced to produce AMPs in the hemolymph.

The septic injury of *Drosophila* results in the activation of several distinct immune responses against microbes in the body cavity (Figure 1.1). The activation of several proteolytic cascades lead to the deposition of melanin at the site of injury and the production of cytotoxic reactive oxygen species (ROS) (Nappi et al., 1995). The surviving microbes are phagocytosed by the hemocytes (Braun et al., 1998; Braun et al., 1997). A systemic immune response is activated that can recognise different classes of invading microbes. The downstream signaling results in the nuclear translocation of the NF-κB transcription factors thus triggering the induction of antimicrobial peptides (AMPs) active against the large categories of microbes (Basset et al., 2000; Dimarcq et al., 1988; Fehlbaum et al., 1994; Ferrandon et al., 2007; Ferrandon et al., 2004; Lemaitre and Hoffmann, 2007). The epithelia of *Drosophila* are protected by various physical and chemical immune barriers, namely cutin and peritrophic matrix, and the production of ROS and AMPs, respectively (Vallet-Gely et al., 2008). Below I describe the different arms of known *Drosophila* immune responses in detail.

Systemic Immune Response

The systemic immune response in *Drosophila* is composed of humoral and cellular responses.

Humoral response

The microbes in the hemocoel are recognized through their structural components, activating signaling cascades, leading to the synthesis and release of AMPs by the fat body, and possibly hemocytes. These processes are described in detail as follows:

Recognition of Microbes

Drosophila immune system detects microbes of various categories through their conserved structural components, namely microbe-associated molecular patterns (MAMPs). Distinct MAMPs are associated with Gram-negative and Gram-positive bacteria and fungi. *Drosophila* detects these microbes respective recognition mechanisms.

Recognition of Gram-negative bacteria via DAP-type PGN

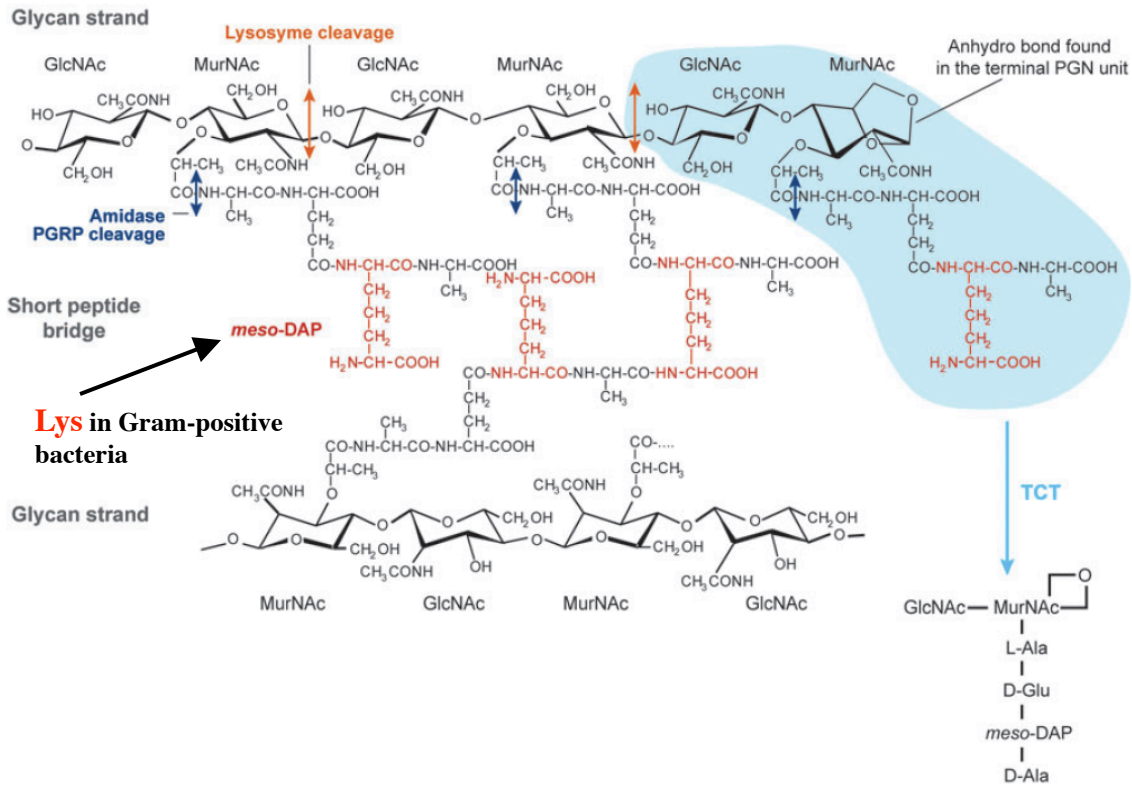

Figure 1.2: **Toll and IMD pathway recognition of microbial structural components**
Peptidoglycans are made up of long chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues connected by short peptide bridges. The third protein residue is a meso-diamiopimelic acid (DAP) in Gram-negative bacteria and a lysine (Lys) in Gram-positive bacteria (shown in *red* and indicated by *black* arrow). A structure akin to TCT (shown in *blue*) is present at the extremity of all glycan strands. Amidase PGRPs remove short peptides from the sugar backbone (*blue arrow*) while lysozymes cleave the β-1,4-glycosidic bond between GlcNAc and MurNAc (*orange arrow*). Modified from (Lemaitre and Hoffmann, 2007).

The Immune Deficiency (IMD) pathway is responsible for the recognition of Gram-negative bacteria and some Gram-positive bacilli (Ferrandon et al., 2007; Lemaitre et al., 1995). Although lipopolysaccharides (LPS) form the outer cell wall of the Gram-negative bacteria and are highly immunogenic in mammals (Beutler et al., 2003a; Beutler et al., 2003b) they are not recognised by *Drosophila* (Kaneko et al., 2004; Leulier et al., 2003).

Peptidoglycan, lying beneath external LPS layer and outer cell membrane, are linear glycan strands cross-linked by short peptides (Rogers, 1980). Alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by β-1→4 bonds form the glycan strands (Vollmer, 2008). A peptide stem, with the composition of L-Ala-γ-D-Glu-meso-diAm-D-Ala-D-Ala in *Escherichia coli* where the last D-Ala residue is lost in the mature macromolecule, substitutes the D-lactoyl group of each MurNAc of the glycan backbone (Figure 1.2) (Vollmer and Bertsche, 2008). These glycan-bound peptide chains are generally cross-linked by the carboxyl group of D-Ala at position 4 and the amino group of the meso-diamiopimelic acid (DAP) at position 3 of the other peptide, either directly or through a short peptide bridge. This DAP residue at position 3 in the cross-linking peptide stems is abundant in Gram-negative bacterial PGN (DAP-type PGN), as well as that of some Gram-positive bacilli. This residue at position 3 is replaced by a lysine (Lys) residue in Gram-positive bacterial PGN (Lys-type PGN) (Vollmer et al., 2008). *Drosophila* PGN-recognition proteins (PGRPs) can discriminate between these two types of PGN (Charroux et al., 2009; Ferrandon et al., 2007). The *Drosophila* genome encodes 13 members of peptidoglycan recognition proteins (PGRPs). They are categorized either as catalytic PGRPs (Mellroth et al., 2003; Mellroth and Steiner, 2006) or recognition PGRPs (Kim et al., 2003) on the basis of their function. The former are evolutionarily related to bacteriophage type II amidases while the later have lost the amino acid residues essential for the catalytic amidase activity.

PGRP-LC and PGRP-LE are the two noncatalytic members that sense DAP-type PGN of Gram-negative bacteria and activate the IMD pathway (Figure 1.3) (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Takehana et al., 2002; Takehana et al., 2004). PGRP-LC, main receptor of the IMD pathway, is a type II transmembrane protein while PGRP-LE is either cleaved to retain only the PGRP domain and secreted in the hemolymph (similar to mammalian CD14) or serves as

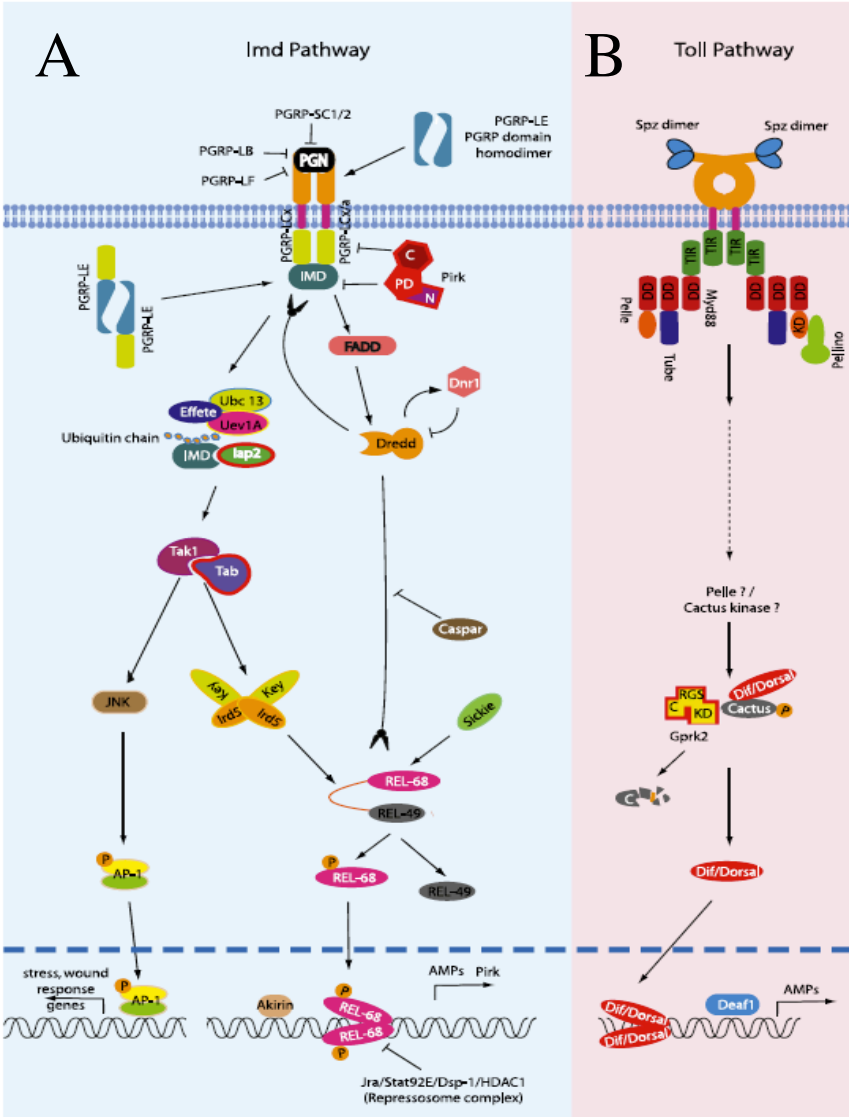

Figure 1.3: **The IMD and Toll pathways**

(A) The IMD pathway is activated by DAP-type PGN that di- or multimerize PGRP-LE or -LC. Some other members of the PGRP family, such as PGRP-LB, -SC, and -LF, negatively regulate the IMD pathway. Activated IMD recruits DREDD through FADD. IMD and Relish are proteolytically cleaved by DREDD. Cleaved IMD is K63 poly ubiquitinated by the E3-ligase IAP2, E2-ubiquitin-conjugating enzyme UEV1a, Bendless (Ubc13), and EFFECTE (Ubc5). This leads to the activation of Relish and and AP-1 triggering AMPs production and stress response, respectively. Akirin is required for Relish function. Prik/rudra/PIMS negatively regulates the IMD pathway at the level of recognition while DNR-1 and Casper block the response in the cytoplasm. (B) Dimerized Toll recruits TIR-domain containing adaptor protein MyD88 that connects with the death domain-containing Tube and Pelle. By a still undiscovered mechanism Cactus is degraded and then releases DIF/Dorsal. These transcription factors move to the nucleus and induce the expression of their taget genes. Adopted from (Valanne et al., 2012).

an intra-cellular receptor in full length where it binds to monomeric peptidoglycans (Kaneko et al., 2004; Kaneko et al., 2006). Both PGRP-LC and PGRP-LE can directly bind to longer or short length DAP-type PGNs, like tracheal cytotoxin (TCT), a naturally existing Gram-negative PGN (Chang et al., 2006; Mellroth et al., 2005).

There are three splice isoforms of PGRP-LC, a, x and y each with different extra-cellular domains (Werner et al., 2000). PGRP-LCx homodimers bind to polymeric DAP-type PGN while shorter length PGNs are sensed by the heterodimers of PGRP-LCx and PGRP-LCa. Detailed studies showed that heterodimerization of PGRP-LCx and PGRP-LCa was induced by TCT (Chang et al., 2006). The on crystal structure of TCT bound to PGRP-LC revealed that the ectodomain of PGRP-LCx binds with TCT and presents it to the ectodomain of PGRP-LCa for recognition since the former lacks the canonical peptidoglycan-docking groove conserved in other PGRPs. For that reason PGRP-LCa alone fails to bind directly to polymeric or monomeric PGNs (Mellroth et al., 2005). On the other hand when TCT is bound to PGRP-LE, a buried ionic interaction is established between the unique carboxyl group of DAP of TCT and an arginine residue of PGRP-LE conserved in all known PGRPs that bind to DAP-type PGN. The PGRP-LE bound TCT is than presented to another PGRP-LE. It has been shown that the contribution of prior TCT binding withh PGRP-LE enhances the affinity of PGRP-LE for the ligand (Lim et al., 2006).

The recognition of TCT initiates downstream signaling throught the IMD pathway. As a result of an activation of the IMD pathway, AMPs are secreted that lyse the bacteria. The lysed bacteria release long chain PGNs which are recognised by PGRP-LC to further activate the IMD pathway to fight the bacterial infection. Of note, overexpressed PGRP-LE is also found to induce melanization by triggering prophenoloxidase (proPO) cascade ((Takehana et al., 2002) in addition to induction of IMD pathway.

PGRP-LC is strictly needed to resist many Gram-negative bacterial species including *Enterobacter cloacae* or *Erwinia carotovora* (Gottar et al., 2002; Takehana et al., 2004), while PGRP-LE mutant flies did not exhibit a profound susceptibility to most Gram-negative bacterial infections (Takehana et al., 2004). However PGRP-LC and PGRP-LE double mutant flies failed to induced IMD pathway upon TCT infection as well as were found susceptible to *Escherichia coli* (Kaneko et al., 2004; Kaneko et al., 2006).

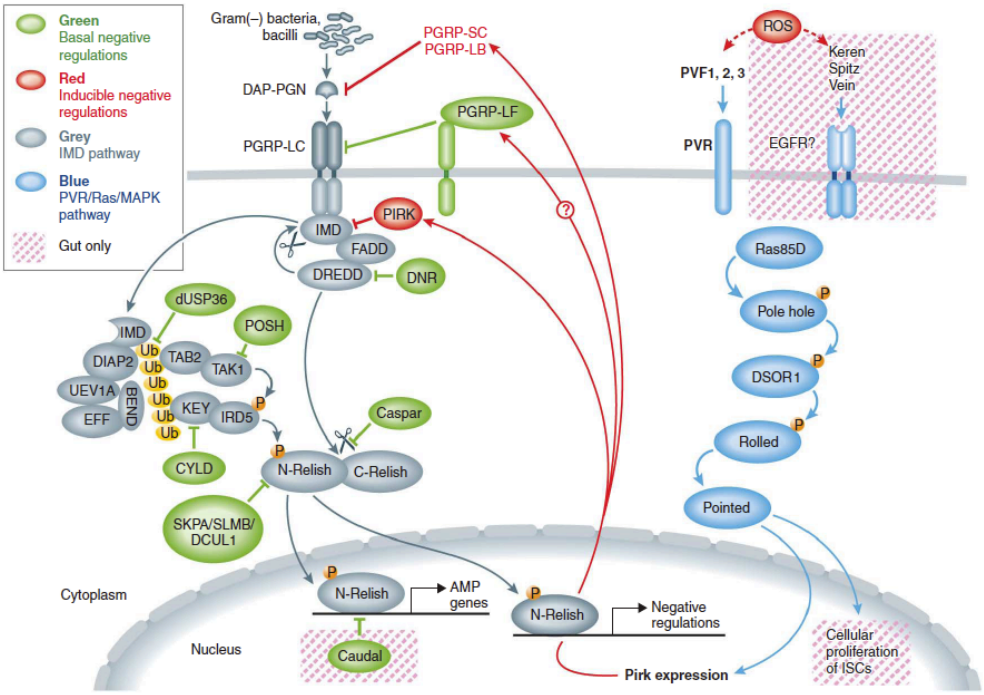

Figure 1.4: **Negative regulation of the IMD pathway**

The prolonged overactivation of the IMD pathway is controlled by the negative regulators (shown in *red*). Constitutive activation of the IMD pathway is prevented by the basal regulators (shown in *green*). Arrows: positive interaction,, dashed arrows: indirect interactions, red boxes: interactions in the *Drosophila* gut, ROS: reactive oxygen species. Taken from (Lee and Ferrandon, 2011).

Catalytic PGRPs (PGRP-SC1, PGRP-SB1 and PGRP-LB) of *Drosophila* retain their PGN degradation function. The elimination of the immunostimulatory properties of PGN by catalysis, thus, negatively controls a long-term IMD pathway activation to avoid potential detrimental effects for the host itself (Mellroth et al., 2003; Paredes et al., 2011). PGRP-LB, for example, is a secreted protein which is induced by the IMD pathway (Zaidman-Remy et al., 2006). It is an amidase that specifically degrades DAP-type PGNs of Gram-negative bacteria, thereby, providing a negative feedback loop to tightly control the activation of the IMD pathway (Paredes et al., 2011).

The transmembrane receptor PGRP-LF, although a non-catalytic PGRP, exhibits inhibitory function to immunity since reduction of PGRP-LF levels, in the absence of an infection, led to some basal IMD pathway activation (Maillet et al., 2008; Persson et al., 2007). PGRP-LF shows high affinity to DAP-type PGN and low affinity to Lys-type PGN (Maillet et al., 2008). It has two PGRP extra-cellular domains, LFz and LFw. None of the two domains of PGRP-LF can bind directly to PGN since they lack the PGN-docking groove found in other PGRP binding domains. Consequently PGRP-LF competes with PGRP-LCa to bind with PGRP-LCx/PGN complex, thus, downregulating the IMD pathway activity (Basbous et al., 2011).

Recognition of Gram-positive bacteria via Lys-type PGN

The cell walls of many Gram-positive bacteria contain Lys-type PGN (see above). The *Drosophila* genome encodes PGRP-SA that preferentially binds to Lys-type PGN (Figure 1.5) (Gobert et al., 2003). Another receptor namely Gram Negative Binding Protein 1 (GNBP1) has also been reported to sense Lys-type PGN (Gobert et al., 2003; Pili-Floury et al., 2004). A protein complex is formed by physical interaction of PGRP-SA and GNBP1 following recognition of Gram-positive bacteria. Activated GNBP1 hydrolyses Lys-type PGN into short di- or tetrameric muropeptides and presents the new glycan reducing ends to PGRP-SA (Wang et al., 2006). In a later report, however, full length GNBP1 did not show an enzymatic activity, and was rather suggested to function as a linker between PGRP-SA and ModSP, a modular serine protease (Buchon et al., 2009). Of note, the co-expression of both PGRP-SA and GNBP1 activates the Toll pathway in the absence of any infection (Gobert et al., 2003). PGRP-SD also senses Lys-type PGN with partial redundancy to the PGRP-SA-GNBP1 complex (Bischoff et al., 2004). The PGN-dependent recognition of the Gram-positive bacteria then activates a protease cascade that ultimately cleaves the

Toll receptor ligand Spätzle (SPZ). Cleaved SPZ then binds to the Toll receptor. The downstream signaling through the Toll pathway triggers the expression of its target genes including AMPs, such as Drosomycin.

Toll pathway activity is negatively regulated by PGRP-SC1B, a catalytic PGRP, that hydrolyzes the lactylamide bond between the glycan strand and the cross-linking peptides in Lys-type PGN thus preventing their detection by the receptors (Figure 1.2) (Bischoff et al., 2006; Mellroth et al., 2003)

Fungal Recognition

Fungi are recognized by the Toll pathway through two different mechanisms (Figure 1.5) (Gottar et al., 2006; Lemaitre and Hoffmann, 2007; Valanne et al., 2011). First, the fungi are detected through their cell wall components β-(1,3)-glucans by the circulating recognition molecules GNBP3, which then activate ModSP-dependent protease cascade (Buchon et al., 2009; Mishima et al., 2009). GNBP3 has also been reported to induce melanization of fungi through triggering a Phenoloxidase protease pathway activation (Gottar et al., 2006; Matskevich et al., 2010). Second, the *Drosophila* zymogen Persephone (PSH) is cleaved by the virulence factors of fungi as well as some Gram-positive bacteria (El Chamy et al., 2008; Gottar et al., 2006; Ligoxygakis et al., 2002). For instance, a fungal protease (PR1) is produced by the entomopathogenic fungus *Beauveria bassiana* initially to digest fly cuticle. This protease, PR1, also cleaves PSH, which subsequently activates the protease cascade eventually triggering the Toll pathway (Gottar et al., 2006).

Toll pathway Activation

Nine members of the family of Toll receptors are encoded by the *Drosophila* genome (Toll, 18-wheeler, Toll3-9), which are evolutionary conserved in mammals (Lund and Delotto, 2011; Moresco et al., 2011). *Drosophila* Toll, however, is the only among nine receptors showing an immune function (Eldon et al., 1994; Gay and Gangloff, 2007; Tauszig et al., 2000). In contrast to mammalian Toll-Like Receptors (TLRs) and *Drosophila* Toll9, *Drosophila* Toll does not sense pathogen factors PAMPs directly. Being a cytokine receptor itself, Toll has numerous leucine-rich repeats that bind to the cytokine SPZ cleaved by a proteolytic cascade activated after recognition of the microbial factors.

There are six members in the SPZ cytokine family. Structural studies reveal that SPZ is similar to neurotrophins. Synthesized as an inactive dimeric precursor (proSPZ) it is secreted in the hemolymph. SPZ is activated and cleaved by the three different pathways in *Drosophila* during immune response and development (Figure 1.5):

i) After recognition of microbial components by PGRP-SA, PGRP-SD, GNBP1 or GNBP3 (see above for details), a modular serine protease ModSP is activated initiating a proteolytic cascade involving several other serine proteases like Grass, Spirit, Sphinx1/2 and Spheroide, eventually activating SPE that ultimately cleaves SPZ (Kambris et al., 2006; Kellenberger et al., 2011).

ii) SPE is also activated directly by PSH, which senses the enzymatic activity from virulence factors from fungi and bacteria (*e.g.* fungal PR1) (Gottar et al., 2006; Jang et al., 2006).

iii) During embryogenesis, *Easter* is activated in response to positional cues, and in turn cleaves full-length SPZ. The activated SPZ than activates the Toll pathway, which is also required for the embryonic dorso-ventral patterning (Chasan and Anderson, 1989; Chasan et al., 1992; Gay and Keith, 1992).

The Toll pathway

The activation of the Toll pathway is initiated by the binding of the cleaved SPZ with the Toll receptor. Of note, the humoral and cellular innate immune responses cooperate at the level of SPZ since knocking down SPZ specifically in hemocytes of *Drosophila* larvae blocked Toll pathway activation after infection (Shia et al., 2009). Once in the hemolymph, the cleavage of the amino-terminal of disulfide-linked dimeric cystine-knot protein SPZ by SPE allows its carboxy-terminal fragement to bind to the amino-terminal of extra-cytoplasmic region of Toll receptor. This interaction forms a complex comprising a SPZ dimer and two Toll receptors thus activating the downstream signaling cascade (Figure 1.3) (Arnot et al., 2010; Hoffmann et al., 2008; Hu et al., 2004; Mizuguchi et al., 1998; Weber et al., 2005; Weber et al., 2003).

The intra-cellular domain of Toll receptor is a 150 amino acid TIR domain that is homologous to the intra-cellular signaling domain of interleukin-1 receptor and all TLRs in mammals (Ferrandon et al., 2007; Gay and Keith, 1991; Hashimoto et al., 1988; Schneider et al., 1991). The activation of Toll leads to dimerization of the two

TIR domains that recruit three death-domain containing proteins, dMyD88, Tube, and Pelle. These proteins, together, form the Toll-induced signaling complex (TISC) (Sun et al., 2004). The cytoplasmic adaptor MyD88 (myeloid differentiation primary-response gene 88) is conserved between vertebrates and invertebrates (Tauszig-Delamasure et al., 2002). It has one TIR domain and one death domain. The TIR domain of MyD88 interacts with the TIR domain of the Toll while the death-domain interacts with the death-domain of Tube. The Death-domain of Tube then binds to the death-domain of Pelle, a member of the IL-1R associated kinase family (IRAK) of serine-threonine kinases (Shelton and Wasserman, 1993; Towb et al., 2009). The substrate of Pelle during an immune response is not known yet. However, *in vitro* assays reveal that the *Drosophila Dichaete* gene, a member of the Sox family of high mobility group (HMG) domain proteins, is a phosphorylation target of Pelle. Phosphorylated Dichaete is required for its proper subcellular distribution in developing oocytes and normal development of the egg chamber (Mutsuddi et al., 2010).

Under noninduced conditions, the nuclear factors Dorsal and/or Dorsal related immunity factor (DIF) are bound to Cactus (the *Drosophila* homolog of I-κB) and remain anchored in the cytoplasm. During Toll pathway activation an uncharacterized mechanism causes the phosphorylation and K48 ubiquitination of Cactus. Cactus is then degraded in the proteasome-dependent manner (Belvin et al., 1995; Fernandez et al., 2001). This releases Dorsal and/or DIF that, upon nuclear localization, bind to the κB-response elements triggering the expression of the target genes, including the AMPs like *Drosomycin* that is widely used as a reporter of Toll pathway activation.

A G-protein (Guanine nucleotide binding protein)-coupled receptor kinase (Gprk)2, CG15737/Toll pathway activation mediating protein, and U-shaped are recently been found to be required for normal *Drosomycin* response *in vivo* (Valanne et al., 2010). Studies on S2 cells reveal an interaction of Gprk2 with Cactus, but Gprk2 is not required for its degradation though. Moreover, the Toll receptor ligand complex might be endocytosed since two important proteins of endocytosis complex, the Myopic (MOP) protein and the Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), are required for Toll pathway activation both in cells and flies. According to epistasis studies, MOP protein functions upstream of the MyD88 adaptor and Pelle kinase. This shows similarities with mammals where TLR4 was proposed to induce

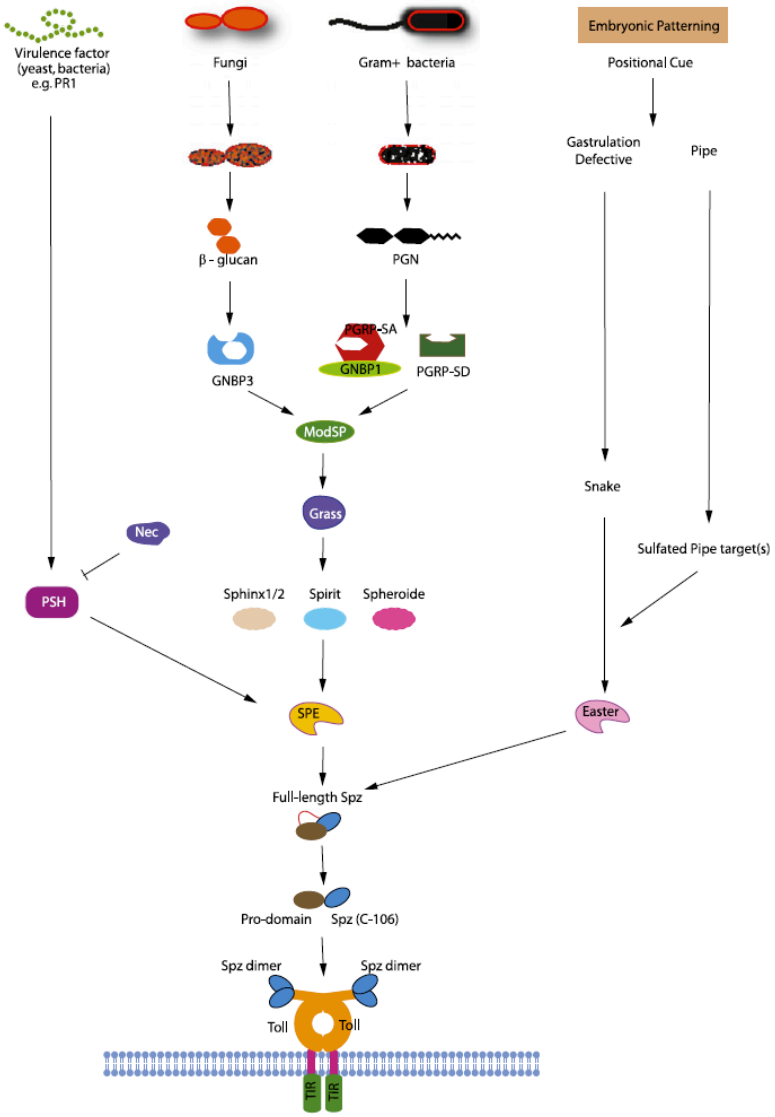

Figure 1.5: **Cleavage of the SPZ cytokine which is required for Toll pathway activation** SPE is activated by three protease cascades during an immune response. Virulence factors from Gram-positive bacteria and fungi activate Persephone (PSH) that in turn activates SPE. The other two cascades sense structural components of Gram-positive bacteria and fungi and converge at ModSP-Grass. The downstream activation of protease cascade induces SPE. Once activated, SPE cleaves the prodomain of SPZ. During early embryogenesis SPZ is cleaved by Easter. After cleavage of SPZ, its C terminal domain is exposed and binds to the transmembrane receptor Toll, thus triggering the downstream Toll pathway activation. Adopted from (Valanne et al., 2012).

TIRAP-MyD88 interaction at the plasma membrane. This interaction then leads to the internalisation of the receptor as a result of the activation of TRAM-TRIF signaling from the early endosomes (Kagan et al., 2008) and CD14-mediated endocytosis (Zanoni et al., 2011).

Negative regulation of Toll pathway

The activation of the Toll pathway mediated by the serine protease PSH is negatively regulated by Necrotic, a serine protease inhibitor of the Serpin family, thus regulating the Toll-mediated immune response (Levashina et al., 1999; Pelte et al., 2006; Robertson et al., 2006; Robertson et al., 2003). The catalytic PGRP-SC1B degrades Lys-type PGN and renders them unavailable for detection (Bischoff et al., 2006; Garver et al., 2006). Moreover, WntD (Wnt inhibitor of Dorsal), which is itself induced in response to the Toll pathway activation, negatively regulates the Toll pathway by preventing the nuclear localization of Dorsal during development. WntD has been shown to work in parallel or downstream of Cactus (Ganguly et al., 2005; Gordon et al., 2008; Gordon et al., 2005; McElwain et al., 2011). In a recent study, where WntD was shown to be involved in lipid metabolism for proper migration of primordial germ cells, *Drosophila* strains deficient for the positive regulators of Toll pathway, such as Toll, Tube, Pelle and MyD88, did not enhance WntD-mediated lethality. This suggests that the Toll pathway has no affect on WntD dependent metabolic affects (McElwain et al., 2011).

The IMD pathway

Both PGRP-LC and PGRP-LE share a RIP homotypic interaction motif (RHIM)-like motif in their amino-terminal domain, which is required for the initiation of signaling (Kaneko et al., 2006). Upon binding with the DAP-type PGN this cytoplasmic domain of PGRPs dimerizes or multimerizes, thus likey recruiting this death-domain containing adaptor IMD (Figure 1.3) (Choe et al., 2005).

The adaptor protein IMD plays a central role and initiates two genetically distinct processes, both aimed at activating the IMD pathway and ultimately leading to the nuclear localization of the main IMD pathway target, phosphorylated Relish, a NF-κB-like transcription factor. Relish is structurally similar to the mammalian p100 and p105 precursors (Ferrandon et al., 2007; Hedengren et al., 1999; Silverman et al., 2000; Stoven et al., 2000). The first process is the phosphorylation of Relish. IMD,

after being activated, recruits FAS-associated death domain (FADD) protein (Hu and Yang, 2000; Leulier et al., 2002) that, in turn, recruits the caspase-8 like protein DREDD (death-related ced-3/Nedd2-like protein) (Chen et al., 1998; Hu and Yang, 2000). This leads to the DREDD-mediated cleavage of IMD (Paquette et al., 2010), exposing the inhibitor of apoptosis (IAP)-binding motif (IBM) of IMD to the RING (really interesting new gene)-finger containing protein *D. melanogaster* inhibitor of apoptosis protein 2 (DIAP2). DIAP2 is thought to be a *Drosophila* E3 ligase for the K63-ubiquitination of IMD (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006). Ubiquitin conjugating E2 enzyme Bendless (*Drosophila* homologue of the mammalian ubiquitin-conjugating enzyme 13 (Ubc13)), Effecte (Ubc5) and ubiquitin-conjugating enzyme E2 variant 1 (Uev1a) also play a role in the ubiquitination of the IMD (Paquette et al., 2010; Zhou et al., 2005). IMD-linked K63-polyubiquitination serves as a scaffold for the assembly of a complex comprising of the MAPKKK transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1), and its regulatory subunit TAK1-binding protein 2 (TAB2). Eventually, the activation of IKK complex that consists of catalytic subunit immune-response deficient 5 (IRD5, *Drosophila* homologue of mammalian IKKβ) and regulatory subunit Kenny (*key*, IKKγ in mammals) phosphorylates Relish (Kleino et al., 2005; Zhou et al., 2005). The phosphorylation of Relish is required for the transactivational properties of Relish, but not for its cleavage (Erturk-Hasdemir et al., 2009). Second process is the cleavage of Relish. After being recruited by the IMD-bound FADD, DREED likey cleaves Relish, a process that is independent of its phosphorylation by the IKK complex (Erturk-Hasdemir et al., 2009; Leulier et al., 2000; Naitza et al., 2002). The phosphorylated and cleaved Relish then moves to the nucleus and induces the expression of target genes mainly AMPs. In a recent study Relish was found to also induce the apoptosis of neuronal in a retinopathy model (Chinchore et al., 2012).

The IMD pathway also interacts with JUN amino-terminal kinase (JNK) pathway. The Hemipterous (*Drosophila* homologue of MKK7/JNKK in mammals) is activated at the level of the TAK1/TAB2 complex (Naitza et al., 2002; Silverman et al., 2003) thus initiating a stress response. The exact role of JNK pathway activation in the systemic immune response remains controversial.

Negative regulation of IMD pathway

Since the hyperactivation of IMD pathway can be detrimental, it is tightly regulated at various stages (Figure 1.4) (Kim et al., 2007; Kim et al., 2006; Ryu et al., 2008). PGRP-SC and PGRP-LB are two amidases that degrade PGNs into nonstimulatory smaller fragments. Moreover, PGRP-LF competes PGRP-LC for dimerization by forming inactive heterodimers (see above). Another inhibitor of IMD pathway is PIRK, also called Rudra or PIMS, that acts at post-PGN recognition by binding to and inhibiting PGRP-LC activity (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008).The fact that the transcription of PIMS is controlled by Ras/MAPK pathway, a mechanism that promotes cell proliferation upon activation, shows an inverse relationship between growth and immunity. The Ras/MAPK pathway exhibits the immune inhibitory function in *Drosophila* hemocytes, fat body and adult intestinal stem cells (Ragab et al., 2011). The activity of these regulators is inducible and they regulate IMD pathway in a negative feedback.

There are many other constitutively expressed intracellular factors that regulate the IMD pathway during noninduced conditions. A transcription factor Caudal blocks AMP transcription in posterior midgut region, but not that of the negative regulators PGRP-LB and PIRK. Casper inhibits DREDD mediated cleavage of Relish. Many other factors (*e.g.* dUSP36, POSH, CYLD, SKPA, SLMB, DCUL1, DNR-1) affect the stability of IMD pathway components influencing their ubiquitination status. The JNK pathway also provides negative feedback. It blocks the expression of certain AMPs, which are induced upon IMD pathway activation. AP-1, which is expressed upon IMD dependent JAK/STAT pathway activation, makes a repressor complex binding to the promoter region of Relish-dependent AMP genes (Davis, 1999; Kim et al., 2007; Kim et al., 2005). Furthermore, the Toll-8 (or Tollo (a member of the Toll pathway receptors)), the ligand SPZ2 and the intracellular TIR domain containing protein SARM negatively regulate the IMD pathway-dependent AMP activation in the respiratory epithelium (Akhouayri et al., 2011).

The JNK pathway

Many physiological processes like cytoprotection, apoptosis, autophagy, cell proliferation, differentiation, tissue repair, regeneration, metabolism and growth are influenced in response to the activation of the JNK pathway. The JNK pathway is activated by ROS, ultra-violet (UV) light and heat stress (Biteau et al., 2011; Boutros et al., 2002; Silverman et al., 2003) as well as by the IMD pathway (see above).

In case of IMD-mediated inflammation TAK1 (see above) activates Hemipterous (dJNKK) that phosphorylates Basket (dJNK) that in turn activates AP-1 (Delaney and Mlodzik, 2006; Geuking et al., 2009; Kim et al., 2007). A complex comprising AP-1, Stat92E, Dsp1 and HDAC1 binds the promoter region of IMD induced AMPs thus negatively regulating the pathway. In another study, however, JNK pathway has been shown to induce some AMPs (Delaney et al., 2006). This indicates the complexity of the role of JNK pathway. Moreover, the PDGF- and VEGF-receptor related (PVR), PDGF- and VEGF-related factor 2 (PVF2) and PVF3 ligands are induced by IMD-induced JNK pathway. PVR then blocks phosphorylation of JNK and Relish in a ERK dependent manner, thus negatively regulating the IMD pathway (Bond and Foley, 2009). In a recent study DREDD has been shown to be essential for the IMD-dependent activation of JNK pathway (Guntermann and Foley, 2011). These studies indicate that in addition to an antimicrobial response, the innate immune responses play a role in the endurance process of the flies where they can also withstand and repair the damages caused by the microbial infections.

Effector molecules of the systemic immune response

Antimicrobial peptides

Following a septic infection, the antimicrobial peptides (AMPs) are secreted in the hemolymph as a result of the Toll and/or IMD pathway activation in the fat body cells. Seven families of AMPs have been identified to date including Diptericins (2 genes), Drosocin, Attacins (4 genes), Drosomycins (7 genes), Metchnikowin, Cecropins (4 genes) and Defensins (2 genes). Dipericins, Drosocin and Attacins are active against Gram-negative bacteria, drosomycins and Metchnikowin against fungi, cecropins against both bacteria and fungi, while defensins are the only AMPs that show specific antimicrobial activity against Gram-positive bacteria. To monitor an activation of the IMD and Toll pathway, respectively. DiptericinA and Drosomycin1 are widely used as readouts.

Tep proteins

The *Drosophila* genome encodes six Thioester-containing proteins (TEP1-6) that are similar to the mammalian complement C3/α2-macroglobulin superfamily (Blandin and Levashina, 2004; Jiggins and Kim, 2006). TEP6, however, lacks the canonical thioester-motif. The fat body cells strongly induced seven TEP genes upon

an immune challenge. Once secreted they might work as opsonins to facilitate phagocytosis (Lagueux et al., 2000). No role of TEP proteins has been found in *Drosophila* in classical infection models (Bou Aoun et al., 2011), however, TEP2 is required for an efficient phagocytosis of *E. coli*, while TEP3 is needed for the phagocytosis of Gram-positive bacteria and might be required in the *Drosophila* gut against Gram-negative bacterium (unpublished data). The thioester lacking TEP6 may function to bind and internalize the fungi. In mosquito hemocyte-specific TEP1 functions as opsonin and promotes phagocytosis of bacteria (Levashina et al., 2001).

Miscellaneous effectors

A few putative immune factors are secreted in the *Drosophila* hemolymph as a result of an immune challenge. These molecules include *Drosophila* immune molecules (DIMs) and Tot proteins (De Gregorio et al., 2001; Ekengren and Hultmark, 2001; Ekengren et al., 2001; Levy et al., 2004; Uttenweiler-Joseph et al., 1998). Other effectors include catalase, transferrin, iron transporter gene and other factors that participate in distinct defense mechanisms like melanization and coagulation (Yoshiga et al., 1999). The exact function of the hundreds of genes induced upon immune challenge remains to be established.

Cellular Immune Response

In *Drosophila*, larval and adult hemocytes are derived from the mesoderm of the developing embryo (Evans and Wood, 2011; Tepass et al., 1994). They remove the apoptotic cells (Franc et al., 1996; Franc et al., 1999). The embryonic hemocytes constitute the major circulating hemocytes in larvae while ones produced in the lymph gland, the hematopoietic organ that proliferates and differentiates throughout the larval period (Grigorian et al., 2011), are not released in the absence of an infection until the onset of metamorphosis when they play a vital role in tissue remodeling. The embryonic and lymph gland produced hemocytes remain in the adult flies, mostly in a sessile form (Lemaitre and Hoffmann, 2007).

Among all three types of hemocytes, plasmatocytes are the largest group (90-95%) of larval hemocytes followed by crystal cells (5%) while lamellocytes are hardly detectable (Lanot et al., 2001). Crystal cells are nonphagocytic and are involved in melanization. They contain large amount of crystals of the hemocyanin-related oxidoreductase, the prophenoloxidase (proPO). They function as storage cells and

release their contents upon injury. Lamellocytes function in encapsulation of smaller foreign particles, oil droplets and parasitoid eggs (see below). Adult flies possess only plasmatocytes. They are phagocytic and their population remains uniform (about 1000-2000 per fly). They do not undergo proliferation or differentiation (Elrod-Erickson et al., 2000; Holz et al., 2003; Lanot et al., 2001).

Phagocytosis

The major function of plasmatocytes is to phagocytose invading microbes including viruses, bacteria and yeast, as well as other foreign particles and damaged self-components *e.g.* apoptotic cells during development. The microbes are recognized then internalized through cytoskeleton modulation. The engulfed microorganisms are enclosed in vacuoles and finally degraded by digestive enzymes in phagolysozymes. The genetic ablation of phagocytes by inducing hemocytes-specific apoptosis (Charroux and Royet, 2009; Defaye et al., 2009) or blockade of phagocytosis by the injection of nondegradable latex beads (Nehme et al., 2011) makes adult flies susceptible to some microbial infections. The genetic ablation of hemocytes in IMD and Toll pathways mutantsresults in larval mortality because of infections by opportunistic microbes (Matova and Anderson, 2006).

Several proteins have been identified that function as phagocytic receptors in *Drosophila*. Eater is an EGF-like repeat containing scavenger receptor (Kocks et al., 2005). Transcriptional downregulation of *eater* resulted in reduced binding and internalization of bacterial cells in cell culture assays. The *eater* null mutant adult flies have enhanced susceptibility to microbes (Charroux and Royet, 2009; Defaye et al., 2009; Nehme et al., 2011). The Nimrod family of EGF-repeat containing proteins, which are actually Eater-like molecules, are putative phagocytic receptors or opsonins (Kurucz et al., 2007). Draper, Croquemort and SIMU have been shown to recognize apoptotic cells to initiate their phagocytosis (Elliott and Ravichandran, 2008; Franc et al., 1996; Kurant et al., 2008; Ryoo and Baehrecke, 2010). A *Drosophila* Junctophilin, Undertaker, associates Draper-mediated phagocytosis and homeostasis (Cuttell et al., 2008). Croquemort, a member of CD36 family of phagocytic receptors, also recognizes bacteria (Stuart et al., 2005). The scavenger receptor, SR, has been demonstrated to recognize bacteria, but not fungi (Ramet et al., 2001). Gram-negative bacteria are also phagocytosed by PGRP-LC, a receptor for the IMD pathway that controls expression of AMPs. More than one thousand

splice forms of the Down syndrome cell adhesion molecule (Dscam) are secreted in the hemolymph and are suggested to mediate bacterial binding with the hemocytes, although these findings lack *in vivo* demonstration (Watson et al., 2005). *phgA* null mutant flies exhibit enhanced sensitivity to bacterial infections because of impaired phagocytic binding of bacteria. The full-of-bacteria (Bergeret et al., 2008), psidins (Brennan et al., 2007), nonaspanins (Bergeret et al., 2008) are proteins involved in phagocytosis in steps downstream of internalization.

Encapsulation

The larval lamellocytes encapsulate eggs of parasitoids, foreign particles and oil droplets (Russo et al., 1996). Soon after the egg deposition by the female parasitoid, plamatocytes bind to the chorion and signal for a rapid release of hemocytes from the lymph glands and form sessile hemocytes by an unknown mechanism. This also triggers the massive proliferation and differentiation of sessile hemocytes into lamellocytes in the sub-epidermis (Jung et al., 2005; Markus et al., 2009). These hemocytes form multilayered capsule around the egg that, later on, becomes melanized. The local production of cytotoxic products including ROS and melanization cascade intermediates play a role in the killing of the parasitoid egg (Eleftherianos and Revenis, 2011; Nappi et al., 2009).

Clotting

Clotting or coagulation is a critical process to rapidly heal the wound upon an injury and regenerate the barrier upon infection. It has been essentially studied in larvae. This process immobilizes the invading pathogens and facilitates their killing (Wang et al., 2010). The clot formed at the site of injury is composed of fibers mainly composed of hemolectin (Goto et al., 2003; Karlsson et al., 2004; Scherfer et al., 2004). The cross-linking of the fiber components is mediated by proteins like transglutaminase and proPO. This fibers networks traps hemocytes and invading microbes (Lemaitre and Hoffmann, 2007; Lindgren et al., 2008; Scherfer et al., 2006).

Melanization

It is the process of melanin deposition that occurs during both encapsulation and clotting. It starts with the recognition of the microbes by PRRs like GNBPs and PGRP-LE, which activates cascades of a series of serine proteases, leading to the activation of prophenoloxidase activating enzyme (PPAE). PPAE than cleaves

prophenoloxidase (proPO) into the enzymatically active form, phenoloxidase (PO). PO then oxidizes mono- and diphenols to orthoquinones that polymerise to melanin (Eleftherianos and Revenis, 2011; Lemaitre and Hoffmann, 2007; Liu et al., 2011). Its role in host defense is a matter of debate (Ayres and Schneider, 2008; Leclerc et al., 2006; Tang et al., 2008).

Other Miscellaneous hemocyte functions

Larval plasmatocytes express immune molecules like hemolectin, the JAK/STAT pathway ligand UPD3 and the Toll ligand SPZ (Agaisse et al., 2003; Goto et al., 2003; Shia et al., 2009). These cytokines are proposed to function as tools of communication between hemocytes and the immune responsive tissues such as fat body, in larvae.

A subset of circulating hemocytes expresses IMD-dependent AMPs expression upon an immune challenge (Reichhart et al., 1992). Moreover, many components of extracellular matrix like Collagen IV and Peroxidasin are secreted by the circulating plasmatocytes. These components are thought to play a role in the formation of basal membranes (Fessler et al., 1994).

Local Immune responses in barrier epithelia

The epithelia or the external surfaces of the animals are the frontiers between the environment and the internal milieu of the animals. These are the places where microbes first interact with the host animal. In *Drosophila*, the trachea (air paths), genital organs and intestine induce important immune responses against microbes (Akhouayri et al., 2011; Ferrandon et al., 1998; Tingvall et al., 2001; Tzou et al., 2000). Local immune responses in the *Drosophila* intestine are discussed in the following section.

1.2.2. The *Drosophila* intestine

The *Drosophila* intestine as a model for human gut infections and biology

Recent advances in the techniques to sequence metagenomes have revolutionized the study of microbiota in the human intestine. A normal human gut hosts as many as 10^{14} bacterial cells belonging to 400-1000 different species. A healthy intestine can maintain such a diverse array of microbes due to a tight control on the intestinal homeostasis, immune responses, specific composition and quantity

of microbiota, eradication of potential pathogens, tissue repair and regeneration. Abnormalities in these processes lead to many diseases of human intestine, such as inflammatory disorders, colorecteral cancer, metabolic imbalance and gastrointestinal infections (Chassaing and Darfeuille-Michaud, 2011; Garrett et al., 2010; Wells et al., 2011). Moreover, many physiological complications, for instance obesity (Kallus and Brandt, 2012), insulin resistance (De Bandt et al., 2011), and inflammation of the intestine are associated with the specific resident microbiota (Caricilli et al., 2012; Serino et al., 2011). This highlights a need to develop robust research approaches for better understanding of the intestinal homeostasis, interactions with resident microbiota and immune responses against potential pathogens. Mammalian models provide good homology to the human biology; however, they are expensive, complex and associated to ethical issues. Also it is difficult to isolate and study individual mechanisms of interest due to the complex nature of these models. Furthermore, high to medium throughput studies are labor-intensive and not cost-effective in mammalian models.

Because it feeds on rotten fruits and vegetables, *D. melanogaster* regularly interacts with a variety of microbes over and over. It can survive over a period of two months in a microbe rich environment. *Drosophila* has evolved distinct mechanisms against microbes. These immune defenses are widely studied and established in flies. Systemic and local immune responses are induced in *Drosophila* against microorganisms in hemolymph and on the surface epithelia, respectively (see *Drosophila* immunity (1.2.1) and below). These systems are quick and easy to be monitored efficiently using genetic techniques developed in *Drosophila*. Also the *Drosophila* genome encodes large number of conserved signaling pathways involved in stem cell proliferation, damage repair, cell death, embryogenesis and development, neural signaling, nutrient metabolism, starvation resistance, autophagy and innate immune system. Moreover, use of *Drosophila* as model organism in different fields of biological research spread over a period of a century has resulted in the development of diverse genetic tools to study these biological mechanisms. For instance, lineages of gut epithelial cells can be genetically marked, traced and analysed using multiple techniques for *in vivo* studies (reviewed in (Apidianakis and Rahme, 2011)). The ease of establishing artificial infection models and the study of biological phenomena of interest in throughput studies add to the advantages of

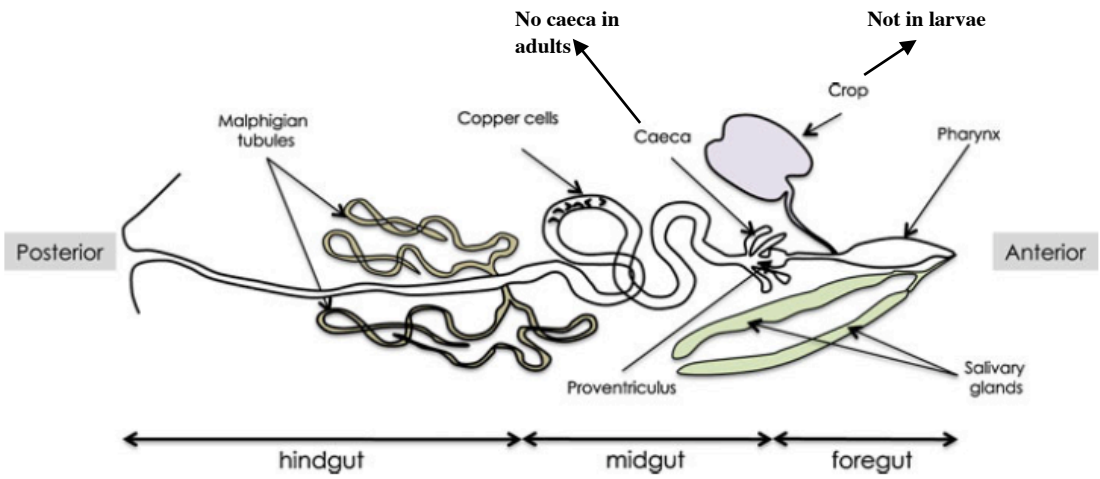

Figure 1.6: **The *Drosophila* midgut**. The crop is a storage organ of adults while the proventriculous is a value at the junction between foregut and midgut. The midgut has a stomach-like acidic section, the copper cell region. Malpighian tubules, functionally analogues of mammalian kidneys, are present at the junction between midgut and hindgut. Note: the caeca are only present in the larvae, but not in adults. Taken from (Royet, 2011).

using *Drosophila* as a model organism for the better understanding of the host-microbe interactions in the intestine.

The intestinal epithelium

The *Drosophila* intestine (Figure 1.6) is the first site of interaction between the host and the ingested microbes. In order to limit microbes inside the lumen and kill potential pathogens, *Drosophila* midgut epithelium has evolved many physical and chemical barriers. A peritrophic matrix, mucous in mammals, is the first line of defense. It is secreted by the proventriculous and also possibly by enterocytes. It is a chitinoproteinaceous layer that lines the inner surface of the epithelium. It functions as a physical barrier to prevent microbes from coming into a direct contact with the epithelial cells and a systemic dissemination into the hemolymph (Kuraishi et al., 2011; Shanbhag and Tripathi, 2009). The *Drosophila* intestinal epithelium is a monolayer composed of three types of cells. The polyploid enterocytes (EC) form the majority of the midgut cell population, followed by hormone secreting enteroendocrine (EE) cells and the proliferating intestinal stem cells (ISC). ECs are absorptive cells but also secrete digestive enzymes in some parts of the gut. The *Drosophila* epithelium is constantly being renewed with a turnover time of one week. During this renewal, ISCs divide asymmetrically to produce a population of non-differentiated enteroblasts (EB) that later differentiate into ECs and EEs (Charroux and Royet, 2010; Cordero and Sansom, 2012). The ISCs are located near to the basal membrane that is further lined by circular muscles. Both of these structures are important for the ISCs stability and maintenance (Bardin et al., 2010; Lin et al., 2010). The *Drosophila* epithelium also contains a medial copper cell region, which is acidic most likely for digestion purposes. Proteases, lipases (LipA), catalytic PGRPs and lysozymes are among the digestive enzymes secreted by the midgut cells (Sieber and Thummel, 2012).

Local AMP expression

The midgut epithelial cells of *Drosophila* secrete AMPs against ingested microbes. This immune response is IMD, and not Toll, pathway dependent (Liehl et al., 2006; Nehme et al., 2007; Ryu et al., 2006). In the absence of an infection *Drosophila* midgut tolerates commensal microbes. This is related to the low amounts of PGN released by the commensals in intestine, which is not sufficient to trigger a strong immune response. Additionally various negative regulators, like Caudal,

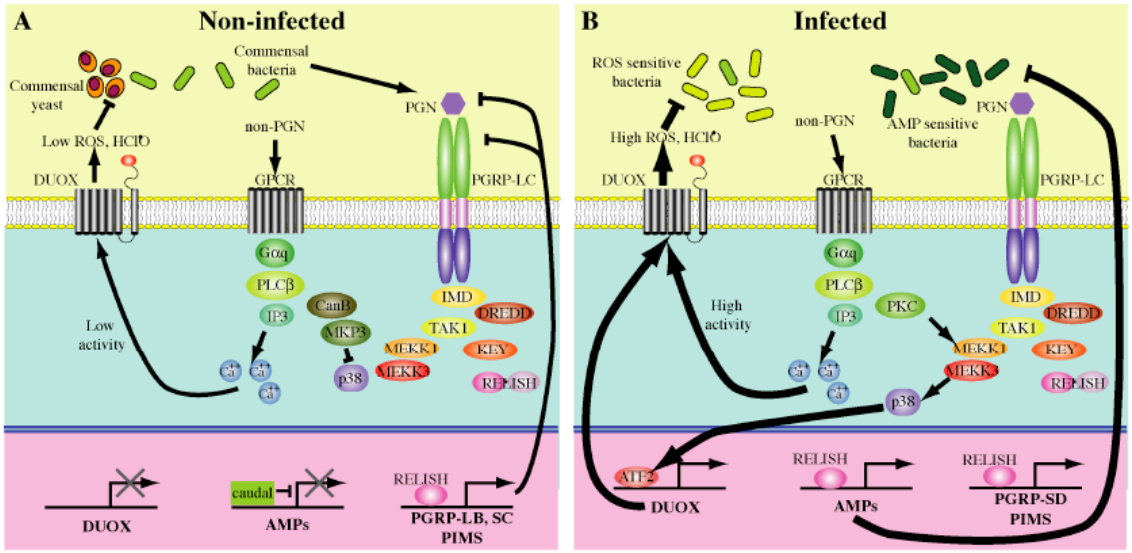

Figure 1.7: **Mechanism of AMPs and ROS regulation in the gut in the presence of commensals and increased microbial burden.** (A) Upon activation of an unknown G protein coupled receptor triggered by non-PGN microbial component(s), PLCβ is activated in Gαq-dependent manner. This activates IP3 (direct biochemical control), thus, secreting ROS at low level. The IMD pathway is activated by bacterial PGN, but is prevented from overactivation by Caudal, PIMS and catalytic PGRPs. (B) In the presence of increased microbial burden, high amounts of structural components of microbes induce IMD pathway that triggers the expression of AMPs. In addition to an increased activation of IP3, PKC and IMD pathway-dependent transcription of the DUOX gene is also induced (transcriptional regulation), thus secreting higher amounts of ROS. Taken from (Limmer et al., 2011).

PGRPs, USP36 and PIRK keep the immune response under control (Charroux and Royet, 2010; Ryu et al., 2008). The expression of IMD pathway induced AMPs is blocked by the DNA-binding repressor, Caudal, in the posterior region of the midgut. Caudal does not inhibit the expression of other IMD-dependent negative regulators such as PGRP-LB or PIRK. In the case of an infection, high amounts of PGN are released in the intestine that can trigger a strong AMP response in the midgut. *Drosophila* intestine can quickly come to the normal homeostasis because many negative regulators are under IMD control (see above).

Oxydative Response

The secretion of reactive oxygen species (ROS) is an important arm of the chemical immune response triggered against microbes in the *Drosophila* gut. Even heat-killed bacteria can induce a strong ROS response. By a non-described, PGN-independent, mechanism an unknown transmembrane G-protein coupled receptor (GPCR) is suggested to activate phospholipase C-β (PLCβ) mediated by a Gα protein (Gαq). PLCβ in turn induces the production of 1,4,5-triphosphate (IP3) causing mobilization of intracellular calcium. This induces the transmembrane protein dual oxidase (DUOX), a member of NADPH family, to produce ROS (Ryu et al., 2010). Under normal conditions, ROS are secreted to moderate levels by a direct biochemical control (Figure 1.7). During infection, however, production of ROS is increased, both biochemically as well at the transcriptional level.

ROS are the free oxygen radicals, such as oxygen ions and peroxidases. They are cytotoxic and lyse the microbes. An overproduction of ROS is neutralized by the secretion of an extracellular immune-related catalase (IRC) to avoid self toxicity that can be lethal for flies (Ha et al., 2005).

Homeostasis of the intestinal epithelium during intestinal infections

The midgut epithelium is damaged during bacterial infections of the intestine. The homeostasis is maintained by compensatory proliferation of ISCs. The JNK ((Ryoo et al., 2004) and see above), Hippo (see below), JAK/STAT, and EGFR pathways are involved in the repair of the damaged midgut epithelium in *Drosophila* (Buchon et al., 2010; Cronin et al., 2009; Jiang and Edgar, 2009; Xu et al., 2011) (Figure 1.8).

The Hippo pathway

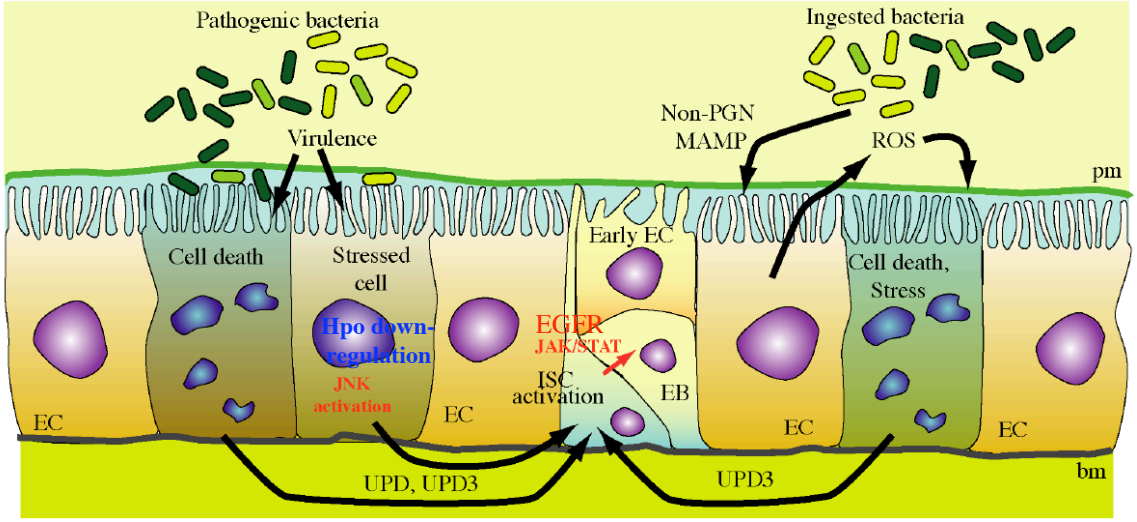

Figure 1.8: **The *Drosophila* midgut homeostasis**. The enterocytes (ECs) in the midgut epithelium are damaged either by the virulence factors of pathogenic bacteria or overactivated host immune response triggered against ingested microbes. The damages to ECs, as well as activation of JNK pathway and downregulation of Hippo (Hpo) pathway in the stressed ECs, lead to the secretion of cytokines, such as unpaired (UPD), and UPD3. After receiving a stress signal, the JAK/STAT and EGFR pathways are induced in ISCs (located in the vicinity of the basal membrane (bm)), thus leading to the compensatory proliferation of ISCs that maintains homeostasis. Modified from (Limmer et al., 2011).

First discovered in *Drosophila*, the conserved Hippo pathway is anticipated to be the major growth control pathway in vertebrates and arthropods (Staley and Irvine, 2012). After being activated by the extracellular ligand Dachsous (Ds) the transmembrane receptor Fat, a large (more than five thousand amino acids) protein containing 34 cadherin repeats in its extracellular domain, activates the Merlin and Expanded (Mer-ex) protein complex. These redundant proteins, in turn, activate the cytoplasmic Hippo kinase cassette. The Hippo kinase cassette is comprised of four core proteins namely Hippo (Hpo), Salvador (Sav), Warts (Wts) and Mob-as-tumor-repressor (Mats). Hpo-dependent phosphorylation and auto-phosphorylation of the kinase Wts leads to the phosphorylation of transcriptional co-activator Yorkie (Yki) at three Ser residues (Huang et al., 2005; Ren et al., 2010b). Yki functions as an oncogene where it promotes cell proliferation and growth. Many more factors interacting with the Hippo pathway are reviewed in (Staley and Irvine, 2012). The Hippo pathway is involved in the compensatory ISCs proliferation in *Drosophila* midgut (Huang et al., 2005; Meignin et al., 2007; Ren et al., 2010a; Shaw et al., 2010).

The JAK/STAT pathway

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is involved in various physiological process including cell proliferation, differentiation, migration, stress response and immunity (Arbouzova and Zeidler, 2006; Ivanenkov et al., 2011; Karsten et al., 2002; Tamiya et al., 2011). It is conserved both in vertebrates and invertebrates. The JAK/STAT pathway has been extensively studied in *Drosophila* (Gregory et al., 2008; Luo and Dearolf, 2001; Muller et al., 2008; Singh et al., 2005; Zeidler et al., 2000). In flies the transmembrane Domeless (Dome) is activated by the circulating cytokines, Unpaired (UPD), UPD2 and UPD3. This induces the dimerization of the receptors and activation of Hopscotch (Hop (the JAK kinase)), which in turn phosphorylates tyrosine-residues of the intracellular domain of the receptor. This domain, thereafter, provides the docking sites for the cytoplasmic STAT proteins (STAT92E), which are also phosphorylated by the JAK kinase before being dimerized and translocated into the nucleus to induce the expression of the target genes. The JAK/STAT pathway is regulated by many factors. For instance the transcription factor STAT92E, main the player of the JAK/STAT pathway, is positively regulated by the JAK/STAT pathway itself

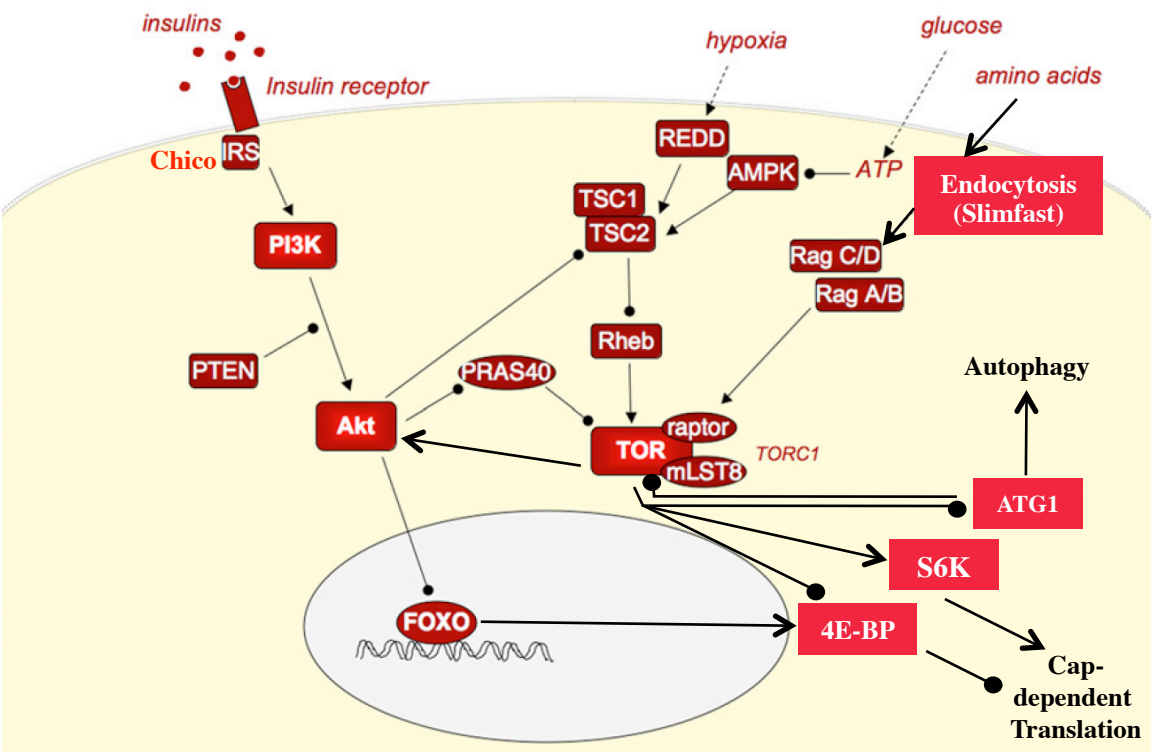

Figure 1.9: **Insulin/TOR signaling in *Drosophila*.** The insulin receptor (INR) is activated by extracellular *Drosophila* insulin-like peptides (DILPs). INR then recruits the insulin receptor substrate (Chico), thus activating PI3K and then Akt, which phosphorylates FOXO, that in turn is restricted to the cytoplasm. The TOR pathway is regulated by three ways: i) Upon activation by extracellular amino acid abundance, Slimfast-dependent endocytosis activates Rag complexes (Rag C/D and A/B complex) that possibly activate TOR, ii) under low ADP/ ATP ratio (energy rich environment; sensed by AMPK) and adequate supply of oxygen,TSC1/2 is inhibited thus increasing TOR activity, and iii) during insulin pathway activation Akt activates TOR by negatively regulating at least two of its negative regulators, TSC2 and PRAS40. TOR activity promotes protein translation by activating S6K and by blocking the activity of 4E-BP, which is initially transcribed by activated FOXO. Autophagy is blocked by TOR activity negatively regulating ATG1. Upon starvation, TOR activity is reduced, which activates ATG1 and thus autophagy. ATG1 also downregulates TOR activity under starvation stress. Modified from (Grewal, 2009).

(Arbouzova and Zeidler, 2006; Xi et al., 2003). Numerous factors like cytokine signaling 36E (SOCS36E), SOCS44A, protein inhibitors of activated STAT (PIAS), BTB/POZ domain containing transcription receptor Ken and Barbie (KEN), phosphatase PTP61F and PP1α96A negatively regulate the JAK/STAT signaling (Callus and Mathey-Prevot, 2002; Muller et al., 2008; Muller et al., 2005; Rawlings et al., 2004; Wormald and Hilton, 2004).

Several immune genes including Tep1 and the turandot (tot) are induced by the JAK/STAT pathway (Agaisse and Perrimon, 2004; Lagueux et al., 2000). In this case, the JAK/STAT pathway interacts with the IMD pathway and the mitogen-activated protein kinase (MAPK) pathway to regulate the expression of the immune genes (Brun et al., 2006). Moreover, the nuclear localization of STAT92E in non-phosphorylated form depends on HP1 protein of the heterochromatin. HP1 is essential for the stability of STAT92E in the nucleus (Brown and Zeidler, 2008; Li, 2008; Shi et al., 2008).

1.2.3. Response to starvation

Living organisms digest, absorb and utilize the nutrients to generate energy when sufficient food resources are available. This energy is used for the routine biological activities like protein synthesis, cell proliferation, growth, development and reproduction. Surplus nutrients are stored in muscles and adipose tissues. Under starvation, however, the requirements are changed. The organism has to halt normal growth and development and redirect the available energy resources to the biological processes vital for survival. The coordination between various biological activities is, therefore, of utmost importance. A successful coordination requires an efficient mechanism of nutrient sensing and signaling to the whole body including tissues far from the sites of nutrient absorption and storage. The insulin pathway and the TOR pathway are responsible for nutrient sensing in *Drosophila*. These pathways are well conserved among other living organisms. Insulin pathway senses and responds to humoral signaling in the whole organism while TOR pathway is responsive to the intra-cellular energy status, especially in the fat body.

Insulin/Igf pathway

The Insulin/Igf-like signaling (IIS) pathway is conserved from yeast to humans. In *Drosophila* the transmembrane insulin receptor (INR) is activated in response to

the humoral signaling by the insulin-like peptides (dilp1-7) (Figure 1.9). The activated INR then recruits an adaptor protein Chico (IRS in mammals) as well as regulatory and catalytic subunits of phosphoinositide 3-kinase (PI3K). Activated PI3-K phosphorylates a serine/threonine protein kinase Akt, which conversely phosphorylates the nuclear factor Forkhead box class o (dFOXO). Under normal conditions FOXO remains phosphorylated and is confined to the cytoplasm. Upon starvation, however, insulin pathway is downregulated leading to the dephosphorylation of FOXO. Once dephosphorylated, FOXO migrates to the nucleus where it induces the expression of its target genes to inhibit cellular growth and development.

The status of the insulin pathway has important implications in ageing, growth and development as well as the innate immune responses in *Drosophila* (Birse et al., 2010; Brogiolo et al., 2001; Dionne et al., 2006; Oldham, 2011; Partridge et al., 2011; Shin et al., 2011; Storelli et al., 2011).

TOR pathway

The target of rapamycin (TOR) pathway is the major intracellular nutrient sensing module. TOR is a kinase, the activity of which is finely regulated by three different mechanisms during stress conditions, such as nutrient deprivation and scarcity of oxygen (Figure 1.9). First, the low availability of amino acids reduces the Slimfast-, an amino acid transporter, dependent bulk endocytosis. This inactivates a complex of two complexes, Rag GTPase C/D and Rag GTPase A/B. The inactive Rag complexes then reduce their interaction with Raptor, an important TOR component, leading to the inactivation of TOR thus decreasing protein synthesis by the ribosomes. Second, a higher AMP/ATP ratio (*i.e.* low energy status during scarcity of glucose or hypoxia) activates the TSC1/2 complex that inactivates a small Ras-related GTPase (Rheb). Inactive Rheb in turn downregulates the activity of TOR. Third, the insulin pathway indirectly regulates TOR activity by downregulating at least two its negative regulators, TSC2 and proline-rich protein Akt substrate (PRAS40). PRAS40 however appears to play a role only during oogenesis.

Consequences of the TOR activity

Active TOR has three major functions:

a) **The synthesis of novel proteins**: The synthesis of novel protein is controlled by the TOR activity in three ways: i) initiation of cap-dependent mRNA translation, ii) rate of elongation of nascent proteins and iii) indirect control of translation of the ribosomal proteins.

b) **The blockade of autophagy**: Active TOR blocks autophagy by phosphorylating an autophagy-related gene, ATG1. Autophagy cannot be induced in the presence of phosphorylated ATG1. Conversely the overexpression of ATG1 is sufficient to induce autophagy under normal conditions.

c) **The onset of endocytosis**: TOR pathway is required for the formation of clathrin-coated vesicles during bulk endocytosis. It has also been shown that the inhibition of endocytic degradation of slimfast, an amino acid transporter, is controlled by TOR activity.

In *Drosophila* starvation studies are mostly performed on larvae (Ballard et al., 2008; Harbison et al., 2005; Hoffmann et al., 2001; Kramer et al., 2003; Kramer et al., 2008; Tettweiler et al., 2005; Wayne et al., 2006; Zinke et al., 2002).

1.3. *Serratia marcescens*

S. marcescens is a Gram-negative proteobacteria that belongs to the family of *Enterobacteriacae*. The bacterial cells are rod-shaped and motile with peritrichous flagella. *Serratia* is a facultative anaerobe and thus can generate energy both by aerobic respiration and fermentation. The bacteria are free living and found on plant and animal surfaces, soil and water. They are saprophytic and can extract nutrients from organic matter (Hejazi and Falkiner, 1997). They can grow and spoil many food products that are rich in starch. Many strains of *Serratia* can produce a red pigment, prodigiosin. People in middle ages associated this red pigment with some “miracle” where food items with a growth of *Serratia* were thought to start “bleeding”.

Serratia has a widespread host range including insects, corals, plants, nematodes, animals and humans (Grimont and Grimont, 1978; Kurz et al., 2003). It is a health hazard and an opportunistic pathogen (Iosifidis et al., 2012; Maltezou et al., 2012). It is thought to be an emerging major cause of nosocomial infections in neonates and immunocompromised patients. It has been found associated with pneumonia, intravenous catheter-associated infections, infection of skin and eye, endocarditis (inflammation in heart) and osteomyelitis (bone infections). Multiple-

antibiotic resistance in *Serratia* makes it difficult to cure these infections (Knowles et al., 2000; Nakamura et al., 2002; Traub, 2000). The production of β-lactamases and active multi drug efflux provide resistance to *Serratia* against antibacterial drugs. Its cells are able to communicate with each other by quorum sensing, a bacterial density depending mechanism studied in many bacterial species. After reaching a threshold, *Serratia* cells secrete N-Acyl-homoserine lactones and autoinducer 2 that can be sensed by other cells. This leads to a collaborative response in the bacterial cells against changed environmental conditions, for instance availability of food source.

The pathogenicity of *Serratia* depends on multiple virulence factors including secreted enzymes, such as nucleases, proteases (including serralysin), lipase, hemolysin as well as swimming and swarming ability of the bacterium (Hejazi and Falkiner, 1997; Kida et al., 2007). These weapons are used by the bacterium to adhere and invade the eukaryotic cells that provokes cytotoxicity and cytolysis.

I have used the *S. marcescens* strains Db11 and Db10 as the wild type controls. Db11 was selected spontaneously from the strain Db10 for its resistance to streptomycin. The Db10 was isolated from moribund flies (Flyg et al., 1980). I also worked with a bank of miniTn5-Sm transposon induced mutants randomly inserted in the genome of the wild-type strain Db10 (Pradel et al., 2007). The genome of Db11 strain of *S. marcescens* has been sequenced by the Sanger Institute (Hinxton, UK) in collaboration with the laboratory of Jonathan Newbank (Marseille, F). The *Serratia* genome contains 5.11 million base pairs with a coding density of 0.92.

S. marcescens* infections in *D. melanogaster

Serratia is a potent pathogen in *Drosophila* in a septic injury model. A few bacteria are sufficient to kill the fly within a day when inoculated in the hemolymph of the flies. IMD pathway-dependent systemic immune response is induced but *Serratia* is resistant to the AMPs because it proliferates to high titers and the flies finally succumb to bacteremia.

An oral infection model has been established by Nadine Nehme (Nehme et al., 2007). The flies in this infection model are fed with *Serratia* mixed in sucrose solution. The bacteria kill the wild type flies within six days. Nadine Nehme did retrieve bacteria from the hemolymph of the infected flies as early as two hours following the oral infection suggesting that *Serratia* is able to cross rapidly the

intestinal barrier. The proliferation of these bacteria was, however, efficiently controlled by the phagocytosis because phagocytosis-deficient, *eater* mutant (Kocks et al., 2005) or latex beads-injected, flies died faster than their wild type controls. The systemic immune response was not induced unless phagocytosis was blocked. The local immune response, however, is induced and has been shown to confer a partial protection to the flies because IMD pathway, *key*, mutants died faster as compared to their wild type controls. Nadine Nehme suggested that the wild-type flies eventually succumb because of severe damages to the gut epithelium that might be caused by the virulence factors of *S. marcescens*. Indeed, the intestinal epithelium of flies in the *cn bw* background became thinner, and the cytoplasm had an abnormal appearance when analyzed by electron microscopy (Nehme et al., 2007).

1.4. *Staphylococcus xylosus*

S. xylosus is a Gram-positive bacterium bacillus. It is a human and animal commensal of mucus and skin (Hariharan et al., 2011; Kloos et al., 1976; Nagase et al., 2002). *S. xylosus* is ubiquitous and is found in various niches like polluted water (Kessie et al., 1998), animal fodder and grains (Pioch et al., 1988), soil and various surfaces (Shale et al., 2005). It forms biofilms (Planchon et al., 2006). The expression of various proteins in sessile *S. xylosus* found in biofilm, from a human skin commensal strain C2a, is up-regulated as compared to its planktonic form (Planchon et al., 2009). These proteins were involved in various metabolic processes, mainly amino acid syntheses, protein translation and protein secretion pathways, indicating active protein trafficking in *S. xylosus* biofilms. *S. xylosus* can adapt to various environmental conditions. It is a natural component of raw meat and milk. It is used as a starter medium in the meat and milk fermentation industry (Kloos and Schleifer, 1986; Talon et al., 2002). Moreover, zinc-dependent metallolipase produced by *S. xylosus* is extensively used in the biotransformation industry (Bertoldo et al., 2011).

S. xylosus is normally considered to be a nonpathogenic *Staphylococcus* but some strains are associated with diseases. These strains behave as the opportunistic pathogens infecting immuno-compromised humans and animals. In humans *S. xylosus* is associated with endocarditis (Conrad and West, 1984),

septicemia (Koksal et al., 2009) and acute pyelonephritis (Tselenis-Kotsowilis et al., 1982). Chronic granulomatous disease (CGD) refers to a collection of genetic disorders in humans where some NADPH oxidase components are defective. CGD patients suffer from recurrent bacterial and fungal infections (Roos et al., 2007). *S. xylosus* is the major cause of death (or euthanasia) in mice deficient in phagocyte superoxide production due to defects in NADPH oxidase (Gozalo et al., 2010). Bacteria migrate to the internal organs, primarily lymph nodes and lungs and, to a lesser extend, muscles, bones and meninges, where they cause abscesses and granulomas in soft tissues. Several other reports have described *S. xylosus* as an opportunistic pathogen in animals (Bingel, 2002; Bradfield et al., 1993; Fthenakis et al., 1994; Jackson et al., 2001; Miedzobrodzki et al., 1989). Using the *S. xylosus* strain C2a as a driver, suppressive and subtractive hybridisation (SSH) analysis performed on pathogenic and nonpathogenic strains of *S. xylosus* led to the identification of two distinct groups of strains with one composed only of the potentially hazardous strains (Dordet-Frisoni et al., 2007).

In my study I used *S. xylosus* strain Argentoratum, which was originally isolated from moribund flies in our laboratory in Strasbourg, France. The dying fly stocks were later on found to be co-infected with microsporidia.

Aims and objectives

Microorganisms constitute the most primitive form of life on earth. They are the most abundant living creatures and display a vast diversity in the their habitats and host range. Not all of these microbes are pathogenic since they are tolerated or killed by the animals, thanks to the innate and adaptive immune responses that the hosts have selected during evolution. Yet, in humans several microbial infections cause severe diseases, some of them being lethal for a significant fraction of population. Many pathogenic bacteria are associated with the inflammatory disorders in the human intestine. Prevailing practices to counter these microbes mostly depend on the use of broad range antibiotics and the development of specific vaccines. Still there are numerous opportunistic and pathogenic microbes that lack proper diagnosis and control. Moreover, outbreaks of antibiotics-resistant strains remain a constant problem. To develop efficient novel antimicrobial drugs against these microorganisms, one needs to understand the complex molecular interactions between these microbes and their hosts. Microbial studies in human cell cultures and mice models provide high similarities to human infections but their advantages are limited for high-throughput investigations as well as the inconvenience of having to deal with multiple arms of the complex immune responses, for instance innate and adaptive defenses.

One of advantages of *D. melanogaster* is the presence of an efficient innate immune responses, and the absence of adaptive immunity. Many of the genetic factors mediating these responses are conserved in humans. The ease of rearing, short life cycle, high rate of offspring and the presence of powerful genetic tools make *Drosophila* an ideal model to study host-microbial relationships.

The immune responses in *Drosophila* upon septic injuries have been studied extensively. These responses are induced specifically against broad categories of microbes. In case of the Gram-negative bacterium *S. marcescens*, a few bacteria kill flies rapidly within a day when introduced directly into the hemolymph. A strong systemic immune response is induced but bacteria are resistant and flies eventually succumb to bacteremia. Yet, after systemic dissemination of *Serratia* in the hemolymph only a few hours after an oral infection, *Drosophila* survives for many days. The hemocytes control the proliferation of bacteria that had escaped into the hemolymph because the flies in which phagocytosis was impaired died faster because of bacteremia. Although bacteria damage the midgut epithelium, the exact

cause of death is not known. So during my PhD I asked the questions such as: what is the exact cause of death of flies during oral infection? What happens when bacteria disseminate in the hemocoel through the gut barrier, since their proliferation is controlled by hemocytes as compared to their exponential growth upon septic injury? Is it a pathogenic specific mechanism where bacteria change their virulence program during their passage through the intestine or is it host mediated? The compensatory proliferation of intestinal stem cells (ISCs) is required to repair the damages induced by *Serratia*. Complex molecular interactions involving the JNK, Hippo, JAK/STAT and EGFR pathways have been shown to mediate damage repair in the gut. I used two approaches to address these questions: i) orally infecting the wild-type and the immunocompromised flies and monitoring whether the damages in the fly gut induced by the bacteria were irreversible and could not be reversed?, and ii) screening mutant bacterial strains in survival experiments by orally infecting phagocytosis-impaired, *eater* mutant, flies to identify virulence factors of *S. marcescens* and factors required for crossing the intestinal barrier.

Most of host-microbe systems of intestinal interactions in *Drosophila* use Gram-negative bacteria. In a second project of my PhD I attempted to develop an oral infection model using a Gram-positive bacterium *Staphylococcus xylosus* strain Argentoratum. While attempting to identify the reason of an unexpected result, my work led me to investigate the relationships between immunity, microbiota, and metabolism.

2. The host factors in oral infections with *Serratia marcescens*

2.1. The host factors

The *D. melanogaster* is adapted to a diverse of microbes that it ingests during feeding on rotten fruits and vegetables. The study of the underlying host mechanisms that provide protection to the flies against hazardous microbes required the establishment of an oral infection model. Therefore, in an attempt to develop an oral infection model of *D. melanogaster* with the entomopathogenic Gram-negative bacterium *S. marcescens* the flies were fed on sucrose solution containing a constant concentration of bacteria as described in Nehme et al., (2007). Briefly, the bacteria were grown overnight in LB at 37°C. Next day, the concentration of bacteria was measured in the overnight bacterial culture, which usually ranged from an optical density of 3-4 at 600nm (OD_{600} 3-4). The bacterial solution was then diluted to an OD of 1 in sterile LB followed by a ten times dilution in sterile sucrose (50mM) solution. This infection solution was then added to the absorbent filters (Millipore) placed at the bottom of the medium fly culture tubes. Finally, flies were added in these tubes. Survival experiments were kept at 25°C.

The wild-type flies in this oral infection model succumbed only 6 days post-infection. The IMD pathway provided partial protection to the flies because *key*, an IMD pathway mutant, flies died almost 2 days earlier than the wild-type flies (Nehme et al., 2007). A significant numbers of bacteria were found in the fly hemolymph only a few hours after the beginning of infection. The proliferation of these bacteria in the hemolymph is controlled by phagocytosis because the flies for which phagocytosis was impaired either by the injection of latex-beads or the deletion of *eater* gene (Kocks et al., 2005) died faster as compared to wild-type the flies. In phagocytosis-impaired flies, the growth of *S. marcescens* in the hemolymph was exponential (Nehme et al., 2007). Taken together these findings indicated that both the IMD pathway and phagocytosis only provide a partial protection to the flies, because eventually the wild-type flies died to *S. marcescens* in this oral infection model. The exact cause of death remained undiscovered, thus raising the issue of the physio-morphological changes that occur in the flies during the course of infection. So the objective of this study was to uncover the host factors, for instance damages that might be induced in response to or by the pathogen, which might play a role in the eventual fly death in this oral infection model.

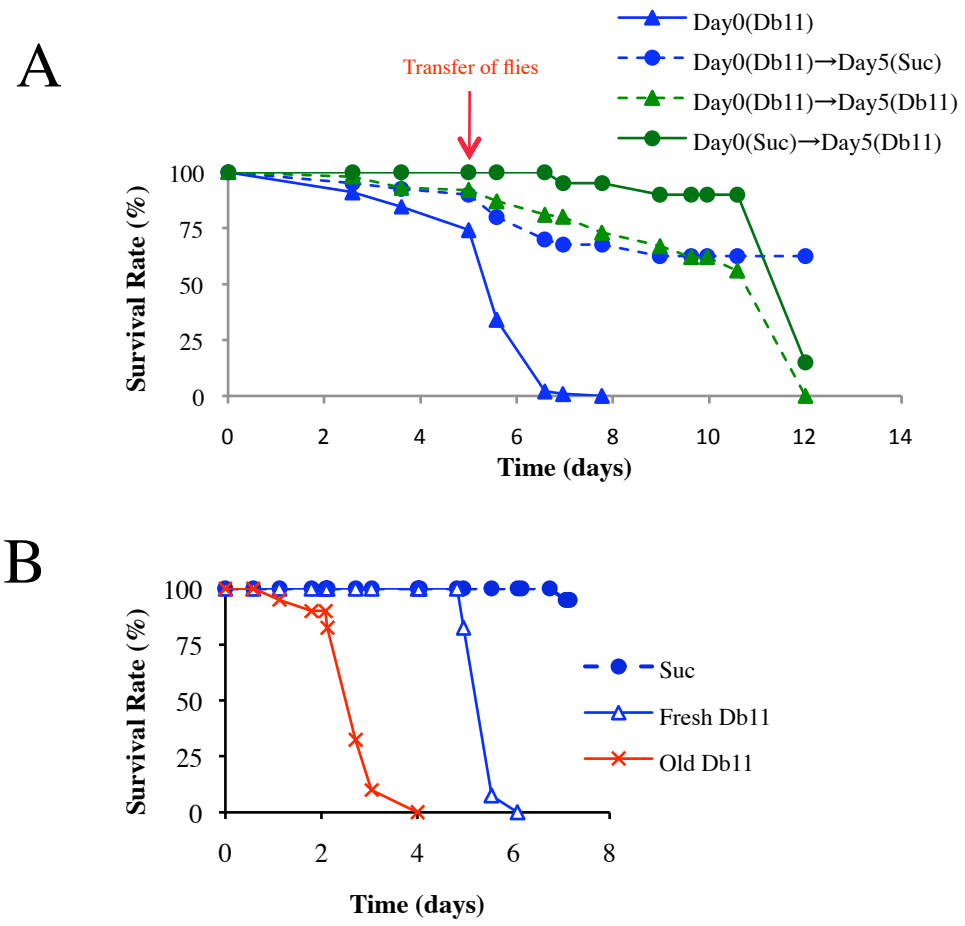

Figure 2.1: **Wild-type flies die due to old bacterial solution**

(A) The wild-type flies orally infected by standard protocol as described by (Nehme et al., 2007) die within 6 days (*blue solid* line). When the infected flies were transferred to sterile sucrose 50mM) solution 5 days post-infection (indicated by *red* arrow), almost 70% flies were still alive (*blue discontinuous* line) until 12 days when the experiment was terminated. Db11-infected flies shifted on fresh infection solution at day 5 post-infection died within 12 days (*green discontinuous* line), similar to flies which had been kept on sucrose solution for 5 days, before transferring them to the fresh infection solution (*green solid* line). (B) Naïve flies were either fed on freshly prepared bacterial solution or 5 days old infection solution on which another batch of wild-type flies had been feeding before being discarded and replaced by the naïve flies.

2.2. Wild-type flies survive a prolonged interaction with *S. marcescens* in the gut

S. marcescens induces damages to the fly gut epithelial cells as shown by the induction a JAK/STAT pathway and the enhanced proliferation of intestinal stem cells (Cronin et al., 2009). One can think that, as a result of a constant feeding of flies on *S. marcescens* for many days, some bacterial virulence factors might have caused severe damages to the host midgut epithelium. These damages may become irreversible in nature and might lead to the death of the flies. So I hypothesized that if these damages are irreversible flies should still die even if I would shift them to a sterile sucrose solution one day prior to their expected death. I therefore infected flies according to the standard procedure where flies were constantly feeding on *S. marcescens* for 5 days. At day 5, however, I shifted them to a sterile sucrose solution (50mM). Interestingly, this treatment completely rescued the flies indicating that the damages were reversible and flies could survive if shifted to the noninfected food source (Figure 2.1A). Moreover in a control experiment, flies feeding on *S. marcescens* for 5 days, were shifted to new infection tubes that were prepared with the fresh infection solution. Surprisingly, these flies died only after around 11-12 days (which is 5-6 days following the shift of the infected flies on the fresh infection solution, the time naïve flies took before eventually succumbing in the oral infection model). Also control flies that had been feeding on the sterile sucrose solution for 5 days and then were shifted to the fresh infection tubes at day 5 died within the same time period (11-12 days). These results suggested that the flies might not have died because of the irreversible damages as a result of consistent feeding on the bacteria and that the fly mortality might be associated to the infection solution present on the filter pads. To test this possibility, I did a modification in the standard infection procedure. I fed flies on the bacterial solution for 5 days according to the standard infection protocol. At day 5, however, the flies feeding on the older pads were discarded and replaced by the naïve flies. Indeed, naïve flies succumbed within just 2-3 days (Figure 2.1 B) strongly suggesting that something was changing in the older infection tubes and caused the death of flies feeding on this solution.

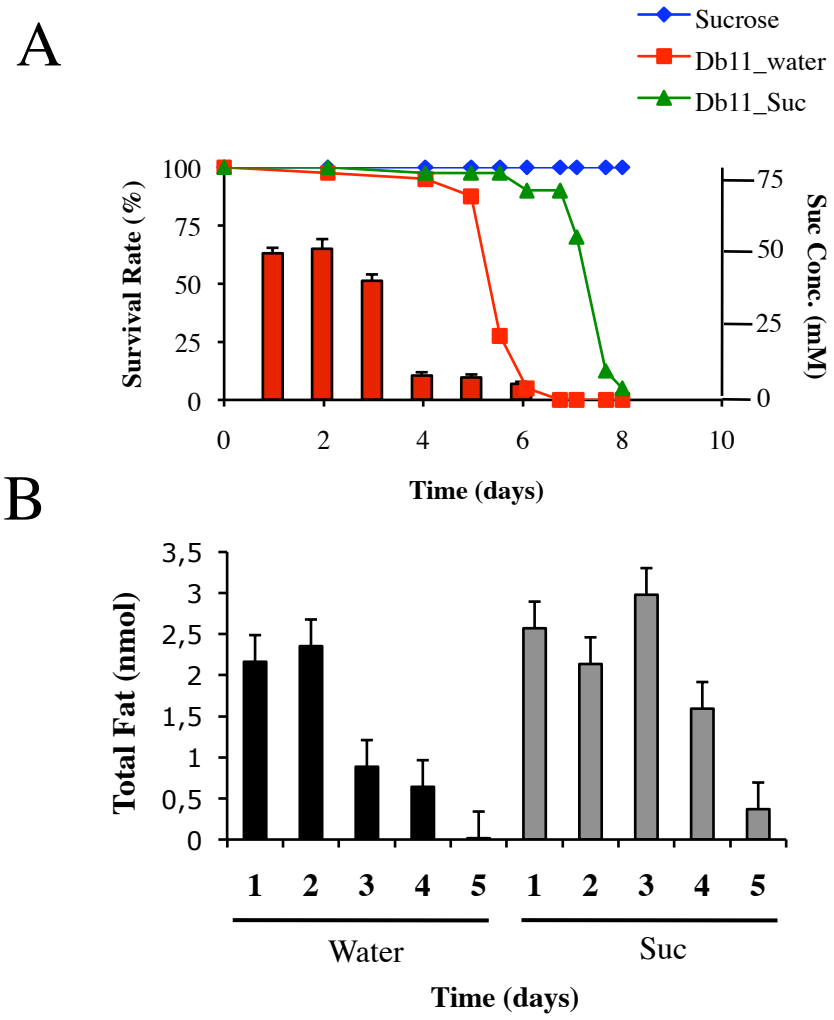

Figure 2.2: **Orally infected flies are starved**

(A) Wild-type flies orally infected by the conventional protocol (Nehme et al., 2007) died within 6 days (*red* line). However Db11-infected flies that also received a supplementation of 200µL of 50mM sucrose solution once after two days (*green* line) survived two days better than the conventionally infected flies. Quantification of sucrose in infection solution (conventional protocol where flies were not provided with additional food source) indicated depletion of sucrose solution to 10mM at day 4, as compared to the original concentration of 50mM. (B) Quantification of total fat reserves in the extracts of flies that were either infected by the conventional protocol (*black* bars) or that received an additional sucrose supply (*gray* bars). The former exhibits severe starvation symptoms at day 3 beginning of infection while the latter are starved at day 5.

2.3. Flies are starved to death

There were at least two possibilities that could explain these findings. First, the bacteria present on old filters for five days might have changed their virulence program at around 4-6 days post-infection. They expressed their full virulence program and, therefore, killed the flies rapidly. Second, there was a competition for food among the bacteria and flies that exhausted sucrose resources. Thus, the flies were actually starved and succumbed to food deprivation. Practically, it was difficult to check the first possibility since it required the study of changes in bacterial expression pattern on filters as well as inside the infected flies during the course of infection. Recovery of sufficient amount of bacteria from the infected flies and the unavailability of the *Serratia*-specific microarray chips for high-throughput studies make it a longterm project. I therefore decided to first check the second possibility. I provided the infected flies with a supplementary source of sucrose solution (addition of 400μL of 50mM sucrose solution after each 2 days) during the oral infection. The control flies received 200μL sterile water, which is usually added to maintain the required humidity in the infection vials. Indeed, sucrose supplementation improved fly survival for two days (Figure 2.2A). These results suggested that there was a deficiency of sucrose on the filters. To confirm these results, I measured the amount of sucrose on the filters that had not received an additional supply of sucrose solution. I collected the infection solution from the filters at day 1-6. In order to keep similar experimental conditions, the wild-type were allowed to feed on the infection solution before collection of the samples. Indeed, the sucrose resources started to deplete from day 3 onwards, while at day 4 only 20% of the original sucrose quantity was left (Figure 2.2A). This provided a direct proof that the flies faced food deficiency and might eventually die of starvation. However, before drawing a conclusive statement, I decided to perform an alternative experiment. If the flies feeding on *S. marcescens* infection solution had been starved, they would lose their fat reserves, which could be monitored as a starvation symptom. So, I measured the total lipid reserves from day 1 to day 5 post-infection in whole fly extracts. I tested two groups of flies: the flies that had received either 200μL of sterile water or sucrose (50mM) solution after each two days during the course of oral infection. As expected, flies receiving only pure water started losing their lipid reserves at day 3 and completely exhausted these stocks at day 5, just a day prior to their death. On the other hand,

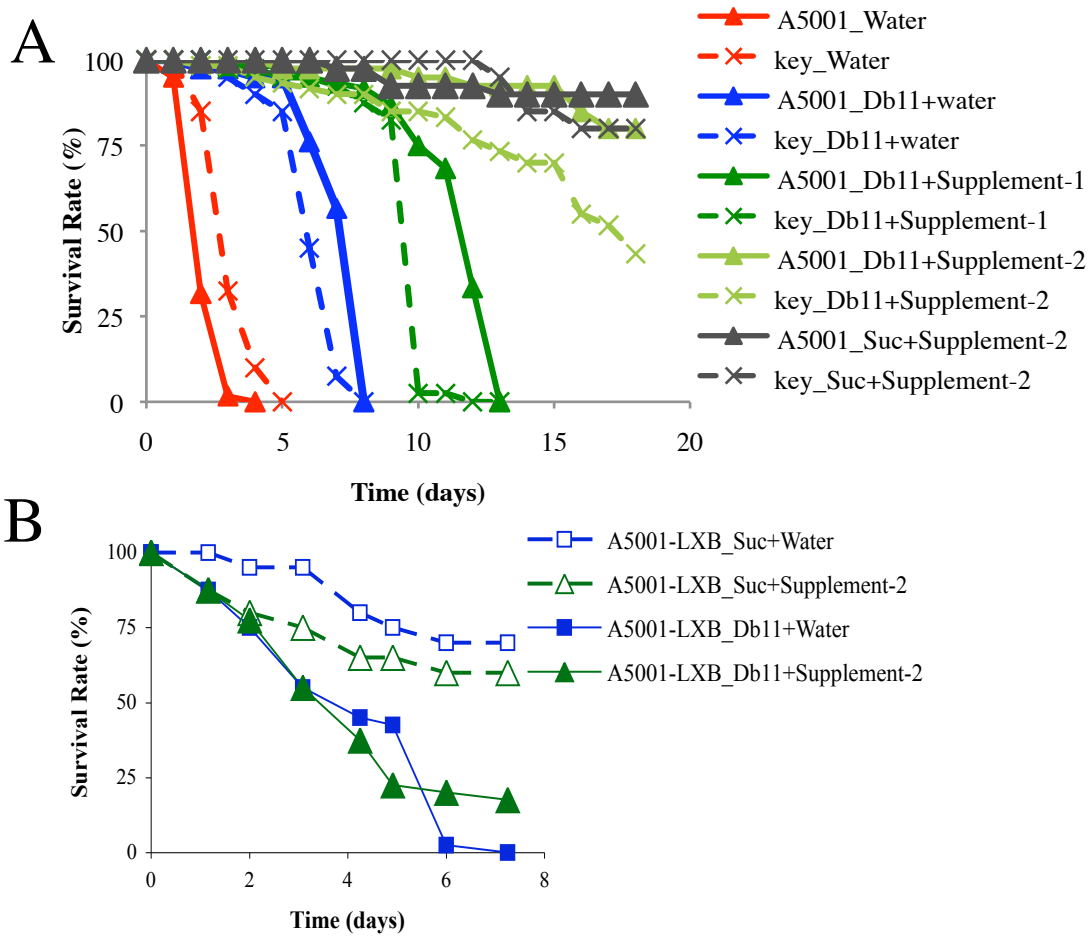

Figure 2.3: ***S. marcescens* kills immunocompromised flies**

(A) The wild-type A5001 flies die within 3 days (*red solid* line), while the *key* mutants survived one day better (*red dashed* line), in a starvation assay where flies were kept on pure water at 25°C. The Db11-infected A5001 flies and *key* mutants received three different treatments on daily basis: a) conventionally infected flies received 200µL pure water (*blue* lines), or b) 200µL of 50mM sucrose solution (supplement-1) (*green* lines), or c) 200µmM of 100mM sucrose solution (supplemented-2) (*light green* lines). In each case the *key* mutants died earlier than the wild-type flies that received the same treatment. The infected wild-type flies as well as noninfected wild-type and *key* mutants that received supplement-2 survived as long as 18 days post-infection. (B) Phagocytosis-deficient (latex beads-injected) flies die to Db11 strain upon oral feeding. Supplementation of sufficient food sources does not rescue these flies suggesting a protective role of hemocytes against the bacteria that have crossed the intestinal barrier.

flies receiving additional sucrose supply started depleting their fat reserves only at day 5 (Figure 2.2B), Taken together, these data suggest that the flies feeding on *S. marcescens* according to the conventional protocol were starved to death.

2.4. *S. marcescens* kills only the immuno-compromised flies

It has been described previously that the IMD pathway provides protection to the flies against *S. marcescens* in the gut because *key* mutants died faster as compared to the wild type controls when orally infected following the conventional protocol. One could argue that in an assay where the wild-type flies succumb due to starvation, the *key* mutants might have died faster because of an enhanced susceptibility to starvation. So, to test this possibility, I decided to starve the *key* mutants as well as the wild-type flies. For this purpose the flies were added to the vials that contained filters soaked with pure water. In parallel, the two fly strains were also infected according to the conventional infection protocol (*blue* lines; Figure 2.3). The *key* mutants actually survived mildly better than the wild-type flies in starvation assay (*red* lines; Figure 2.3). As it had been shown earlier (Nehme et al., 2007), *key* mutants infected by the conventional protocol died faster as compared to the wild-type controls. These results indicate that starvation does not play a role in the susceptibility of *key* mutants observed in the conventional infection model. Of note, the wild-type flies died within 3 days in the starvation assay, which is the time it took to kill naïve flies transferred on 5 days old infection pads (Figure 2.1B (note that the flies died within 3 days)).

I further tested the susceptibility of the *key* mutants and wild-type flies to orally fed *S. marcescens* in food supplementation assays. The *key* mutants and the wild-type flies were infected by the standard procedure; however, both fly strains were provided with the additional source of sucrose during the course of oral infection. The sucrose solution was supplemented by one of the two methods: addition of 400μL of 50mM sucrose solution every other day (*supplement-1*), or daily supplementation of 200μL of 100mM sucrose solution (*supplement-2*). The wild-type flies that received *supplement-1* died at day 13 post-infection while the ones that received *supplement-2* did not die 18 days post-infection the when experiment was terminated. Moreover, another investigator, Dr Matthieu Lestradet, monitored the titer of live *S. marcescens*

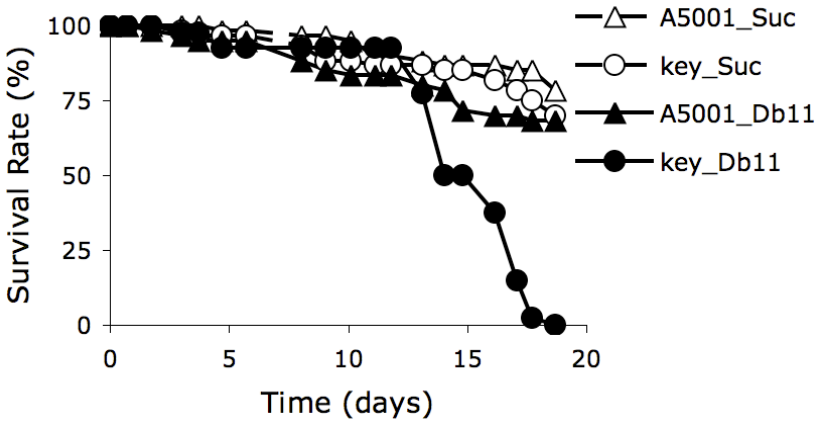

Figure 2.4: **The new oral infection model**

The wild-type *S. marcescens* strain Db11 was grown in LB overnight at 37°C. Next day, the bacterial culture was centrifuged at 5000 rpm for 15 minutes. Supernatant was discarded and the bacterial pellet was resuspended in fresh LB to a final optical density of 10 (OD_{600} 10). The bacterial suspension was then diluted ten times in sucrose (50mM) and 2mL of the infection was used to soak two absorbent filters in medium sized fly culture tubes. 20 flies were added in each tube and survival experiment was performed at 25°C. 200uL of sterile sucrose (100mM) solution was added to the filters on daily basis. Survival was monitored at least once per day. The wild-type do not die to this treatment while the IMD pathway, *key*, mutant flies die within about 16 days.

in the infection solution on filters that received *supplement-2*, with wild-type flies feeding on them. He concluded that the original bacteria titer was maintained till day 15 post-infection when he terminated the experiment. These data indicate that despite the stress symptoms observed by the induction of JAK/STAT pathway and compensatory stem cell proliferation (Cronin et al., 2009), the wild-type flies are able to repair damages and are resistant to the oral infection of *S. marcescens*. Moreover, the addition of sucrose solution by *supplement-2* is sufficient to avoid starvation, both in flies and bacteria.

Interestingly, the *key* mutant flies always died faster than the wild-type flies. Even in the presence of sufficient food supply, more than 50% of the *key* mutants succumb to *S. marcescens* (Fig. 2.3A). These data re-establishes that the IMD pathway is critical in the *Drosophila* gut against *S. marcescens* oral infection. The role of phagocytosis against an oral infection of *Serratia* was also retested. The flies where phagocytosis was impaired by the injection of latex beads, died even in the presence of sufficient food sources (Fig. 2.3B) This reconfirmed that the phagocytosis protects flies from systemic bacteremia during oral infection with *S. marcescens*. Taken together I have shown that the IMD pathway and the phagocytosis are essential to provide full protection to the flies against the orally fed *S. marcescens*.

2.5. The new infection model

Since wild-type flies died due to starvation during infection following conventional protocol, there was a need to develop an alternative oral infection assay to further investigate pathogenesis of the *S. marcescens* in the *Drosophila m*idgut. Although the addition of sucrose by *supplement-2* was sufficient to avoid starvation, the immuno-compromised *key* flies died very slowly (only 50% dead after 18 days of infection). So, I tested different initial concentrations of *S. marcescens* (it was a collaborative work with two other investigators, Dr Matthieu Lestradet and Dr Kwang-Zin Lee).

When an initial concentration of bacteria was increased ten times (*i.e.* OD 1 at 600nm (instead of OD_{600} 0.1) in 50mM sucrose solution) and flies were provided with 200µL of 100mM sucrose solution on daily basis (*supplement-2*), wild-type flies did

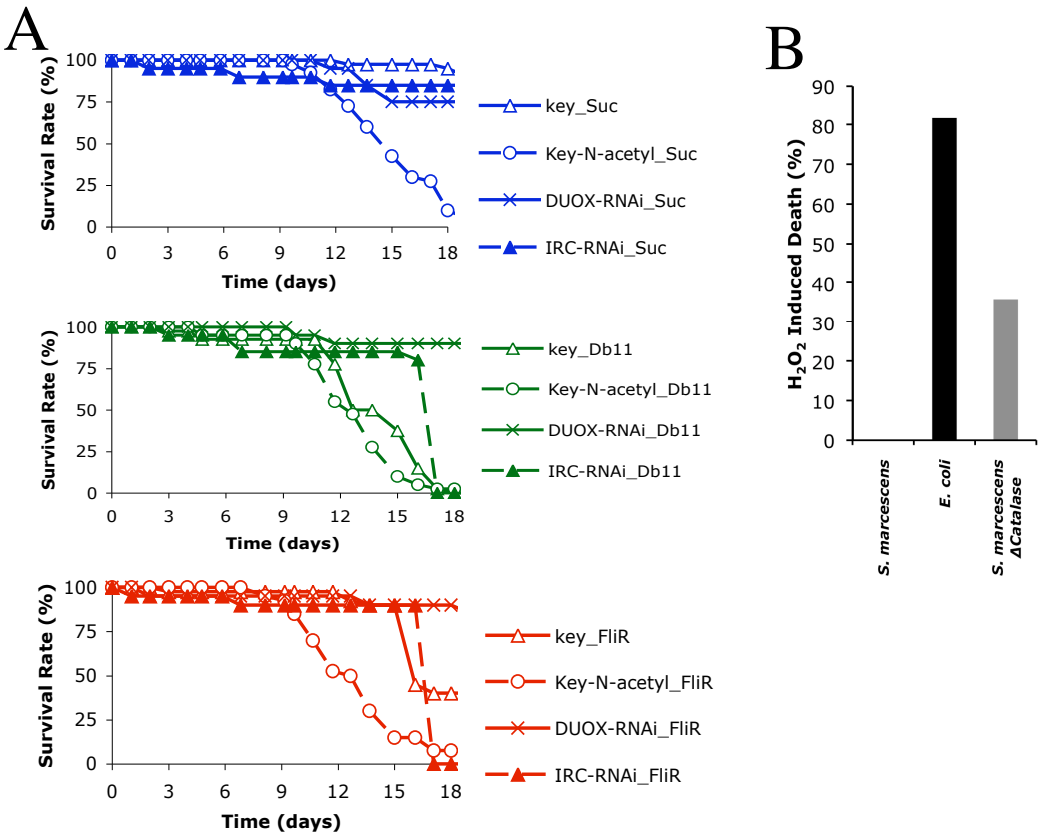

Figure 2.5: ***S. marcescens* resists to ROS in *Drosophila* gut**

(A) The knockdown of DUOX and IRC was achieved by crossing DUOX-RNAi and IRC-RNAi lines, respectively, with the gut specific, NP1-*Gal4*/*80*, driver at 25°C. 3-8 days old flies were then incubated at 29°C for 2 days before infecting with Db11 strain or *fliR* mutants following the newly developed oral infection model (see Fig. 2.4). It is possible that in the experiment with the *fliR* mutants, the *key* flies actually succumb to a secondary infection from some component of the microbiota as is likely the case for key flees fed only on sucrose supplemented with N-acetylcystein. (B) Bacterial strains were grown in LB overnight at 37°C. Next day LB was added to the overnight cultures to bring the bacterial concentration to optical density of 1 (OD_{600} 1). Finally, the bacterial suspension was diluted 10,000 times in 0.01% H_2O_2 (diluted in pure water) solution and incubated for 30 minutes at room temperature before plating on LB plates. Death rate was calculated by the following formulae:

Death rate (%) = 100 – ((number of H_2O_2 treated bacteria/ number of non-treated bacteria) x 100)

not die until day 20 when the experiment was terminated (Fig. 2.4). All *key* mutants, however, died within 16 days post-infection. So we concluded that the flies orally infected with *S. marcescens* following this protocol could be used to further investigate the host-pathogenic interactions between *D. melanogaster* and *S. marcescens*. The *key* mutants die at very different rates (and slopes), depending on the availability of nutrients. Thus, a defective IMD-dependent response leads to nutrient-dependent detrimental effects. The provision of adequate food supply may partially compensate immune response.

2.6. *S. marcescens* resists to ROS in *Drosophila* intestine

Another important arm of the intestinal immune defense of *D. melanogaster* is the secretion of reactive oxygen species (ROS). ROS are produced by a transmembrane dual oxidase (DUOX) enzyme in response to ingested microbes or inhabiting commensals. ROS are free oxygen radicals, such as the superoxide, or peroxide, or hydroxyl radicals, which are cytotoxic and kill the microbes in the gut. The overproduction of ROS, however, is toxic to the flies themselves. The excessive amount of ROS is neutralized by the immune response catalase (IRC) in the *Drosophila* intestine. I utilized the newly established infection model to study a role of the oxidative immune response against *S. marcescens* in the *Drosophila* intestine. I used four types of flies to performed survival experiments: i) *key* mutants, ii) *key* mutants minus ROS response (by treatment with N-acetylcysteine that supposedly neutralizes ROS), iii) DUOX-downregulated mutants (by overexpressing DUOX-RNAi using gut specific NP1-Gal4 driver), and iv) IRC-downregulated flies (IRC-RNAi driven by NP1-Gal4). All four types of flies were either fed on sterile sucrose solution or orally infected with wild-type *S. marcescens* strain Db11 or *fliR* mutant, a nonmotile strain mutated for *fliR* gene that encodes a protein which is essential for the biosynthesis and function of flagella.

As expected the *key* flies infected with Db11 strain died within 16 days (Fig. 2.5A *green*). *fliR* mutants did not kill flies as rapidly as Db11 (please see Ayyaz et al., *in preparation* (Chapter 4) for discussion) (Fig. 2.5A *red*). The infected flies in which DUOX was downregulated did not die to Db11 strain or *fliR* mutants suggesting that the ROS response does not provide protection to the flies against *S. marcescens* in

the fly gut. However, the IRC-deficient flies infected with Db11 or *fliR* died within 17 days. These results suggest that a mild ROS response is induced in the gut against *S. marcescens*. It should however be reversible by N-acetylcystein treatment. The wild-type flies efficiently control the overproduction of ROS using IRC to avoid self-toxicity. Finally the *key* flies in which ROS response was neutralized by mixing N-acetylcysteine in food source died in non-infected control experiments where these flies were feeding only on sucrose solution (Fig. 2.5A *blue*). These flies are deficient for both major immune responses (i.e. AMPs and ROS production) so the mortality in these flies might be the result of outbreak of some potentially hazardous microbial population(s) residing in the *Drosophila* intestine. Of note, ROS-deficient *key* flies infected with Db11 strain or *fliR* mutants died about two days earlier, again suggesting a protective role of IMD pathway against *S. marcescens* in the gut.

We know that the *S. marcescens* genome encodes one catalase. So I hypothesized that if *Serratia* could neutralize ROS response in the fly gut, it should be resistant to H_2O_2 *in vitro*. I included a nonpathogenic *E. coli* strain and the catalase mutant of *S. marcescens*, created in strain Db10 by my former supervisor, Dr Philippe Giammarinaro. Indeed, no death was observed in *S. marcescens* to the same concentration of H_2O_2 where about 80% of the *E. coli* cells died (Figure 2.5B). Surprisingly, only about 35% of the catalase mutants of *S. marcescens* were killed. To further verify that the catalase mutants do not neutralize the H_2O_2 at all, I dipped these mutants and Db11 strain in a concentrated solution of H_2O_2 (30%) with the help of wooden tooth pick. I did not observe the production of bubbles that were immediately produced in large amounts by Db11 strain upon dipping in the H_2O_2. These results indicate that *Serratia* has multiple mechanisms to neutralize and tolerate hostile environments. To definitely establish that *S. marcescens* catalase helps to protect against host generated ROS, I shall challenge *key* mutant flies with the catalase bacterial mutant, with or without N-acetylcystein.

3. The virulence factors of *Serratia marcescens*

List of Potential Mutants Isolated from Screen											
Strain	**Fly Death**	**Strain**	**Fly Death**	**Strain**	**Fly Death**	**Strain**	**Fly Death**	**Strain**	**Fly Death**	**Strain**	**Fly Death**
10 A2	**Slow**	16 B1	**Slow**	18 H11	**Slow**	63 E9	**Fast**	63 H2	**Fast**	59 B10	**Fast**
10 A3	**Slow**	16 C1	**Slow**	19 E9	**Slow**	63 A8	**Slow**	58 G1	**Slow**	59 C10	**Slow**
10 C3	**Slow**	17 A8	**Slow**	19 E11	**Slow**	63 B11	**Slow**	58 H6	**Slow**	55 C1	**Fast**
10 E2	**Slow**	17 E6	**Slow**	19 D12	**Slow**	63 C7	**Slow**	58 G9	**Fast**	55 E1	**Fast**
10 H2	**Slow**	17 C3	**Slow**	19 E12	**Slow**	63C11	**Slow**	58 B12	**Slow**	55 H2	**Slow**
11 A9	**Slow**	17 B11	**Slow**	19 G12	**Slow**	63 D9	**Slow**	60 A2	**Slow**	55 E8	**Slow**
11 H12	**Slow**	17 A3	**Slow**	19 H12	**Slow**	63 D10	**Slow**	60 C4	**Slow**	57 F1	**Fast**
11 E3	**Slow**	18 F8	**Slow**	62 E1	**Slow**	63 H12	**Fast**	60 C6	**Fast**	57 F2	**Slow**
16 D5	**Slow**	18 G8	**Slow**	62 E2	**Slow**	63 F11	**Fast**	59 B4	**Fast**		
16C9	**Slow**	18 D7	**slow**	62 H9	**Slow**	63 C12	**Fast**	59 A11	**Slow**		

Table 3.1: **Internal annotation numbers of the potential transposon insertion mutants with attenuated virulence in *eater* mutant flies**

eater mutant flies were infected, in batches of 10, with one of the 48 *S. marcescens* mutant strains in each set of survival experiments (protocol for oral infection is described in (Nehme et al., 2007)). Multiple control survivals were also included where *eater* mutants were infected with the wild-type *S. marcescens* strain Db11. Fly survivals were monitored once per day. After the termination of survival experiments, LT50 (time a bacterial strain takes to kill 50% flies) were calculated from each survival curve. The bacteria mutant strains whose LT50 values were more than $\pm$ 2 standard deviation (SD) from the mean value were considered potential mutants. The LT50s calculated from the Db11 infected controls were usually similar to the mean LT50 values.

Note: The six underlined strains were reconfirmed in second round of survival experiments (see Table 2A).

3.1. Rationale for the screens

One interesting feature of the *S. marcescens* intestinal model is that bacteria are able to cross the digestive tract very early on during the infection. Yet, bacteria that have escaped into the hemocoel appear to be quiescent with little if any proliferation and are controlled by the cellular immune response, namely phagocytosis by plasmatocytes. Thus, they do not appear to contribute to pathogenesis in this model. When the cellular immune response is impaired, either through the loss of the phagocytic receptor Eater or by saturation of the phagocytic apparatus by the prior injection of latex beads, *S. marcescens* proliferates to high levels in the hemocoel, which likely provokes the early demise of infected flies. We therefore surmised that mutant bacteria with altered virulence or bacteria impaired in their ability to cross the intestinal epithelium would display a slower death rate in *eater* null mutant flies. Alternatively, bacteria mutant for negative regulators of bacterial virulence might exhibit a higher killing rate. Thus, we screened a *S. marcescens* Db10 miniTn5 transposon insertion library previously generated in another laboratory (Pradel et al., 2007).

As proteases have been reported to participate in *S. marcescens* virulence in *Drosophila* (Flyg and Xanthopoulos, 1983; Kaska et al., 1976), we also tested this library on milk plates. Wild-type *S. marcescens* colonies form a clear zone at their periphery. In contrast, mutants with defective protease function or secretion were characterized by a hazy halo.

3.2. Results from the screens

We thus fed batches of 10 *eater* null flies with one bacterial mutant strain. In fact, 48 mutant strains were challenged in parallel, with two to three additional vials of flies challenged with wild-type *S. marcescens* Db11 strain, which has the same antibiotics markers as the transposon insertion mutants in the Db10 strain. In a first round, we tested 1348 strains, 58 of which caused an altered survival profile of infected *eater* flies, that is over 4% of the strains tested (Table 3.1). Twelve strains led to an apparent increased virulence, that is about 20% of the putative mutants. Only five strains displayed the original phenotype when retested at least three times, that is that *eater* mutant flies that had ingested these mutants (Table 3.2A) died more slowly to infection than flies that had ingested wild-type *S. marcescens* Db11.

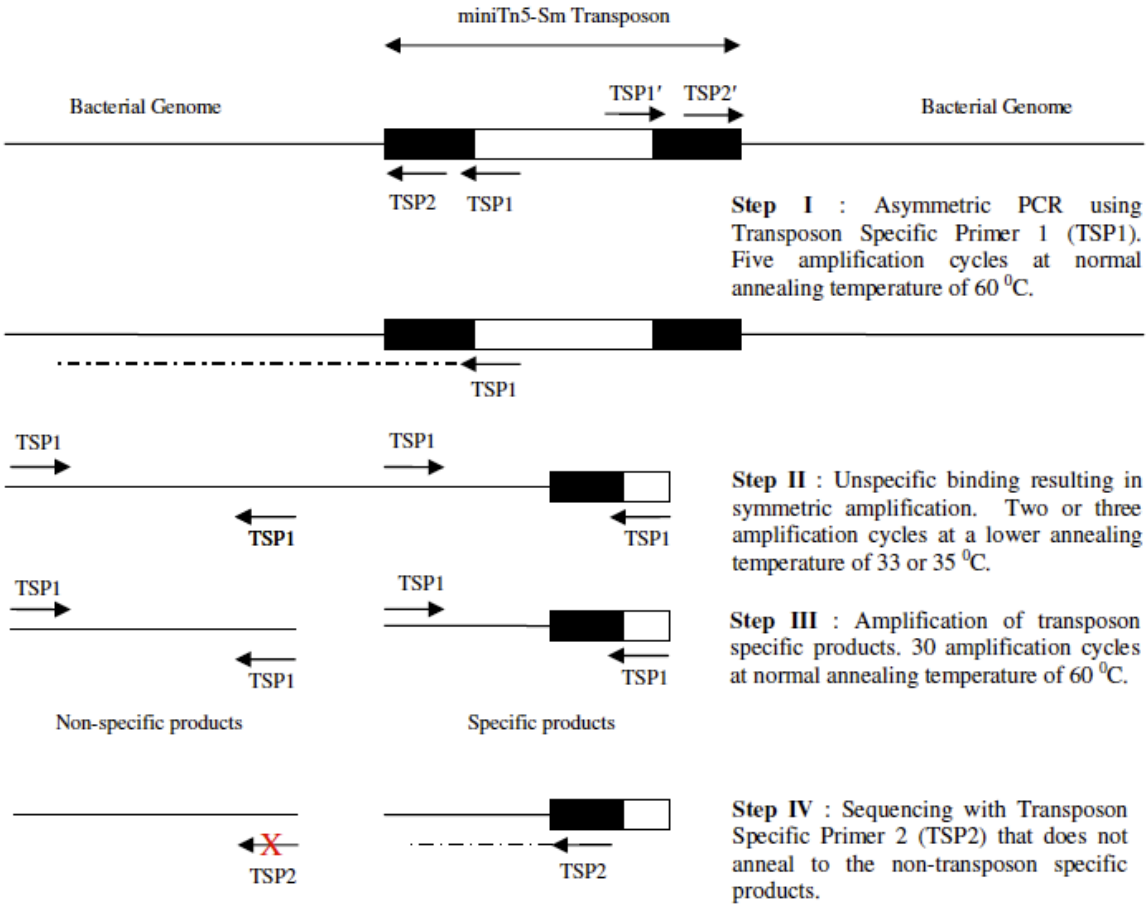

Figure 3.1: **Identification of transposon insertion site in the genome of *S. marcescens* by single primer PCR.** Two transposon-specific primers on left side: (TSP1: 5'ctaggcggccagatctgatcaa3', and TSP2: 5'gctgttcttctacggcaagg3') and two on right side (TSP1': 5'caccaaggtagtcggcaaat3', and TSP2': 5'cgaacttgtgtataagagtcag3') of the mini-Tn5-Sm transposon were designed, respectively. The DNA fragments were amplified in a three step PCR reaction: i) Step I: TSP1 and TSP1' were used separately to amplify single strand transposon-specific DNA fragments that also contained part of *S. marcescens* genome-specific DNA sequence, ii) Step II: The melting temperature (Tm) was decreased to generate double-stranded sequences as a result of non-specific binding of the primers, and iii) Step III: double stranded DNA fragments were then amplified at normal Tm. Finally, in Step IV, the amplified fragments were purified from PCR reaction mixtures and sequenced using remaining two transposon-specific primers (TSP2 for TSP1-, while TSP2' for TSP1'-amplified fragments) that yielded part of transposon-specific and part of *S. marcescens* genome-specific DNA sequences. These sequences were blasted with genome of *S. marcescens* using BioEdit software. After the identification of the site of insertion of the transposon, primers were designed in the genome of *S. marcescens* in the vicinity of the transposon insertion site to perform a normal PCR reaction for reconfirmation of the results obtained from the single primer PCR.

In the milk plate screen, only two mutant colonies were retrieved in the first round (Table 3.2A). These were then confirmed several times on milk plates, and then by zymogram electrophoretic analysis (Ayyaz et al., *in preparation* (Chapter 4)). The two proteases we could consistently detect on the zymograms were missing for both mutant strains. Interestingly, one of the mutant strains had also been picked up and confirmed in the *in vivo* screen.

Next, we determined the insertion site of the transposon in the mutant strains by single primer PCR ((Karlyshev et al., 2000) and Figure 3.2). We did not succeed in mapping one mutant strain. The two protease-deficient mutants mapped to the same operon, the extracellular Lipase secretion system, which is described to secrete a lipase (LipA) and the surface layer protein (SlaA). Next, we generated independent mutants of the identified mutant loci in the *S. marcescens* Db10 strain by plasmid-mediated site-directed insertional mutagenesis. In addition, we also generated mutants for four potential virulence factors, namely in the single catalase (*catA*) gene present in the Db11 genome, in the *gacA* gene, which encodes a virulence regulator in *Pseudomonas entomophila* (Liehl et al., 2006), and in a gene encoding a putative metalloprotease. These mutants were then tested again in the *in vivo* assay. Unexpectedly, the phenotype was not conserved for any of the mutants, except for the insertion in the *fliR* gene. The reason for the discrepancy is not clear. However, we note that all of these mutants were coming from the same half of a 96-well plate in which the library had been replated. Thus, bacteria present on that plate may have been somehow damaged during handling.

3.3. Secreted proteins of *S. marcescens*

S. marcescens secretes various extracellular proteins including proteases, chitinases, lipase and a nuclease (Hines et al., 1988). In an attempt to identify the secreted proteins of *S. marcescens*, we performed mass spectrometry analysis on proteins purified from supernatants of overnight cultures of Db10 strain. We identified 37 proteins with low rate of reproducibility. Four proteins were consistently found in these experiments namely a secreted nuclease, a chitinase (ChiB), a putative antioxidant and a catalase (Figure 3.3).

The *S. marcescens* extracellular nuclease (NucA) is encoded by $nucA_{Sm}$ gene. It is considered to be a virulence factor. The *S. marcescens* nuclease is nonspecific

A

Name of Strain	Annotation Number	Gene mutated	Gene Function	Survival Screen ($LT50_{ave}$)		Protease deficient
19 H12	sma2207	*fliR*	Type III flagellar protein export system	**5,7**	***	No
19 G12	sma0865	*lipB*	*lip* Operon	**6,9**	***	Yes
19 E11	sma4383	*thiH*	Biotin and Thiamin synthesis/ Thiazole biosynthesis	**7,9**	***	No
19 E9	sma3959	*sma3959*	Two component signal transduction system	**7,0**	***	No
19 D12			Uncharacterized	**7,7**	***	No
19 E12	sma0866	*lipC*	*lip* Operon	**4.4**	ns	Yes
Db11			Wild-type	**4,7**		No

B

Name of Strain	Annotation Number	Gene mutated	Gene Function	Attenuation of virulence
FliR	sma2207	*fliR*	Type III flagellar protein export system	Yes
LipB	sma0865	*lipB*	*lip* Operon	No
KatA	sma2675	*catA*	Catalase	No
gacA	sma1098	*grrA/gacA*	Regulator for protease and chitinase production	No
sma1606	sma1606	*sma1606*	Extracellular hemolysin-type zinc-binding metalloproteinase	No
LipC	sma0866	*lipC*	*lip* Operon	No

Table 3.2: **Characterization of potential mutants of *S. marcescens***

(A) Potential mutants identified in the first round of screen were reconfirmed in *eater* fly survival experiments and protease activity assays (milk plates). LT50s of each survival curve values were compared with that of Db11 by LogRank test of Prism software; ($p > 0.05$ for (ns), and $p < 0.0001$ for (***)). Only two mutants strains, 19G12 and 19E12 were found deficient for protease production on milk plates. (B) Homologous recombination based site-specific plasmid insertion mutants were created and tested for attenuation of their virulence in survival experiments of *eater* mutants as well as wild-type flies. Only one mutant strain mutated for *fliR* gene killed flies slower than the wild-type Db11.

Annotation	Function	M.Wt./PI	Exp. 1	Exp. 2
SMA1061	Secreted nuclease	26921/6.23	X	X
SMA2875	Chitinase	55661/6.09	X	X
SMA0310	Putative antioxidant	22353/5.33	X	X
SMA2675	Catalase	55277/5.89	X	X
SMA2962	Fructose-bisphosphate aldolase	38324/6.51	X	
SMA1461	Pyruvate kinase	50837/5.81	X	
SMA3345	Fructose-bisphosphate aldolase	39088/5.81	X	
SMA2877	Chitin binding protein (cbp21)	21801/8.36	X	
SMA3966	Putative spheroplast protein	18674/9.37	X	
SMA2601	Phage tail sheath protein	53230/5.3	X	
SMA0206	Phosphoheptose isomerase	21029/5.79	X	
SMA3263	Quorum-sensing related protein	23913/5.55	X	
SMA4189	Superoxide dismutase (sodA)	23164/6.05	X	
SMA2606	Putative phase major capsid protein	45917/5.23	X	
	Ampicillinase	25177/5.27	X	
SMA1997	Glyceraldehyde 3-phosphate dehydrogenase A, glyceraldehyde 3-phosphate dehydrogenase	33678/6.27	X	
SMA2263	Conserved hypothetical protein	17010/6.06	X	
sma2904	Dihydrodipicolinate synthase	31285/5.59		X
sma3339	No match prediction	20256/8.54		X
sma2843	Cystein synthase A	34417/5.16		X
sma1323	No match prediction	16517/4.88		X
sma3181	2,3,4,5-tetrahydropyridine-2-carboxylate	30039/5.55		X
sma1037	Outer membrane protein A	38516/8.27		X
sma0261	Hypothetical protein of isochorismatase			X
sma1701	Phospholipase D	56143/6.19		X
sma2481	Alpha/beta hydrolase	30372/6.18		X
sma1014	Outer membrane protein	40781/4.51		X
sma2846	Glucose specific PTS system	15623/4.59		X
sma2659	Outer membrane protein	41414/4.74		X
sma4311	Serralysin (Metalloprotease)	53974/4.55		X
sma1037	Outer membrane protein A	38516/8.27		X
sma1037	Outer membrane protein A	38516/8.27		X
sma2606	Phage capsid with phosphopantetheine attachement site	46059/5.33		X
sma1367	Metalloproteinase	50462/4.55		X
sma0761	Outer membrane protein	18537/6.06		X
sma1542	Glycosyl hydrolase	65621/6.51		X
sma1859	Dioxygenase superfamily protein	14592/5.16		X

Table 3.3: **Secreted proteins of *S. marcescens***

Please turn the page for details…

Table 3.3: **Secreted proteins of *S. marcescens***

Fresh Agar culture plates were prepared by plating *S. marcescens* strain Db10 and incubating overnight at 37°C. Next day a liquid culture of *S. marcescens* was grown in LB at 370C overnight. The supernatant was centrifuged, filtered, added to pre-coold acetone in a ratio of 1:4 and incubated overnight at –20−C. After centrifugation pellets were washed with acetone and ethanol. Sephadex G25 was use to remove salts and impurities and 2D buffer [Urea (7M), Thiourea (2M), CHAPS (4%) and Tris (30mM)] was used to dissolve samples. Amounts of total proteins in samples were quantified by Standard Bradford method. Samples were then dissolved in rehydration buffer [2D buffer (90.5µL), DTT (6µL), Ampholyte (3µL) and of bromophenol blue (0.5µL)], spread on Nonlinear pH (pH range: 3-10) strip and incubated overnight at small current of 50 volts. Isoelectric focusing (horizontal protein spreading based on their iso-electric point) was performed by providing a series of different voltage as follows:

	Voltage	**Rise in voltage**	**Duration**	**Temperature**
Stage 1	250V	Rapid	15min	20°C
Stage 2	8000V	Progressive	5h-8h	20°C
Stage 3	8000V	Rapid	volts x hours 60,000-80,000	20°C
Stage 4	500V	Rapid	12h	20°C

The strips were then treated with denaturation solution (urea = 3.6 g, Tris HCL (1.5M, pH: 8.8) = 2.5 mL, Water milliQ = 1 mL SDS 10% = 2 mL, and Glycerol = 2 mL). DTT was used to reduce S=S bridges, iodoacetamide for alkylation, and bromophenol blue (0.5%) for visualization of the limit of migration on the SDS-PAGE. The SDS PAGE was prepared by Tris (1.5M, pH:8.8), Acryl (40%), SDS (10%), APS (10%) and Temed (50µL). The migrated proteins were stained using Commassie Brilliant Blue G-250 (colloidal blue) solution. Prominent spots were analyzed and protein sequence was determined by mass spectrometry at Plateforme Protéomique Strasbourg Esplanade, Strasbourg, France.

and its catalytic activity is 34 times greater than that of DNase I (Benedik and Strych, 1998). High rate of mortality was observed in porcine fetal fibroblasts expressing NucA (Caballero and Piedrahita, 2009). Interestingly, the *flhDC* operon that controls the synthesis and regulation of flagella, is required for the secretion of nuclease, as well as a phospholipase that was also identified in this study. We have shown that a flagellar protein, FliR, was required to invade and damage enterocytes in *Drosophila* midgut epithelium (Ayyaz et al., *in preparation* (Chapter 4)), therefore, it will be highly interesting to test the ability of a nuclease mutant *S. marcescens* strain to invade, damage, and escape the midgut barrier of *Drosophila*. The chitinase of *Serratia* (Brurberg et al., 1995; Harpster and Dunsmuir, 1989) degrades chitin (Vorgias et al., 1996). The fact that the peritrophic matrix, the first line of defense in the *Drosophila* midgut, is partially composed of chitin makes it highly interesting to test the ability of a *Serratia* mutant for chitinase to cross the peritrophic matrix. In addition to the catalase and a putative antioxidant, a third antioxidative protein, superoxide dismutase, was also secreted indicating the presence of multiple mechanisms in *Serratia* to neutralize the oxidative response in the *D. melanogaster* digestive tract.

Interestingly two metalloproteinases were also found in the supernatants of *S. marcescens*. Serralysin has previously been described as an insecticidal protein in locust (Tao et al., 2006), while in humans it induces the inflammatory response after being sensed by the protease-activated receptor 2 (Kida et al., 2007). Interestingly, two protease-deficient mutant strains, 19G12 and 19E12 with mutations in *lip* operon, were found deficient for the production of two active proteases, the molecular weight of one of them (54 kDa) corresponding to that of serralysin. This suggests that the *lip* operon of *S. marcescens* might be required for the extracellular secretion of serralysin, in addition to lipase A (LipA) and surface layer protein (SlaA).

4. *Serratia marcescens* needs *fliR* gene to cross intestinal barrier

Serratia marcescens needs the flagellar component FliR to invade and damage the *Drosophila* midgut epithelium

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Introduction

The antimicrobial defense in *Drosophila melanogaster* depends on innate immunity, the cellular and humoral responses being the major defense mechanisms. Phagocytes engulf circulating microbes while fat body cells secrete antimicrobial peptides (AMPs) upon recognition of the structural components of microbes or the detection of virulence factors enzymatic activity (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). Gram-negative bacteria and some Gram-positive bacilli are sensed through their meso-diamiopimelic acid-containing peptidoglycans (DAP-type PGNs), while often Gram-positive bacteria and fungi are recognised through lysine-containing (Lys-type) PGNs and glucans, respectively. The *Drosophila* genome encodes Pattern Recognition Receptors (PRRs) that sense these microbes. The Peptidoglycan Recognition Protein-LC (PGRP-LC) and PGRP-LE bind to DAP-type PGNs. PGRP-SA, GNBP1 and PGRP-SD sense the Lys-type PGNs while GNBP3 is activated by glucans. Two genetically conserved signaling cascades, namely the Immune deficiency (IMD) pathway (activated by Gram-negative bacteria) and the Toll pathway (turned on by Gram-positive bacteria and fungi), control the expression of partially overlapping sets of AMPs, generally active against the respective categories of microbes. The AMPs are either systemically produced by the fat body cells against microbes disseminated in the hemocoel or secreted locally by barrier epithelia (Akhouayri et al., 2011; Ferrandon et al., 1998; Han et al., 2004; Tzou et al., 2000) against environmental, commensal, or pathogenic microbes.

The *Drosophila* intestine provides a niche for inhabiting commensals. Hazardous microbes and occasional pathogens are contained by physical and chemical barriers. The pertrophic matrix lines the gut epithelium and restricts microorganisms to the lumen of the digestive tract, that is the endoperitrophic space (Kuraishi et al., 2011). The transmembrane protein Dual Oxidase (DUOX) of midgut epithelia cells secretes cytotoxic Reactive oxygen species (ROS) in response to microbes through an unknown sensing mechanism (Bae et al.,

2010; Ha et al., 2005a; Ha et al., 2005b). AMPs are also secreted by the midgut epithelial cells, however, in an IMD pathway-, and not Toll pathway-, dependent manner (Liehl et al., 2006; Nehme et al., 2007; Ryu et al., 2006). Despite the sophisticated immune responses in the *D. melanogaster* intestine, some microbes are able to resist, survive, damage and cross the intestinal barrier and then may cause systemic infections (Limmer et al., 2011; Nehme et al., 2007).

Serratia marcescens is a Gram-negative bacterium. It is an opportunistic pathogen and is becoming a major cause of nosocomial infections in humans (Iosifidis et al., 2012; Maltezou et al., 2012; Voelz et al., 2010). It is also found associated with inflammatory diseases of the human intestine (Carlisle et al., 2011; Filius et al., 2005; Heidemann et al., 2003). *S. marcescens* is a potent pathogen in a septic injury model in *Drosophila* (Nehme et al., 2007). A few bacterial cells kill the flies within a day when introduced directly into the hemolymph. A strong systemic immune response is induced, yet the bacteria are resistant to the AMPs and the flies succumb to the massive proliferation of bacteria in the hemolymph. Interestingly, the flies do not succumb in an oral feeding assay with *Serratia* for many days, although low but significant numbers of bacteria are retrieved from the hemolymph of the flies only a few hours post-infection suggesting that it is able to cross rapidly the intestinal barrier. The midgut epithelium shows signs of stress and damage upon oral feeding of *S. marcescens* (Cronin et al., 2009; Nehme et al., 2007). Homeostasis in the gut epithelium is maintained by compensatory proliferation of intestinal stem cells (ISCs) driven partially by the JAK/STAT pathway (Buchon et al., 2009; Chatterjee and Ip, 2009; Cronin et al., 2009). The IMD pathway mutant flis for *kenny* (*key*) died faster as compared to the wild-type flies suggesting a protective role of the IMD pathway against *S. marcescens* in the gut (Nehme et al., 2007). Phagocytosis controls the proliferation of *S. marcescens* in the hemolymph. Flies mutant for *eater*, which encodes a phagocytic receptor for *S. marcescens* (Kocks et al., 2005),

die faster in the oral infection due to uncontrolled bacterial proliferation in the hemolymph, that is bacteremia.

The virulence factors of *S. marcescens* that induce damages in the *Drosophila* midgut as well as the strategy the bacteria employ to cross the intestinal barrier are not known. We therefore performed a genetic screen in which the *eater* mutant flies were orally infected with single *S. marcescens* mutants taken from a bank created by random insertions of miniTn5 transposon in the bacterial genome (Pradel et al., 2007). The potential bacterial mutants showing attenuation of virulence in *eater* fly survival assays were selected for further analysis. In this screen, we isolated a nonmotile *S. marcescens* strain. This strain carried a transposon insertion in the *fliR* gene, which is required for the biosynthesis and function of the flagella. The reduced virulence appears to be related to a decreased ability to cross the digestive tract. By generating an independent *fliR* mutant strain by plasmid-mediated site directed mutagenesis, we have demonstrated that *S. marcescens* needs *fliR* gene to invade the enterocytes of the *Drosophila* intestinal epithelium. Our data suggest that damage to enterocytes requires FliR-mediated bacterial invasion of these epithelial cells.

Materials and Methods

Fly lines

Flies were reared at 25°C on standard corneal-agar medium. A5001 (Thibault et al., 2004) were wild-type controls for *keyC0*, a P-element insertion null mutant allele of *key*[1] (Rutschmann et al., 2000) and *eater* mutants. The *eater* null mutant flies with two overlapping deficiencies on homologous chromosomes were obtained by crossing two heterozygous fly strains: Df(3R) D605/TM3, Sb[1] Ser [1] and Df(3R) T1-I, e[1]/TM3, Ser[1] (Kocks et al., 2005). The NP1-Gal4 driver, UAS-ATG1, and UAS-ATG1^{DN} lines were obtained from DGRC, Japan. All crosses to generate transgenetic rescue fly lines were performed at 25°C.

Bacterial strains

The *S. marcescens* strain Db11 is a spontaneous streptomycin resistant strain, while the bank of *Serratia* mutants was established by inducing miniTn5 transposon insertions randomly into the genome of the *S. marcescens* wild-type strain Db10 (Pradel et al., 2007). The Db strain was originally isolated from moribund flies (Flyg et al., 1980). DsRed derivatives of the wild-type strain Db11 and *fliR* mutants were obtained by transforming them with plasmid pEP933 (Nehme et al., 2007). Transposon insertion sites in the genome of *S. marcescens* were mapped using one-primer method (Karlyshev et al., 2000). Briefly, two pairs of nested transposon-specific primers were designed, one on each side of the transposon. The inner primes on each side of the transposon were used for unspecific amplifications (5 amplification cycles at Tm 60°C, 3 cycles at Tm 33°C, and finally 30 cycles at 60°C), followed by the sequencing using two outer primers on each side. For plasmid-mediated site-specific mutagenesis, a fragment from *fliR* gene was amplified by PCR using primers

5′gctctagacctgtggctggcggttc3′ and 5′ccgctcgagtcatcatgcccagcgt3′ and cloned in plasmid pHG201spn. The wild-type Db10 strain was then transformed. The *fliR* mutant generated by homologous recombination was then selected for antibiotic resistance, and verified by PCR and motility assay.

Oral infection

Bacteria were grown overnight in LB at 37°C. Fresh LB was used to dilute the bacterial culture to an optical density of 1 (OD_{600} 1) before finally diluting the resulting solution ten times in sucrose (50mM) solution. This infection solution was then used to soak filter pads (Millipore) placed at the bottom of fly culture vials before adding about 20 flies (10 flies for the screen). All survival experiments were performed at 25°C and flies were supplemented with 200µL of sterile sucrose (100mM) solution on a daily basis. Survival was monitored at least once per day.

Dithiothreitol (DTT) treatment of flies

3-8 days old female flies were added to the vials containing filters soaked with DTT (10mM) dissolved in sucrose (50mM) solution. Thee vials were incubated at 25°C for 18 hours. Finally, the flies were shifted to the vials with filters containing infection solution (see above) and incubated for 6 hours at 25°C. Hemolymph was extracted as described in (Nehme et al., 2007).

Peritrophic matrix permeability assay

FITC-labelled dextrans (70kDa) were mixed in sucrose (50mM) solution. Next, the wild-type strain Db11 or *fliR* mutant (final OD 0.1) or DTT (10mM) was added to the final mix and used to soak filter pads placed at the bottom of the fly culture vials. Flies were then added to

these vials and incubated for about 7 hours at 25°C. Guts were dissected in PBS, fixed in 4% PFA for 30 minutes and stained for actin using Texas Red-labelled Phalloidin (Invitrogen). The stained guts were then mounted using DAPI containing Vectashield and observed by confocal microscopy.

Blockade of phagocytosis and histochemistry:

Blockade of phagocytosis was achieved by injecting non-degradable latex beads with the NanojectII (Drummond). Gut dissections, fixation, immunostaining, microscopy and imaging as well as collection of hemolymph to monitor bacterial titre were performed as described in (Nehme et al., 2007).

Bacterial counts in gut epithelium

Flies were orally infected as above. Following one day post-infection flies were shifted to the fly culture vials containing filters that were soaked with Gentamicin (100μg/mL) solution in PBS. After 2 hours, flies were fed on sterile sucrose (100mM) solution for 30 minutes, two times, to wash away any remaining antibiotics. The dissected guts were shortly (almost a second) dipped in 70% ethanol, crushed in PBS and plated on Streptomycin (100μg/mL) containing bacteria culture plates.

Results

We have tested 1348 miniTn5 transposon insertion mutants generated in the strain *S. marcescens* for: i) decreased proteolytic activity of colonies on milk plates; ii) altered survival pattern of phagocytosis-deficient *eater* mutant flies that had ingested the mutant bacteria. *eater* mutant flies succumb to bacteremia caused by bacteria that have crossed the intestinal barrier. In this screen, we found two independent mutants in which the proteolytic activity was reproducibly decreased. Interestingly, the transposon insertions map to two genes in the same operon, namely the *lipase secretion system* (*lip*) operon (Akatsuka et al., 1995), which has been shown to be required for the secretion of lipase A (LipA) and the surface layer protein (SlaA) (Akatsuka et al., 1994; Kawai et al., 1998). This phenotype was confirmed by the direct inactivation of the *lipB* gene by the site-specific insertion of a plasmid. We confirmed by zymogram analysis that the two original mutant strains produce strongly reduced amounts of the two proteases that were reproducibly observed with wild-type *S. marcescens* Db11 (Fig. S1). As regards the *in vivo* screen, we only confirmed one insertion mutant, 19H12, which reproducibly led to a lower virulence in the intestinal infection model with *eater* flies, that is a slower death rate. This transposon mapped to the *fliR* gene, which is the last gene of the *fliL* operon, the seven genes of which are involved in the biosynthesis and functioning of the flagellar organelle (Malakooti et al., 1994). We therefore generated an independent mutant by plasmid-mediated site directed insertion mutagenesis. This mutant did not move as measured by a motility assay (Fig. S2). As shown in Fig. 1A, this *fliR* mutant reproducibly killed *eater* flies less rapidly than the control *S. marcescens* Db11 strain in a recently developed model of *S. marcescens* intestinal infection in which only flies with altered immune response or homeostasis succumb. Data obtained with our classical model of infection are shown in Fig. S3. Note that the difference in survival curves was much more

pronounced with the site directed mutant than with the original insertion mutant (Fig. S3). We checked that *fliR* was as virulent as *S. marcescens* Db11 in the septic injury assay, thus demonstrating that this mutant is not impaired for virulence factors required to overcome the systemic host defense (Fig. S4). To confirm the dependency of the *fliR* mutant phenotype on the cellular immune response, we impaired phagocytosis by the prior injection of latex beads (Fig. 1B). The results that we obtained were similar to those observed using *eater* mutants. As expected, like *S. marcescens* Db11 and *Escherichia coli*, ingested *fliR* bacteria did not specifically kill wild-type A5001 flies (Fig. 1C). In contrast, the IMD pathway mutant flies *kenny* (*key*) were sensitive to ingested *S. marcescens* Db11, even though they have supposedly a normal cellular immune response. Strikingly, *fliR* mutants behaved as nonpathogenic *E. coli* (Fig. 1D).

***fliR* mutants are not able to efficiently cross the intestinal barrier**

The reduced death rate observed in phagocytosis-impaired flies that have ingested *fliR* mutant bacteria might be due either to a reduced virulence once the bacteria are present in the hemocoel, or an altered ability to escape from the digestive tract. The finding that *fliR S. marcescens* does kill flies as efficiently as wild-type bacteria when directly introduced in the hemocoel suggests that the phenotype is not due to a decreased virulence of the mutant. Thus, we monitored the rate of passage of the ingested bacteria through the intestinal barrier. To increase the sensitivity of the assay, we collected hemolymph from latex-bead injected flies, so as to prevent phagocytosis of bacteria that have reached the hemocoel (Nehme et al., 2007). Fig. 2A shows that the rate of passage of *fliR* bacteria was strongly decreased as compared to wild-type bacteria, with actually no bacteria being retrieved in some experiments. The next question we addressed was whether the mutant bacteria were still able to cross the peritrophic matrix, which is the first barrier that bacteria have to cross. By confocal microscopy, we did find bacteria present in the ectoperitrophic space, in close

vicinity to the intestinal epithelium (Fig. S5). We further addressed this problem by two complementary approaches. We first asked whether *S. marcescens* is able to alter the permeability of the peritrophic membrane. To answer that question, we added FITC-labeled dextrans of high molecular weight (70 kD) to the food of flies. As shown in Fig. 2B, the dextrans remained confined to the lumen of the gut (intraperitrophic space) in flies on a sucrose regimen. In contrast, after seven hours of exposure to ingested *S. marcescens* the dextrans were no longer confined to the intraperitrophic space in the intestinal lumen, whether the bacteria were wild-type or mutant for *fliR*. This suggests that *fliR* mutants have intact abilities to alter the permeability of the peritrophic matrix and possibly may cross the peritrophic matrix as efficiently as wild-type bacteria. To complement these findings, we therefore destroyed the peritrophic matrix by feeding the flies with 10mM DTT (Edwards and Jacobs-Lorena, 2000). As expected this treatment allowed the FITC dextrans to diffuse within the whole gut (Fig. 2B). In keeping with a protective role of the peritrophic matrix, we noted that the hemolymph titer we measured with an intact peritrophic matrix after 24 hours of infection was reached here just after six hours. Under these favorable conditions, *fliR* bacteria were still unable to cross the intestinal barrier (Fig. 1A). We conclude that *fliR* mutant bacteria likely are unable to cross the intestinal epithelium itself.

fliR bacteria do not invade enterocytes

Next, we asked whether these mutant bacteria were able to penetrate inside intestinal cells, as had been previously reported for wild-type *S. marcescens* (Nehme et al., 2007). Indeed, we did observe wild-type bacteria inside enterocytes, although it was a relatively rare occurrence. In contrast, we never observed any intracellular *fliR* bacteria (Fig. 3 and Fig. S5). To confirm these data, we used another, more quantitative, approach. Flies were fed with bacteria for one day. Then, they were fed on gentamicin for two hours, a treatment that kills only extracellular bacteria. The flies were fed afterwards on sucrose for one hour to wash

away the antibiotics. This treatment indeed cleared GFP-labeled *S. marcescens* from the gut lumen (data not shown). We next dissected the guts and plated extracts on plates. Fig. 3B shows that hardly any *fliR* bacteria were retrieved from wild-type guts, suggesting that they have a decreased ability to invade intestinal cells. Similar results were obtained in *key* mutants, although the absolute titer for both wild-type and *fliR* bacteria was one Log higher than in wild-type flies (Fig. 3B). We confirmed these data in a cell culture gentamicin protection assay. To prevent host-mediated internalization of bacteria through phagocytosis, we pretreated the cells with cytochalasin D. *fliR* bacteria were as inefficient as nonpathogenic *E. coli* in invading *Drosophila* S2 cells (Fig. 3C), in keeping with results obtained with mammalian cell lines (Fedrigo et al., 2011). In a preliminary series of experiments, we asked whether *fliR* bacteria would be less adherent to S2 cells and found that it was not the case (Fig. S6).

It has been suggested that the autophagic machinery is required for the intracellular protection and proliferation of invading bacteria. We therefore attempted to block autophagy in enterocytes by overexpressing a dominant-negative construct of the ATG1 protein. This treatment did not alter the titer of wild-type *S. marcescens* within the intestinal epithelium. Interestingly however, inducing autophagy by overexpressing wild-type ATG1 did increase this titer (Fig. 3B).

***fliR* mutants do not damage extensively the intestinal epithelium**

Ingested *S. marcescens* damages the intestinal epithelium by attacking enterocytes, which apparently undergo apoptosis (Cronin et al., 2009). The homeostasis of the intestinal epithelium is maintained through the compensatory proliferation of intestinal stem cells (ISCs). Thus, monitoring ISC proliferation by phosphohistone H3 (PHH3) staining yields an indirect measurement of the extent of the damage that the intestinal epithelium undergoes.

First, we checked that the DUOX-mediated ROS response is not causing damage under our conditions of a relatively low titer of ingested *S. marcescens* Db11. Indeed, treatment with N-acetylcysteine did not alter the PHH3 counts (Fig. 4). Interestingly, the PHH3 count in flies that have ingested *fliR* mutant bacteria was as low as that measured in control flies feeding on a sucrose solution. These data suggest that the invasion of enterocytes by *S. marcescens* is required to damage the intestinal epithelium.

Discussion

One interesting feature of the *S. marcescens* intestinal infection model is the ability of these bacteria to rapidly cross the intestinal barrier, even though the bacteria downregulate their virulence program in the process. They however have not lost their virulence properties since they proliferate rapidly when the cellular host defenses are impaired. Here, we have performed a screen to identify bacterial factors that are required for the traversal of the intestinal epithelium.

In addition to this screen, we also identified a couple of mutants with an impaired secretion of proteases, as judged from milk plate assay and zymogram analysis. The molecular weight of one of these proteases is 54 kDa, evocative of the previously described serralysin (Park and Ming, 2002; Tao et al., 2006). Interestingly, the identity of a 54 kDa protein spot on 2D gels loaded with bacterial supernatant was confirmed to be serralysin by mass spectrometry analysis (data not shown). Proteases are thought to be important virulence factors of *S. marcescens*. Indeed, they are likely to degrade AMPs, although this property was not as essential as the presence of the LPS-O-antigen in accounting for the resistance of *S. marcescens* Db11 against the IMD-mediated systemic immune response in a septic injury model (Nehme et al., 2007). In the context of an intestinal infection model, proteases might actually trigger damages to enterocytes. They may also be required to attack the

chitinoproteinaceous peritrophic matrix. However, mutants with altered protease secretion did not exhibit a decreased virulence in the *eater* intestinal infection model. This suggests that the two proteases we have detected may not be required. Of note, the *S. marcescens* Db11 genome encodes another five proteases that were not detected here. The finding that two independent mutants map to the *lip* operon and that the phenotype was reproduced in an independent mutant in *lipB* strongly suggests that this operon is required for the secretion of these two protease. This operon has been reported to be required for the secretion of lipase A and of the surface layer protein (SlaA). Thus, it may also be required for the secretion of other proteins, including proteases.

The mode of passage of *S. marcescens* through the intestinal epithelium remains uncharacterized at present. The bacterium is able to cross the peritrophic matrix, yet does not appear to affect it, at least at the ultrastructural level as observed by electron microscopy (Nehme et al., 2007). Here, we have shown that it is nevertheless able to increase its permeability (Fig. 2), a process that may involve proteinases or chitinases. While this property may not be sufficient to allow the passage of the bacteria as a relatively low molecular weight dextran was used, it may nevertheless facilitate the passage of secreted virulence factors such as a phospholipase, a nuclease, or hemolysin. Indeed, most *S. marcescens* bacteria appear to be contained within the endoperitrophic space when examined by confocal or electron microscopy. We note that the rate of passage appeared to be enhanced after the disrution of the peritrophic matrix by DTT treatment (Fig. 2A).

Two strategies are possible to cross the intestinal epithelium: extracellular passage by going inbetween the closely apposed enterocytes, the epithelium being sealed by septate junctions, the insect equivalent of tight junctions. The alternate strategy is a passage through the epithelial cells. Some *S. marcescens* bacteria were observed by electron microscopy apparently attempting to cross the intestinal epithelium in between enterocytes at their apical

face, only after three days of ingestion (Nehme et al., 2007). To date, we have not observed them transiting or exiting on the basal side. Furthermore, passage may occur as early as two hours after the beginning of the ingestion, a time at which we have never observed pictures evocative of a possible paracellular path. We have also observed intracellular bacteria, especially in *key* mutants. However, we could not exclude that these bacteria were actually being killed by host intracellular defenses, namely autophagy or lysophagosomes. The finding that FliR is required both for the passage of bacteria through the digestive tract and that it is required for internalization of *S. marcescens* in S2 cells suggests an intracellular mode of passage, possibly through transcytosis. However, we cannot exclude that FliR may be required for other processes as discussed further below. A recent publication reported that *S. marcescens* is able to survive and proliferate within autophagic-like vacuoles in cultured Chinese Hamster Ovary (CHO) cells, which are formed in a noncanonical manner (Fedrigo et al., 2011). The bacteria are apparently able to prevent the fusion of lysosomes to autophagic-like vacuoles. Interestingly, internalization of *S. marcescens* depended on the autophagy factor *atg5*. We have attempted here to disrupt autophagy by overexpressing a dominant-negative form of ATG1, which has been reported to inhibit autophagy. This did apparently not influence the level of intracellular bacteria retrieved in the intestinal epithelium (Fig. 3B). It is not clear whether the autophagic process triggered by *S. marcescens* involves ATG1. On the other hand, had autophagy been a major intracellular host defense, then the intracellular titer of *S. marcescens* would have significantly increased. However, as the bacteria might be released on the basal side rapidly, these experiments should be repeated monitoring the bacterial titer in the hemolymph before a firm conclusion can be drawn. The overexpression of ATG1 did increase the intracellular titer of *S. marcescens* in enterocytes. One possibility is that an increased induction of autophagy might hinder the passage to the basal side. Alternatively, it might favorize the invasion of enterocytes. Again, the bacterial titer in the

hemolymph should be measured. Also, the integrity of the midgut should be assessed, as ATG1 overexpression has been reported to induce a caspase-dependent form of cell death during development (Chang and Neufeld, 2009; Juhasz and Neufeld, 2008; Scott et al., 2007). Thus, we cannot yet exclude that this genetic manipulation might alter the overall properties of the epithelium.

The flagellum is a *bona fide* virulence factor. In the gut, it allows movement and may be important for colonization as it may counteract peristaltic movements. We did not notice an obvious depletion of *fliR* mutant bacteria in the gut and observed mutant labelled bacteria in the vicinity of the epithelium (Fig. 3A), although we cannot exclude a quantitative effect. By the same token, we do not have the means to demonstrate formally that FliR is not required for crossing the peritrophic matrix. Yet, when this barrier is removed, *fliR* bacteria remain deficient in their ability to pass the intestinal epithelial border. Flagella have been reported to be required for the invasion of host cells by several bacteria (Claret et al., 2007; Gavin et al., 2003; Grant et al., 1993; Parthasarathy et al., 2007; Pichon et al., 2009; Tasteyre et al., 2001; Young et al., 2000). Our finding of a role for FliR in the passage of *S. marcescens* through the gut and favorizing the invasion of enterocytes and S2 cells is consistent with a recent report on a clinical strain of *S. marcescens*, in which the flagellin regulator operon *flhD* was shown to be required for the early steps of *S. marcescens* interaction with cultured epithelial cells (Fedrigo et al., 2011). However, we did not find a requirement for *fliR* in adherence to S2 cells. One possibility is that this is due to a difference between mammalian and insect cells. Our data are compatible with the hypothesis that FliR is directly required for invasion through a role of the flagellum. However, as the role of FliR is in the export of proteins through the flagellin type III secretion system, we cannot exclude the possibility that this effect is mediated by secreted proteins distinct from flagellin, for instance a phospholipase (PhlA) or a nuclease (NucA), both of whom are regulated by the *flhDC* operon

(Givskov et al., 1995; Liu et al., 2000). They might either be secreted by a FliR-dependent process or alternatively be regulated by this operon in a *FliR*-independent manner. It will be thus interesting to determine whether *phlA* or *nucA* mutants share the *fliR* mutant phenotype.

An important aspect of the *S. marcescens* intestinal infection model is that this pathogen actually causes damages to the intestinal epithelium (Cronin et al., 2009; Nehme et al., 2007). Obvious candidates were secreted virulence factors such as hemolysin, which indeed provokes important injuries early on during the infection (unpublished data). This work opens the possibility that enterocytes may be damaged only as a result of enterocyte invasion. However, as noted above, we cannot exclude at present that enterocytes are actually attacked by either the PhlA phospholipase or the NucA nuclease, which has been shown to kill transgenic porcine fibroblasts (Caballero and Piedrahita, 2009).

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FIGURE LEGENDS

Fig. 1 : *fliR* mutants kill the immunocompromised flies more slowly than the wild-type bacteria

eater null mutants (A), latex beads (LXB)-injected (B), nonLXB-injected (C) wild-type A5001 flies, and *key* mutants (D) were orally infected with an *E. coli* strain, *S. marcescens* wild-type strain Db11, or *fliR* mutants. Survival curves were compared by LogRank test using Prism statistical software. (A) *Serratia* strain Db11 killed *eater* flies faster than the *fliR* mutants (p values: sucrose *vs. E. coli*: $p > 0.05$ (ns), sucrose *vs.* Db11: $p < 0.0001$ (***), sucrose *vs.* FliR: $p < 0.0001$ (***), Db11 *vs.* FliR: $p < 0.0001$ (***), with n = 5 (Db11), n = 6 (FliR), n = 2 (sucrose and *E. coli*). (B) 50% of phagocytosis-deficient flies died within 3 days when infected wild-type Db11 strain while *fliR* mutants killed these flies within 6 days (Db11 *vs.* FliR: $p = 0.0004$ (***), n = 9). (C) Wild-type flies did not die from feeding on *S. marcescens* when a sucrose solution is added daily during the experiment ($p > 0.05$ for each pair, and n = 8 (Db11 and sucrose), n = 10 (FliR), n = 7 (*E. coli*)). (D) Half of *key* mutant flies infected with strain Db11 died within 14 days while *fliR* mutants did not kill these flies (p values: sucrose *vs. E. coli*: $p > 0.05$ (ns), sucrose *vs.* Db11: $p < 0.0001$ (***), sucrose *vs.* FliR: $p > 0.05$ (ns), Db11 *vs.* FliR: $p < 0.0001$ (***), with n = 9 (Db11 and *E. coli*), n = 8 (FliR), n = 6 (sucrose). Error bars represent standard errors in each panel.

Fig. 2 : *fliR* mutants are unable to cross the intestinal barrier but appear not to be hindered by the peritrophic matrix

(A) More than two logs higher titer of wild-type Db11 bacteria, as compared to *fliR* mutants, were retrieved in the hemolymph of LXB-injected A5001 flies, one day post-infection (p values = 0.004 n = 7 (**)). A similar titer of Db11 was observed 6 hours post-infection in the

hemolymph of flies that had been preincubated with DTT (10mM) for 18 hours, while we failed to retrieve any *fliR* mutants in these flies. Error bars represent standard errors. (B) Wild-type flies were fed with FITC-labeled dextrans (70kDa) (green) either by mixing with sucrose (50mM) solution alone or also containing wild-type Db11 strain, *fliR* mutants or DTT (10mM). Guts were dissected 7 hours post-infection, fixed, stained for actin (red) and nuclei (blue), and observed by confocal microscopy. The figures are representative of two independent experiments. Note that fluorescent dextrans were retained only in flies fed on sucrose suggesting that *S. marcescens* alters the permeability of the peritrophic matrix.

Fig. 3: *fliR* mutants do not invade *Drosophila* cells *in vivo* or *ex-vivo*

(A) DsRed derivatives of bacteria (red) are found in the vicinity of the intestinal epithelial cells of wild-type flies, 24 hours post-infection. The intra-cellular Db11-DsRed are indicated by *arrows* and the brush border of epithelial cells is shown by *arrow heads*. (B) Fewer *fliR* mutants were found invading the gut epithelium of the wild-type A5001 flies and *key* mutants in a gentamicin protection assay; (*for A5001*: $p < 0.05$ n = 5; *for key mutants*: $p < 0.01$ n = 6). NP1-Gal4 (midgut enterocyte-specific) driver fly line was the wild-type control to monitor Db11 titer in the gut epithelium of autophagy mutants: NP1-*Gal4*/UAS-ATG1^{DN} (downregulation of autophagy in enterocytes of gut epithelium (NP-ATG1-DN)) and NP-*Gal4*/UAS-ATG1 (over-expression of autophagy in enterocytes of gut epithelium (NP1-ATG1)); (p values: NP-ATG1^{DN}: $p > 0.05$ n = 4 (ns), NP-ATG1: $p < 0.01$ n = 4 (**)). There was no statistical difference between the Db11 titers of the two wild-type, A5001 and NP1-Gal4, fly strains: ($p = 0.07$, n = 4 (ns)). (C) Like nonpathogenic *E. coli*, *fliR* mutants were less efficient in invading S2 cells both at multiplicity of infection (MOI) of 5 and 10 in a gentamicin protection assay; (*for MOI 5*: Db11 vs FliR: $p = 0.0005$ (***), FliR vs *E. coli*: $p = 0.34$ (ns); for *MOI 10*: Db11 vs FliR: $p = 0.008$ (**), FliR vs *E. coli*: $p = 0.26$ (ns); n=9 for all

data sets). Of note, S2 cells had been pretreated with cytochalasin D to neutralize phagocytosis-mediated internalization. Error bars in B and C represent standard errors.

Fig. 4 : *fliR* mutants do not damage the gut epithelial cells

The extent of damage to enterocytes was through measurement of the compensatory proliferation of intestinal stem cells (ISCs) by counting the number of phosphohistone-H3 positive cells in midgut of wild-type flies, 24 hours post-infection; the non-parametric Mann Whitney test was applied for statistical analyses (Db11 vs Db11_N-ac: $p = 0.6$ (ns), Db11 vs FliR: $p = 0.007$ (**), Db11 vs sucrose: $p = 0.0007$ (***), Db11_N-ac vs FliR: $p = 0.03$ (*); while n=24 (Db11), n=16 (Db11_N-ac), n=23 (FliR), and n=21 (sucrose). Mean values are shown.

Supplementary Materials and Methods

Protease milk assay

The milk culture plates contained 3% (w/v) milk powder, 2.5% LB and 1.5% Agar. After stirring, culture plates were incubated at room temperature overnight.

Zymogram

Bacteria were grown overnight at 37°C with constant shaking in the basal salts and buffer (BSB that contained per liter: 0.375g KH_2PO_4, 0.325g $(NH_4)_2SO_4$, 0.25g NaCl, 0.125g $MgSO_4$, and 33.35g HEPES buffer) medium that also contained glycerol (0.62%), calcium chloride (1mM), and Leucine (10mM) (Bromke and Venuti, 1999). The supernatants from overnight cultures were then filtered and proteins were separated by electrophoresis. The separating gel was prepared with 4 ml Gelatin solution (0.5%), 3.3 ml of 30% Acrylamide mix, 2.5 ml of 1.5M Tris (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% APS, 0.004 ml of TEMED, while the stacking gel contained 3.4 ml sterile de-ionized water, 0.83 ml of 30% Acrylamide mix, 0.63 ml of 1.0M Tris (pH 6.8), 0.05 ml of 10% SDS, 0.05 ml of 10% APS, and 0.005 ml of TEMED. After the transfer of proteins at 30mA for 3 hours, the gel was shaken for 1 hour in 2.5% Triton X-100 solution. The gel was developed in 1 mM Dithiothreitol (DTT) at 37°C for 3 hours. Finally, the gel was stained in Coomassie blue C-250 (0.01% (w/v)) solution containing 10% acetic acid and 40% methanol.

Swimming Assay

Fresh culture plates were made with loose agar (0.3% agar). A few bacterial colonies were then picked up with the help of sterile toothpick and placed in the middle of the loose agar plate. These plates were than incubated for 8 hours at 37°C. Mobile bacteria formed a circle around their colonies while the sessile cells did not move.

Oral infection of *eater* mutants

Bacteria were grown overnight in LB at 37°C. Then fresh LB was used to dilute the bacterial culture to an optical density of 1 (OD_{600} 1) before finally diluting ten times in sucrose (50mM) solution. The infection solution was then used to soak filter pads (Millipore) placed at the bottom of fly culture vials before adding 10 to 20 flies. All survival experiments were performed at 25°C and flies were supplemented with 200µL of sterile water. Survival was monitored at least once per day.

Pricking infection model

96nL of DMSO was injected in hemolymph of the flies with the NanojectII (Drummond). Flies were allowed to settle for almost a day at 25°C. Flies were then pricked with a needle, which was previous dipped either in the bacterial solution (final OD_{600} 0.1 in PBS) or sterile PBS. Survival experiment was performed at 25°C.

Supplementary Figure Legends

Fig. S1 : Protease activity assays

(A) The bacteria were streaked on milk plates and incubated overnight at room temperature. Wild-type *S. marcescens* strain Db11 secretes active proteases, thus forming a clear halo around its colonies. Note the hazy halo in the periphery of the mutant strains 12 E12 and 19 G12 indicating that these strains are deficient in protease secretion/ activity. Non-pathogenic *E. coli* does not produce active proteases; therefore, no halo was formed in the periphery of its growing colonies. (B) The supernatants from the overnight bacterial cultures were filtered and tested for the presence of active proteases in protease zymograms (see supplementary materials and methods). Two proteases were detected in the supernatant of wild-type Db11

strain, which were not present in that of the protease mutants as well as the nonpathogenic *E. coli* strain.

Fig. S2 : Bacteria mutant for the *fliR* gene are non-motile

The DsRed derivative of wild-type *S. marcescens* strain Db11, as well as the spontaneous revertant strain 12 $H12^{RT}$, are motile and form a circle around its growing colonies. The *fliR* mutants and the original transposon 19 H12 mutants, however, are nonmotile and do not form such circle consisting of the swimming bacteria.

Fig. S3 : *fliR* mutants slowly kill phagocytosis-deficient *eater* mutants flies

The survival experiment was performed in orally infected *eater* mutant flies following the protocol described in (Nehme et al., 2007). In this protocol no sucrose is added to the vials during the infection. The *fliR* mutants as well the strain 19 H12 killed flies slower than the wild-type *S. marcescens* strain Db11. However, the strain 19 H12 killed flies much faster as compared to *fliR* mutants. This observation may be accounted for by a high rate of spontaneous reversion that was observed in 19 H12 mutants in motility assays.

Fig. S4 : *fliR* mutants are as pathogenic as wild-type *Serratia* in septic injury model

Wild-type flies succumbed to *fliR* mutants as fast as to wild-type *S. marcescens* strain Db11 in the pricking assay, suggesting that nonmotile *fliR* mutants are still pathogenic when introduced directly in the hemocoel.

Fig. S5 : Wild-type Db11, and not *fliR* mutants, invade the midgut epithelium

(A-D2) The DsRed derivatives of the wild-type *S. marcescens* strain Db11 crossed the brush border and invaded the enterocytes of the *Drosophila* midgut epithelium. (a-d2) *fliR* mutants,

however, were found only in the vicinity of the epithelial cells. Note the colocalization of fluorescent bacteria (*red* (A and a)) and the nuclear stain DAPI (*blue* (B and b)), confirming the presence of live bacterial cells.

Fig. S6 : Wild-type Db11, *E. coli* and *fliR* mutants adhere to S2 cells to the same extent

S2 cells were incubated with bacteria followed by washing without the gentamicin treatment. This yielded a number of total bacteria including the ones that had invaded the cells. Therefore, the number of bacterial cells that had invaded the S2 cells (Fig. 3C) was then subtracted from the total number of bacteria to obtain the number of bacteria sticking to the surface of cells.

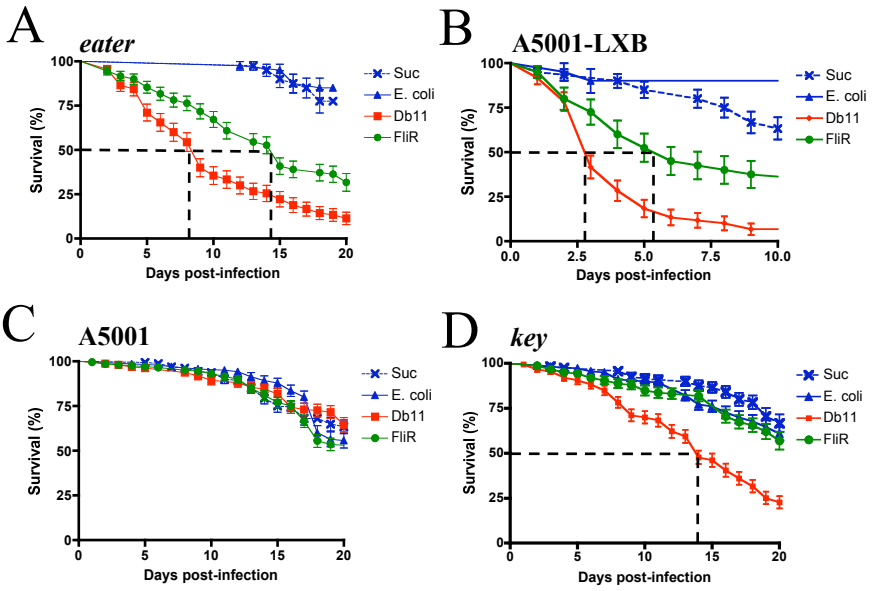

A
eater
Survival (%)
Days post-infection
Suc
E. coli
Db11
FliR
B
A5001-LXB
Survival (%)
Days post-infection
Suc
E. coli
Db11
FliR
C
A5001
Survival (%)
Days post-infection
Suc
E. coli
Db11
FliR
D
key
Survival (%)
Days post-infection
Suc
E. coli
Db11
FliR

Fig. 1

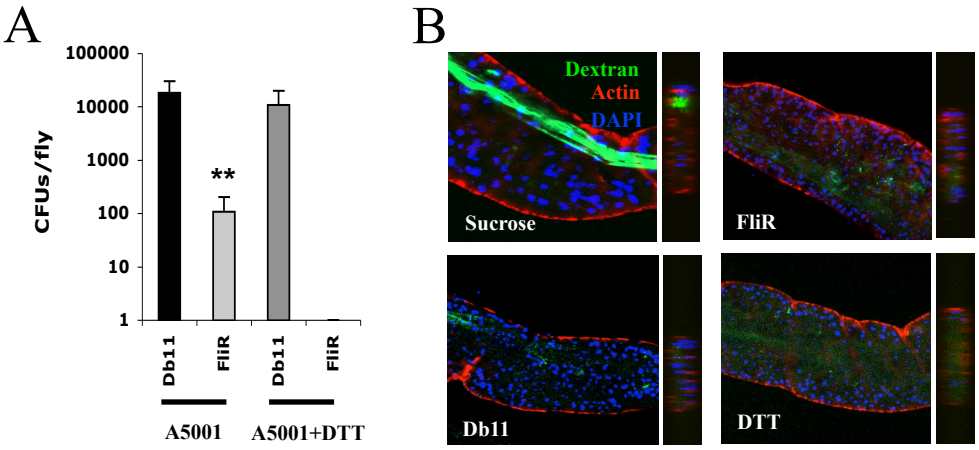
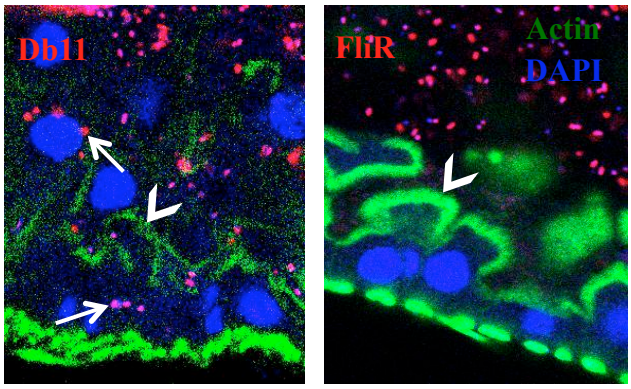
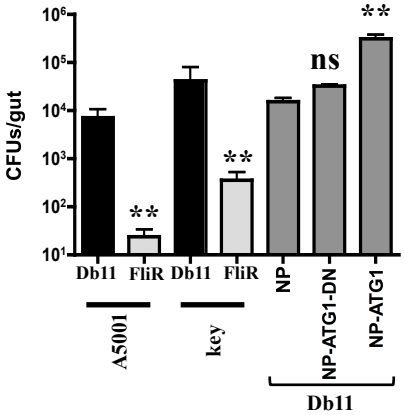

A
100000
10000
1000
100
10
1
CFUs/fly
**
Db11
FliR
Db11
FliR
A5001
A5001+DTT
B
Dextran
Actin
DAPI
Sucrose
FliR
Db11
DTT

Fig. 2

A


Db11
FliR
Actin
DAPI

B


CFUs/gut
10^6
10^5
10^4
10^3
10^2
10^1
**
**
ns
**
Db11
FliR
Db11
FliR
NP
NP-ATG1-DN
NP-ATG1
A5001
key
Db11

C

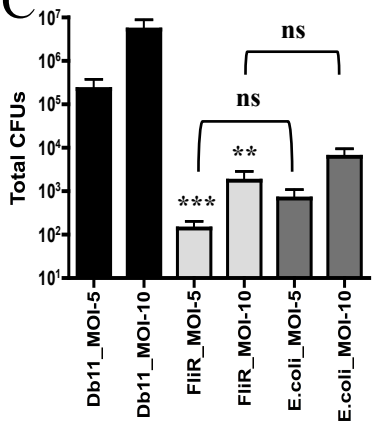

Total CFUs
10^7
10^6
10^5
10^4
10^3
10^2
10^1
ns
ns
***
**
Db11_MOI-5
Db11_MOI-10
FliR_MOI-5
FliR_MOI-10
E.coli_MOI-5
E.coli_MOI-10

Fig. 3

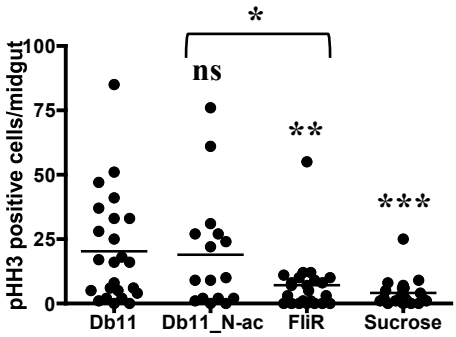

*
ns
**
***
pHH3 positive cells/midgut
100
75
50
25
0
Db11
Db11_N-ac
FliR
Sucrose

Fig. 4

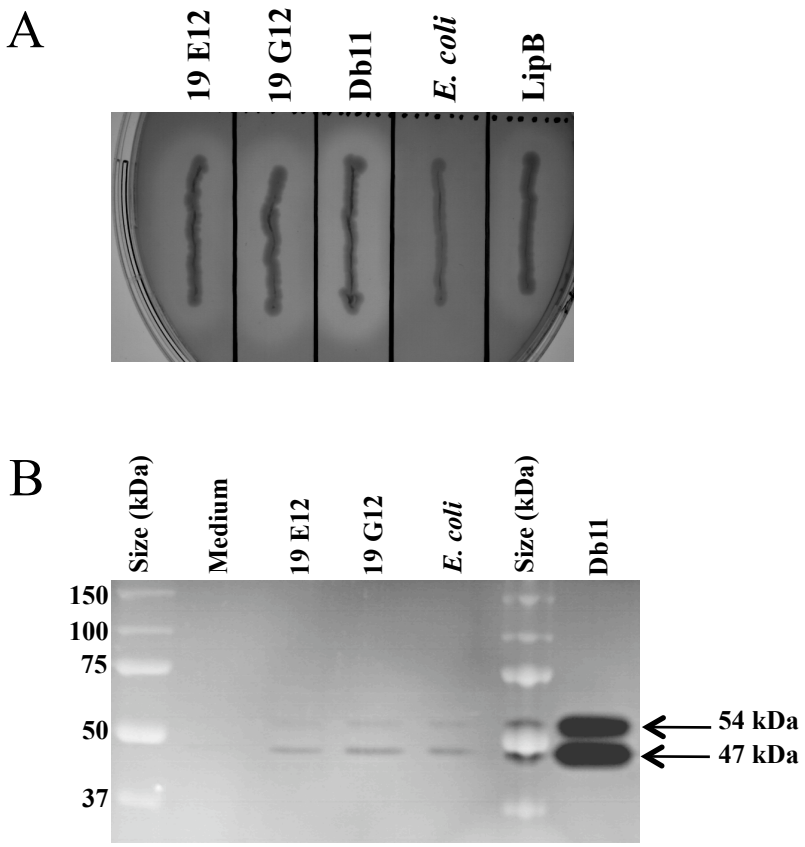

A
19 E12
19 G12
Db11
E. coli
LipB
B
Size (kDa)
Medium
19 E12
19 G12
E. coli
Size (kDa)
Db11
150
100
75
50
37
54 kDa
47 kDa

Fig. S1

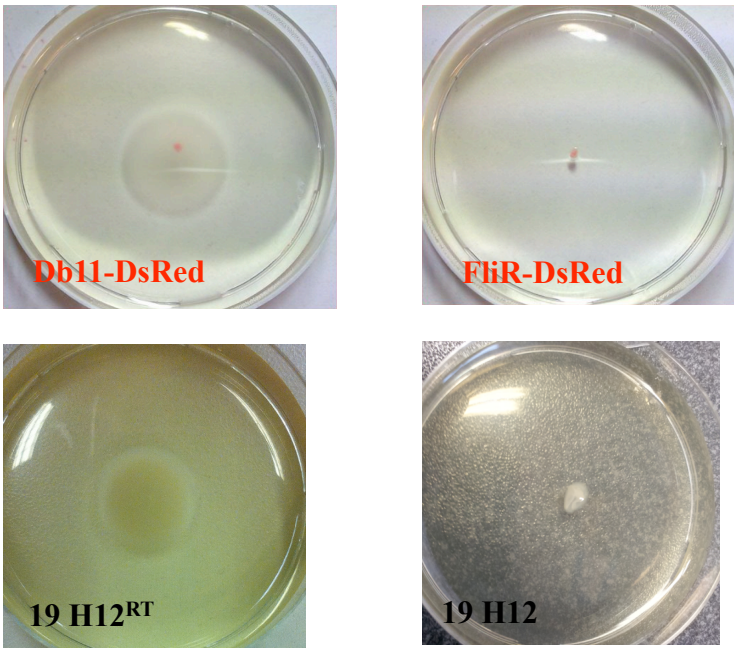


Fig. S2

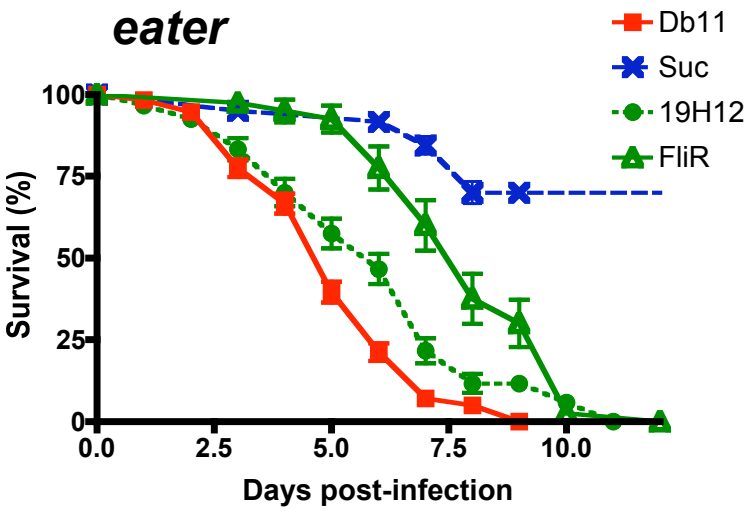

eater
Db11
Suc
19H12
FliR
Survival (%)
100
75
50
25
0
0.0
2.5
5.0
7.5
10.0
Days post-infection

Fig. S3

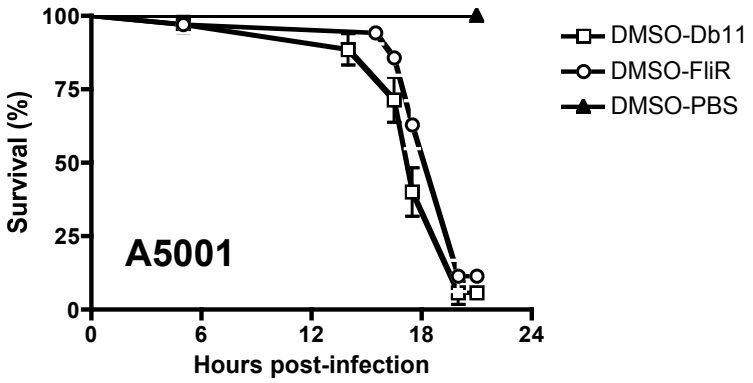

100
75
50
25
0
Survival (%)
A5001
0
6
12
18
24
Hours post-infection
DMSO-Db11
DMSO-FliR
DMSO-PBS

Fig. S4

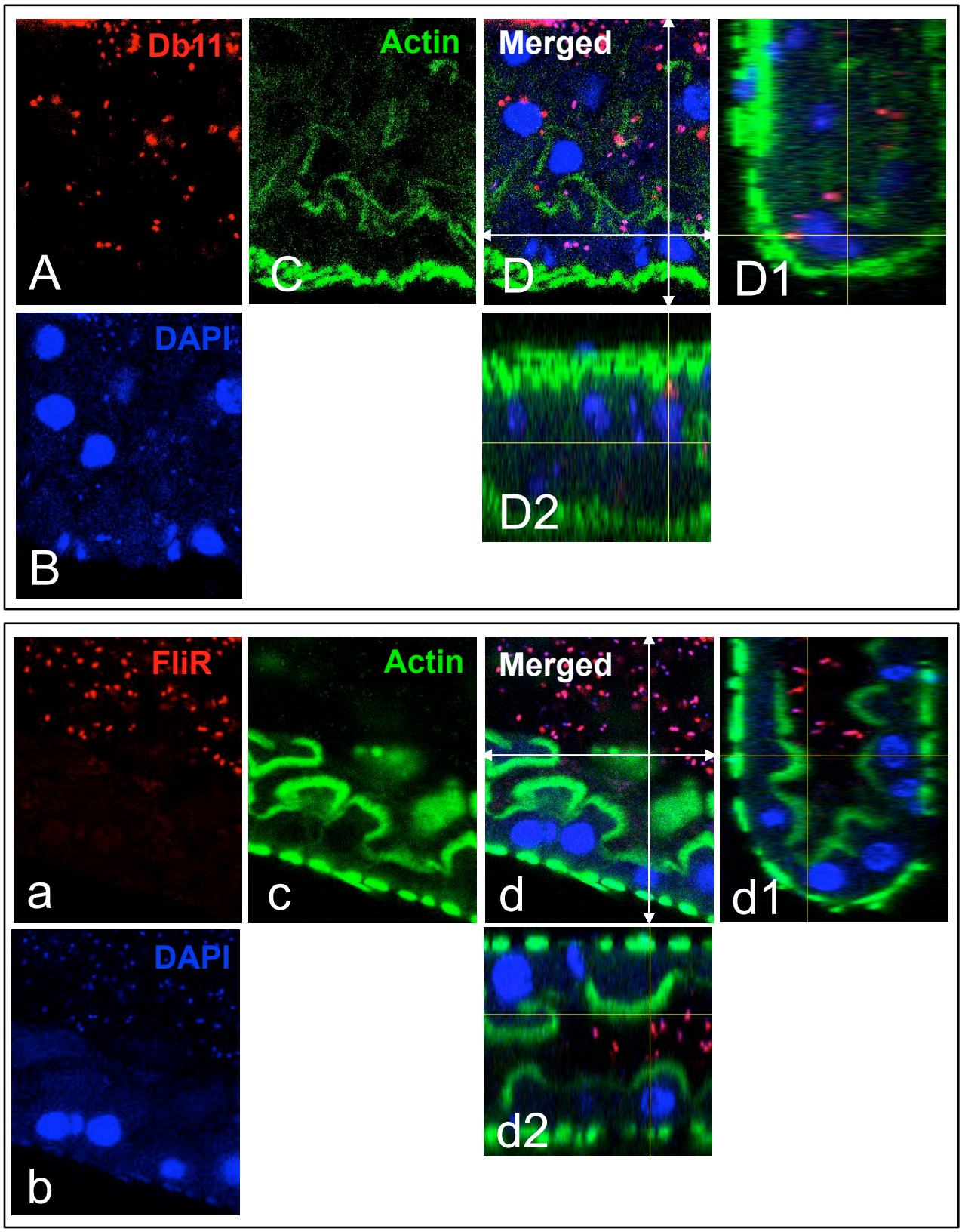

Db11
Actin
Merged
A
C
D
D1
DAPI
D2
B
FliR
Actin
Merged
a
c
d
d1
DAPI
d2
b

Fig. S5

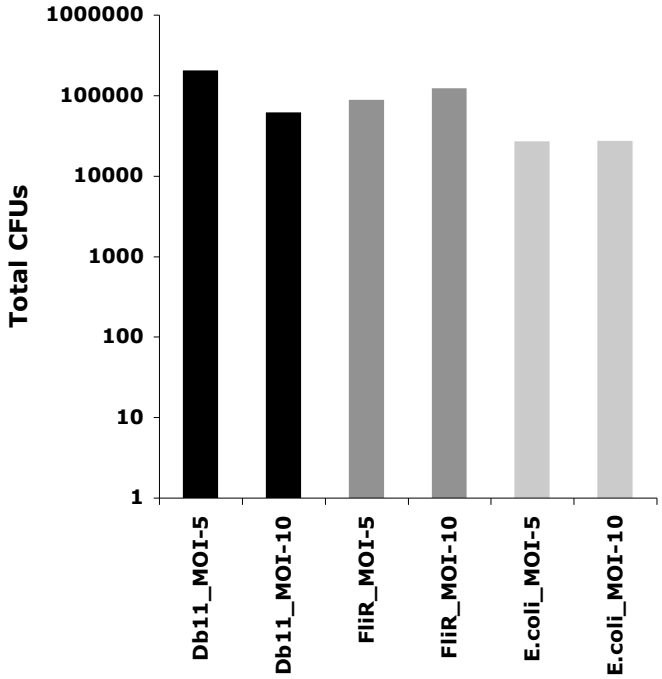

1000000
100000
10000
1000
100
10
1
Total CFUs
Db11_MOI-5
Db11_MOI-10
FliR_MOI-5
FliR_MOI-10
E.coli_MOI-5
E.coli_MOI-10

Fig. S6

5. The Toll pathway: from immunity to metabolism

5.1. *MyD88* protective during septic injury but enhances susceptibility to starvation

Preface

The Toll pathway is activated after septic injury with Gram-positive bacteria, providing protection to the flies against this category of microbes. The local immune responses, however, are IMD pathway-, and not Toll pathway-, dependent. The antimicrobial defense in the *Drosophila* intestine against Gram-positive bacteria remains undiscovered. We therefore used a Gram-positive bacterium *Staphylococcus xylosus* strain Argentoratum, which was originally isolated from moribund flies in our laboratory at UPR9022, IBMC, Strasbourg, to investigate a role of the Toll pathway in the *Drosophila* intestine. In septic infections, *S. xylosus* was pathogenic in a concentration-dependent manner. The Toll pathway as well as phagocytosis provided protection to the flies against *S. xylosus*. Surprisingly, when provided orally to the flies the Toll pathway, *MyD88*, mutant flies survived better than the wild-type controls. Later on, we demonstrated that the flies actually died because of starvation and that the *MyD88* flies were more resistant to starvation. Moreover, following one day of starvation, wild-type flies lost more than 80% of total fat content while only 20% of such loss was observed in *MyD88* mutants suggesting that the starvation resistance phenotype observed in *MyD88* flies was mediated through fat metabolism. We thus discovered a novel role of *MyD88* gene in addition to its known functions during early development and immunity.

A negative role of *MyD88* in the resistance to starvation as revealed in an intestinal infection of *Drosophila melanogaster* with the Gram-positive bacterium *Staphylococcus xylosus*

Running Head: *MyD88* in starvation resistance

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Key words: *Drosophila*, immunity, lipid, metabolism, *MyD88*, *Staphylococcus*, starvation.

Abbreviations:

AMP: Antimicrobial peptides

CGD: Chronic granulomatous

DIF: Dorsal-related immunity factor

FOXO: Forkhead box O

GNBP3: Gram negative protein 3

IMD: Immune deficiency

ISC: Intestinal stem cell

IRC: Immune response catalase

JAK/STAT: Janus kinase/signal transducers and activators of transcription

NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase

PGN: Peptidoglycan

PGRP: Peptidoglycan recognition protein

ROS: Reactive oxygen species

PRR: Pattern recognition receptor

SPZ: Spätzle

TLR: Toll like receptor

UV: Ultra violet

Abstract

Drosophila melanogaster is a useful model to investigate mucosal immunity. The immune response to intestinal infections is mediated partly by the Immune deficiency (IMD) pathway, which gets activated by structural compounds that are missing in several medically-important Gram-positive bacterial species such as *Staphylococcus*. Thus, the intestinal host defense against such bacterial strains remains poorly known. Here, we have used *S. xylosus* to develop a model of intestinal infections by Gram-positive bacteria. *S. xylosus* behaves as an opportunistic pathogen in a septic injury model, being able to kill only flies immunodeficient either for the Toll pathway or the cellular response. When ingested, it is controlled by IMD-independent host intestinal defenses, yet flies eventually die. Having excluded an overreaction of the immune response and the action of toxins, we find that flies actually succumb to starvation, likely as a result of a competition for sucrose between the bacteria and the flies. Fat stores of wild-type flies are severely reduced within a day, a period when sucrose is not yet exhausted in the feeding solution. Interestingly, the Toll pathway mutant *MyD88* is more resistant to the ingestion of *S. xylosus* and to starvation than wild-type flies. *MyD88* flies do not rapidly deplete their fat stores when starved, in contrast to wild-type flies. Thus, we have uncovered a novel function of *MyD88* in the regulation of metabolism that appears to be independent of its known roles in immunity and development.

Introduction

Drosophila melanogaster feeds on decaying fruits and vegetables and thus lives in a microbe-rich environment. As a result of constant interactions with its septic environment, *Drosophila* has evolved a sophisticated host defense that generally allows it to contain potentially hazardous microorganisms. The phagocytosis of microbes by circulating hemocytes and the secretion of antimicrobial peptides (AMPs), respectively the cellular and humoral immune responses, constitute the major *Drosophila* defense mechanisms against infections (Ferrandon et al, 2007; Lemaitre & Hoffmann, 2007). AMPs are either secreted systemically or can be produced locally by contact epithelia (Akhouayri et al, 2011; Ferrandon et al, 1998; Han et al, 2004; Tzou et al, 2000).

Following a septic injury, AMPs are secreted by the fat body, a functional analog of the mammalian liver, into the fly hemolymph. Microbes are either recognized through their structural components or alternatively by the enzymatic activity of microbial virulence factors (Gottar et al, 2006). On the basis of differences in the chemical properties of microbial structural compounds, *Drosophila* is able to distinguish between different categories of microbes and, to some extent, activate the relevant antimicrobial response (reviewed by (Ferrandon et al, 2007)). Gram-negative bacteria, for instance, are recognized by the Pattern Recognition Receptors (PRRs) Peptidoglycan Recognition Protein-LC (PGRP-LC) and PGRP-LE. These receptors sense Gram-negative bacteria and Gram-positive bacilli through their meso-diaminopimelic acid-containing peptidoglycans (DAP-type PGNs) and subsequently activate the Immune deficiency (IMD) pathway, which ultimately leads to the nuclear localization of Relish, a NF-κB family transcription factor. Nuclear Relish transcribes AMP genes such as *Diptericin*, *Drosocin*, *Attacins*, and *Cecropins* that are active against this category of microbes. Fungi and some Gram-positive bacteria are sensed via their ß-

(1,3)glucans or via their Lysine type peptidoglycans (Lys-type PGNs). This recognition event involves respectively Gram Negative Protein 3 (GNBP3) for fungi and a complex comprising the secreted proteins PGRP-SA, PGRP-SD, and GNBP1 for the Gram-positive bacteria. The detection of these microbes activates proteolytic cascades that ultimately lead to the cleavage of the Spätzle cytokine (SPZ) into a ligand of the transmembrane Toll receptor. Toll activation triggers the nuclear localization of DIF in a DmelMyD88-dependent manner. The NF-kappaB transcription factor DIF in turn transcribes AMP genes encoding antifungal peptides such as Drosomycin and Metchnikowin. Interestingly, the only AMP active against Gram-positive bacteria is Defensin, the expression of which can be induced by IMD and Toll pathway activation (Dimarcq et al, 1994; Imler & Bulet, 2005; Nehme et al, 2011).

The *Drosophila* gut is equipped with physical and chemical barriers that contain microbes within the digestive tract. The peritrophic matrix is the first line of defense restricting the microbes to the lumen and prevents their direct contact with epithelial cells (Kuraishi et al, 2011). It thus fulfils a function analogous to that of mucus in vertebrates. AMPs are also secreted by the epithelial cells. However, this local AMPs secretion is IMD pathway-, and not *Toll* pathway-dependent (Liehl et al, 2006; Nehme et al, 2007; Ryu et al, 2006). A finely regulated induction of reactive oxygen species (ROS) is also triggered against ingested microrganisms (Bae et al, 2010; Ha et al, 2005a; Ha et al, 2005b). In addition to its resistance to microbes, *Drosophila* has developed endurance mechanisms to withstand and repair the damages caused by pathogenic bacteria. Gut homeostasis, in this case, is maintained by the compensatory proliferation of intestinal stem cell (ISC) (Biteau & Jasper, 2011; Buchon et al, 2009a; Buchon et al, 2010; Buchon et al, 2009b; Cronin et al, 2009; Jiang et al, 2011; Jiang et al, 2009). Most of these studies, however, have been performed using Gram-negative bacterial species.

The human intestine harbours hundreds of bacterial species (Qin et al, 2010). Any change in balanced interactions between intestinal microbes and the host immune system can lead to inflammatory disorders (Chassaing & Darfeuille-Michaud, 2011; Wells et al, 2011). Moreover, Firmicutes, a phylum that mostly consists of Gram-positive bacteria, is a major microbial population inhabiting the human intestine. In mammals, many physiological complications like obesity (Kallus & Brandt, 2012), insulin resistance (De Bandt et al, 2011), and Toll like receptors (TLRs)-mediated inflammation have been found to be associated with an abnormal proportion of Firmicutes (Caricilli et al, 2012; Serino et al, 2011). The *Drosophila* microbiota is made up of only 5 to 20 bacterial species. Interestingly, the microbiota is mainly dominated by Firmicutes, such as *Enterococcus faecalis* and *Lactobacillus plantarum*, and Proteobacter like *Acetobacter pomorum* (Cox & Gilmore, 2007; Ren et al, 2007; Roh et al, 2008; Shin et al, 2011; Storelli et al, 2011; Wong et al, 2011).

Staphylococcus xylosus is a Lys-type PGN containing Gram-positive bacterium that belongs to the phylum Firmicutes. It is a commensal of mucus and skin in mammals (Hariharan et al, 2011; Kloos & Schleifer, 1986; Kloos et al, 1976; Nagase et al, 2002). *S. xylosus* can be found in various niches like polluted water (Kessie et al, 1998), animal fodder and grains (Pioch et al, 1988), soil and various surfaces (Shale et al, 2005). It can form biofilms (Planchon et al, 2009; Planchon et al, 2006) and can adapt to various environmental conditions. *S. xylosus* is a natural component of raw meat and milk. It is used as a starter medium in the meat and milk fermentation industry (Kloos & Schleifer, 1986; Talon et al, 2002). Moreover, the zinc-dependent metallolipase produced by *S. xylosus* is extensively used by the biotransformation industry (Bertoldo et al, 2011).

S. xylosus is normally considered to be a nonpathogenic *Staphylococcus* but some strains are opportunistic in humans and animals (Bingel, 2002; Bradfield et al, 1993; Fthenakis et al, 1994; Jackson et al, 2001; Miedzobrodzki et al, 1989). In humans *S. xylosus*

has been found associated with endocarditis (Conrad & West, 1984), septicemia (Koksal et al, 2009), acute pyelonephritis (Tselenis-Kotsowilis et al, 1982) and chronic granulomatous disease (CGD) (Gozalo et al, 2010). CGD is caused by genetic disorders in humans that affect one component of NADPH oxidase and lead to recurrent bacterial and fungal infections (Roos et al, 2007). Indeed, *S. xylosus* was reported to be the major cause of death for mice deficient in NADPH oxidase (Gozalo et al, 2010). Genetic variation observed between 24 different strains of *S. xylosus* divided them into two distinct groups based on their potential to become opportunistic pathogens (Dordet-Frisoni et al, 2007a).

Our knowledge about the *Drosophila* gut defense responses against Lys-type PGN Gram-positive bacteria is very limited. Indeed, the major AMP response described in the intestinal epithelium is controlled by the IMD pathway, which cannot be activated by these bacteria. We therefore used a *S. xylosus* strain Argentoratum originally isolated from microsporidia-infected fly stocks found in our laboratory in Strasbourg. In this work, we first found that *S. xylosus* behaves as a classical Gram-positive bacterium upon septic injury. Second, we showed that flies fed on a *S. xylosus* containing solution succumbed faster than uninfected controls. We than discovered that the Toll pathway mutant *MyD88* were more resistant to an oral challenge with *S. xylosus* as compared to wild-type flies and to starvation. These data allow us to uncover a potential link between innate immune genes and lipid metabolism.

Materials and methods

Fly strains.

Flies were reared at 25°C on standard corneal-agar medium. *cn bw* flies were used as wild-type control for *Dif* mutants flies (Rutschmann et al, 2000) while A5001 were wild-type controls for *MyD88*^{c03881} (Tauszig-Delamasure et al, 2002), *key*, and rescue strains with a UAS-MyD88^{+} transgene (Tauszig-Delamasure et al, 2002). The NP1-Gal4 driver line was obtained from DGRC, Japan. All crosses to generate transgenetic rescue fly lines were performed at 25°C.

Bacterial strains and growth conditions

S. xylosus strain named Argentoratum was isolated from moribund Oregon flies at UPR9022, IBMC. The flies were later found to be infected by microsporidia as well. Another *S. xylosus* strain C2a is described in (Dordet-Frisoni et al, 2007a; Dordet-Frisoni et al, 2007b). Colonies naturally resistant to streptomycin (100µg/mL) were selected to establish stock in 20% glycerol stored at –80°C. Before infection bacteria were grown at 37°C overnight in LB containing Streptomycin (100µg/mL).

Pricking assay:

Bacteria were pelleted to the equivalent of an optical density of about 200 at 600nm (OD_{600}) from an overnight culture grown at 37°C in Luria-Bertani broth (LB). A tungsten needle was either directly dipped in this pellet or the bacteria were first diluted to an optical density of 6 before challenging flies (20 flies/survival experiment) by pricking with the contaminated needle. Survival experiments were performed at 25°C and survival data was collected at least once a day. Survival curves were statistically analysed using the Log Rank

test as implemented in Prism software. Each survival curve is a representative of at least three survival experiments.

Oral infection

Bacteria were grown overnight in LB to an OD of 4, diluted to an OD of 0.4 (equivalent to approximately $6x10^7$ bacteria/mL) in sucrose (50mM) solution containing Streptomycin (100μg/mL). The bacterial solution was then used to soak filter pads (Millipore) placed at the bottom of fly culture vials before adding the flies. To block the effects of the ROS response, N-acetylcysteine (Sigma) was added to the bacterial solution to a final concentration of 20mM. All survival experiments were performed at 25°C and survival was monitored at least once per day. LT50 (logEC50) values were calculated using Prism software.

Starvation

Sterile water was directly added to filter pads in medium sized tubes before transferring flies. Experiments were kept at 25°C and fly survival was monitored twice per day.

Fat Quantification

Male flies were crushed in 0.05% tween (Sigma), heated at 70°C for 5 minutes and centrifuged at 5000 rpm to remove debris. Samples were then mixed with Thermo Infinity Trig. Solution and incubated at 37°C for 5 minutes. The Infinity Trig. Solution contains many reagents including lipase that first converts triglycerides into glycerol. A series of chemical reactions convert glycerol to hydrogen peroxide that in turn produces a red dye. The absorbance of this dye is measured at 570 nm at Mithras LB 940 (Berthold Technologies),

which is proportional to the concentration of the total fats in the sample. Percentage fat loss was calculated by the following formula:

Fat Loss (%) = $[(F_{0h} - F_{24h}) / F_{0h}] \times 100$, where F_{0h} is the initial fat content and F_{24h} is the fat content after 24 hours of beginning of oral feeding with *S. xylosus* (Fig. 3A) or food starvation (Fig. 4B) at 25°C.

Glycogen Quantification

Male flies were crushed in 0.05% tween (Sigma) and centrifuged. Supernatants were treated with freshly prepared Amyloglucosidase (Sigma; 0.1mg/mL) and incubated at 37°C for 3 minutes to covert polysaccharide chains of glycogen into simple glucose molecules. The samples were then added to Infinity Glucose Reagent (Thermo) in a ratio of 1:100 and incubated for 5 minutes at 37°C. This converts glucose to reduced Nicotinamide Adenine Dinucleotide (NADH). Amount of NADH produced is measured at fluorescence at F355/F460 (excitation and emission respectively) on a Mithras LB 940 (Berthold Technologies), which is proportional to concentration of glucose in the sample. Percentage glycogen loss was calculated by the following formula:

Glycogen Loss (%) = $[(G_{0h} - G_{24h})/G_{0h}] \times 100$, where G_{0h} is the initial glycogen contents and G_{24h} is the glycogen contents after 24 hours of starvation at 25°C.

Sucrose Quantification

Samples directly collected from infected filters were mixed with Invertase (Sigma) in final concentration of 2mg/mL and incubated for 2 hours at 55°C. This converts disaccharide sucrose molecules into fructose and glucose (monosaccharides). Samples were then directly added to Infinity Glucose Reagent (Thermo) in a ratio of 1:100, and further treated as described in glycogen quantification.

Blockade of phagocytosis:

Blockade of phagocytosis was achieved by injecting non-degradable latex beads with the NanojectII (Drummond). Gut dissections and collection of hemolymph to monitor bacterial titre were also performed as described in (Nehme et al, 2007).

Results

S. xylosus *Argentoratum is a virulent pathogen in the septic injury model*

To determine whether *S. xylosus* is a virulent bacterium able to overcome *Drosophila* host defenses, we first challenged flies in the septic injury model using a needle that had been first dipped into a concentrated bacterial pellet. Both wild-type and Toll pathway mutants such as *MyD88* and *Dif* mutant flies succumbed to the infection within two days (Fig. 1A). However, when challenged with a needle dipped into a diluted bacterial solution (equivalent to OD6) wild-type flies were much more resistant to the infection than Toll pathway mutants (Fig. 1A). This result is in keeping with results gained with other LYS-type PGN bacteria (Michel et al, 2001; Rutschmann et al, 2002). Both wild-type flies and Toll pathway mutants resisted an infection with about 10 bacteria (corresponding to a needle dipped into a bacterial solution of OD 0.6). Of note, *Toll* pathway mutants challenged with a nonpathogenic strain of *S. xylosus* (needle dipped into an OD6 equivalent), C2a, resisted the infection like wild-type flies (data not shown). The expression of *Drosomycin-GFP*, a classical transgenic read-out of the systemic activation of the Toll pathway, was induced after infection with *S. xylosus,* which suggests that the bacterium is efficiently detected by the fly immune system (Fig. 1B). Next, we assessed the importance of the cellular immune response. When phagocytosis had been saturated by the prior injection of nondegradable latex-beads, flies succumbed to about ten bacteria in about two days, independently of the host genotype (data not shown). In keeping with this result, *S. xylosus* were able to proliferate only when the cellular immune response was impaired, either by latex beads or in mutants for the phagocytic receptor *Eater* (Kocks et al, 2005) (Fig. 1C). However, C2a proliferated to a 100-fold lower level when tested under the same conditions (data not shown). These results underscore the importance of phagocytosis in controlling virulent *S. xylosus* systemic infections, as described previously for

other Gram-positive bacteria (Charroux & Royet, 2009; Defaye et al, 2009; Nehme et al, 2011). Taken together, these experiments indicate that virulent *S. xylosus* behaves rather like *Enterococcus faecalis* in the septic injury model (Michel et al, 2001; Rutschmann et al, 2002).

S. xylosus *is less virulent in an intestinal infection model and apparently kills* MyD88 *mutants less rapidly than wild-type flies*

We asked whether *S. xylosus* might be used to model intestinal infections by Gram-positive bacteria. Thus, we assessed the behavior of *S. xylosus* after oral infection, in which overnight bacterial cultures are diluted one to ten in a sucrose solution and then placed on a filter onto which flies feed. Moisture was kept constant usually by adding water to the vials everyday. Wild-type and *kenny* (IMD pathway mutant) flies succumbed at the same rate (Fig. 2A) with 50% of flies dying by five days (LT50=4.9). However, *MyD88* flies were surprisingly killed more than one and a half day later (LT50=6.6) (Fig. 2B). The IMD pathway is a major defense against Gram-negative bacterial infections in barrier epithelia. In keeping with the *kenny* survival data (Fig. 2A), we failed to detect a consistent induction of the IMD pathway reporter transgene *Dipt-LacZ* in the digestive tract of *S. xylosus*-infected flies (Fig. S1). As some bacteria are able to cross the intestinal barrier, we asked if this were the case for *S. xylosus* and whether bacteria proliferating in the hemocoel might provoke the death of the fly, as observed with *Pseudomonas aeruginosa* (Limmer et al, 2011). While we consistently retrieved very few bacteria from the hemolymph of orally-infected flies, the titer remained constant throughout the infection in wild-type flies (Fig. 2C). To further test a possible involvement of hemolymphatic *S. xylosus*, we neutralized phagocytosis with latex beads. Wild-type flies displayed a weakly enhanced susceptibility to ingested *S. xylosus* when phagocytosis was impaired (LT50=4.3) while *MyD88* mutant resistance was unchanged (LT50=6.6) (Fig. 2D). Furthermore, the bacterial titer in the hemolymph remained moderate

in phagocytosis-impaired flies as compared to untreated flies (only a tenfold increase and no exponential growth as observed with other models (Limmer et al, 2011; Nehme et al, 2007)) (Fig. 2E). Interestingly, there was no significative difference of the hemolymphatic bacterial titer between wild-type and *MyD88* mutant flies after the first day of infection. These results show that *MyD88* mutants are more resistant than wild-type flies in a *S. xylosus* intestinal infection model.

To identify the tissue in which the wild-type *MyD88* gene might be acting to sensitize flies to ingested *S. xylosus*, we attempted to reverse the *MyD88* enhanced survival phenotype by overexpressing a wild-type version of the gene specifically in either of two immune-relevant tissues, hemocytes or enterocytes, in *MyD88* mutant flies. This was achieved by using a transgenic construct, the expression of which was driven using the Gal4/UAS system and either the *hemolectin Delta*-Gal4 driver (hemocytes) or the *NP1*-Gal4 driver (enterocytes). We found that the *MyD88* mutant phenotype was reversed only when the transgene was expressed in the midgut (Fig. S2). We thus dissected midguts at various stages and stained them with phalloidin-FITC to reveal the morphology of enterocytes when observed by confocal microscopy. We failed to detect any striking morphological differences between *S. xylosus*-orally infected wild-type flies and control flies fed on a sucrose solution (Fig. S3). In contrast to flies orally infected with *Serratia marcescens* in which the bacterial titer in the gut increases throughout the infection, we found that the *S. xylosus* titer in dissected guts was constantly low (Fig. S4) and, for instance, was 500-fold lower than the *S. marcescens* titer after five days of infection. This suggested that *S. xylosus* was kept under control by midgut host defenses. As the IMD pathway is apparently not induced, the major known remaining defense is the microbial reactive oxygen species (ROS) response, generated by DUOX (Ha et al, 2005a). The *immune response catalase* (IRC) is necessary in the gut to protect the host against detrimental effects of ROS: in fact ROS are able to kill the IRC

deficient flies, even when triggered by dead bacteria (Ha et al, 2005b). We therefore asked whether too strong a ROS-response might be causing the death of flies that have ingested *S. xylosus*. Adding N-acetyl cysteine to the food solution has been reported to alleviate the ROS response (Ha et al, 2005a). This treatment is indeed able to effectively prevent the oxidative response since it inhibits the activation of a transgenic bacterial catalase reporter (Fig. S5). Then, we fed flies with a mixture of *S. xylosus* and N-acetyl cysteine. This treatment did not significantly improve the survival of flies to ingested *S. xylosus* (Fig. 2F). The ROS response is also able to cause severe damage to the gut within a few hours when strongly triggered by the Gram-negative bacterium *Erwinia carotovora carotovora*. However, gut damages are repaired within 48 hours through the compensatory proliferation of intestinal stem cells, a process that is partially dependent on the JAK-STAT pathway (Bach et al, 2007). We thus monitored the expression of a transgenic construct in which GFP expression is regulated by a promoter that contains ten STAT binding elements. This construct is activated in several gut damage models (Buchon et al, 2009a; Buchon et al, 2009b; Cronin et al, 2009; Jiang et al, 2009). However, w failed to observe any consistent induction of this reporter in flies that had ingested *S. xylosus* (Fig. S6). Taken together, these experiments suggest that midgut damages are not the cause of the demise of the infected flies.

Next, we asked whether flies might be killed by a toxin secreted by the bacteria. Flies fed on bacteria in which the culture supernatant had been removed by centrifugation died at the same rate as flies fed on bacteria according to our standard protocol, while flies fed on the supernatant alone did not die (Fig. S7). Flies fed on UV-killed or heat-killed bacteria also survived the experiment (Fig. S7). Moreover, our *S. xylosus* strain tested negative by PCR for the presence of enterotoxin A to H. In conclusion, flies were apparently not killed by a secreted toxin.

Flies fed on* S. xylosus *likely die from starvation

To monitor the general physiological state of the flies, we measured the total fat in flies that had fed on *S. xylosus*. Wild-type flies lost about 70% of their fat reserves already 24 hours after the beginning of the oral infection; total fat remained constant thereafter (Fig. 3A). This suggested that such a drastic loss might at least be partially caused by starvation. Flies appeared to feed normally as monitored by adding dextran-blue (1%) to the bacterial solution (data not shown). We supplemented the vials daily with a sucrose solution (sucrose regimen) or water (water regimen). The sucrose regimen rescued the survival of the flies independently of their genotypes after an oral infection (Fig. 3C: compare *MyD88* to wild-type flies under sucrose and water). LT50 data are shown in Fig. 3D for flies under the water regimen (they could not be computed for flies under sucrose regimen since they were not dead when we stopped the experiment after 12 days). We checked that the sucrose concentration was significantly declining on the filters after 3 days only in the water regimen, and not in sucrose regimen (Fig. 3B). Interestingly, flies on the sucrose regimen regained their fat stores three to four days after the beginning of the infection (Fig. 3A). These experiments strongly suggest that flies actually succumb to starvation and not to the infection.

MyD88 *mutant flies resist starvation better than wild-type flies*

If death were indeed due to starvation, one would expect that *MyD88* mutants would display a higher resistance to starvation. We therefore deprived flies of food by placing them on filters moistened only with pure sterile water. *MyD88* males and females succumbed about one and a half day later than wild-type flies (males: 1.2 days; females: 1.5 days based on LT50s) (Fig. 4A and data not shown). We then revisited the tissue-specific genetic rescue experiments of the *MyD88* phenotype to determine whether the resistance to starvation was due to a *MyD88* function limited to the midgut. We added additional controls to the

genotypes: *UAS-MyD88*$^{+}$*/+; MyD88/MyD88; hml-Gal4/+* (1xUAS-XChr-hml) for the rescue in hemocytes (transgene heterozygous, one copy on the X) and *UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*; MyD88/MyD88, NP1-Gal4* (2xUAS-XChr-NP1) for the rescue in the gut. Thus, the female flies for which there was a rescue when challenged with *S. xylosus* (*NP1* midgut driver) were actually homozygous for the *UAS-MyD88* transgene insertion on the X chromosome (two copies of the transgene). We thus asked whether the rescue was dependent on the number of copies of the transgene. We first analyzed the survival to starvation of *MyD88* flies carrying two copies of the wild-type *UAS-MyD88* on the X chromosome and of flies containing only one copy, both carrying also the NP1 midgut driver. As shown in Fig. 4A and Fig. S8, whereas the former did rescue the *MyD88* starvation phenotype, the latter did not. However, this result was unlikely to be due to the presence of two copies of the transgene in the genotype as flies carrying also two copies of the transgene, this time on two distinct chromosomes (X and 2nd) did not rescue the phenotype (Fig. S8). Thus, the alternate interpretation is that the transgene inserted on the X chromosome inactivates a resident gene, which would be a recessive suppressor of *MyD88*. Indeed, flies in which the trangene insertion on X is homozygous (females) or hemizygous (males) do display the reversed phenotype (Figs. 4, S8, and data not shown). If this interpretation were correct, then one would expect that the rescue is independent of the expression of the transgene, that is, a rescue should be still observed in the absence of the NP1 driver. This is the result we actually observed as shown on Fig. 4 (compare 2xUAS-XChr-NP1 to 2xUAS-XChr). The homozygous insertion of the transgene on the second chromosome did not rescue the *MyD88* starvation phenotype (Figs. 4 and S8). We conclude that the transgene insertion on the X chromosome inactivates a gene that behaves as a recessive suppressor of the *MyD88* starvation resistance phenotype. This suppressor is specific of the food deprivation phenotype

as *MyD88* flies that carry the homozygous transgene insertion on the X remained sterile and were still sensitive to a challenge with *E. faecalis* (data not shown).

Finally, we further investigated the *MyD88* starvation resistance phenotype. To this end, we measured metabolic stores in males that are wild-type, *MyD88*, and hemizygous for the X chromosome suppressor (*UAS-MyD88*$^{+}$*/Y; MyD88/MyD88, NP1-Gal4*), which partially reverse the starvation resistance phenotype in terms of survival (data not shown) after 24 hours of starvation. While glycogen stores were depleted for all genotypes (Fig. 4B), we found that total fat loss was much less important (20%) in *MyD88* flies than in wild-type and transgenic rescue flies that succumb rapidly to starvation (Fig. 4B and C). We conclude that the wild-type *MyD88* gene is directly or indirectly required for the depletion of fat stores when flies are starved.

Discussion

While attempting to model intestinal infections by opportunistic Gram-positive bacteria in *Drosophila*, we have discovered that flies continuously ingesting *S. xylosus* were actually succumbing to starvation in a process that is facilitated by *MyD88*, a Toll pathway intracellular adapter component. Strikingly, food deprivation led to a drastic and rapid *MyD88*-dependent loss of metabolic fat stores.

S. xylosus Argentoratum behaves like a classical Gram-positive opportunistic pathogen (*e.g.*, *E. faecalis*) in the septic injury model as it efficiently kills Toll pathway mutants or phagocytosis-impaired flies, but not wild-type flies (Michel et al, 2001; Nehme et al, 2011; Rutschmann et al, 2002). Interestingly, another strain of *S. xylosus*, which has been described as nonpathogenic, C2a, is also not an opportunistic pathogen in immunodeficient flies. When ingested, very few *S. xylosus* Argentoratum bacteria manage to cross the gut. We have previously described other intestinal infection models in which Gram-negative bacteria such

as *S. marcescens* escape from the gut. Phagocytosis of *S. marcescens* in the hemocoel by hemocytes is sufficient to prevent their proliferation (Kocks et al, 2005; Nehme et al, 2007). Surprisingly, although the injection of ten *S. xylosus* bacteria in latex bead-treated *MyD88* flies kill those flies, an equivalent number of *S. xylosus* fail to proliferate under the same conditions after their passage through the intestinal barrier (Fig. 1C). One possibility is that bacteria that have crossed the gut have changed their virulence program. Alternatively, they may have come to be weak, for instance after undergoing alterations in their cell wall as a consequence of midgut host defenses. Consequently, once arrived in the hemocoel, they would become sensitive to another arm of the host defense, *e.g.,* melanization (Matskevich et al, 2010), which normally is not sufficient to kill them.

A striking finding is that known epithelial immune responses are mediated via the IMD pathway (Ferrandon et al, 1998; Han et al, 2004; Tzou et al, 2000). This pathway is induced by DAP-type PGN, which is not found in *Staphylococci*. Thus, it is not unexpected that the IMD pathway is not triggered by an oral challenge with *S. xylosus*. However, we find that the number of viable *S. xylosus* found in the digestive tract remains low throughout most of the infection, which suggests that some host defense is able to control it. These host defenses may include digestive enzymes such as lysozymes (Daffre et al, 1994; Hultmark, 1994) or the DUOX-mediated ROS response. Of note, we failed to find any indication of an overreaction of the oxidative immune response that would damage the gut (Figs. S1, S3, & S6), as has been described in other systems (Buchon et al, 2009a). However, we note that we are using a much lower amount of bacteria in our infection model; thus, we might not have reached the threshold that leads to a destructive response.

We did not find any evidence for the action of a toxin. Indeed, simply adding sucrose to the vials was sufficient to rescue the lethality observed in our model. In addition, we did not observe any obvious modification of the digestive epithelium (Fig. S3) nor an induction of

the JAK-STAT pathway (Fig. S6), which is induced by many stresses. As control flies feeding on a sucrose solution survive at least for 12 days, it is likely that there is a competition for sucrose uptake between bacteria and flies, a phenomenon we have observed with other bacteria (unpublished data). Furthermore, our experiments suggest that, even though flies express enzymes able to degrade the bacterial cell wall such as lyzozymes and amidases, they nevertheless are not able to feed on them. We note that sucrose depletion begins around day 4 (Fig. 3B) and that flies succumb one to two days later, which is approximately the time it takes them to die from starvation. The striking result is that fat reserves are depleted very rapidly 24 hours after an oral challenge with *S. xylosus* (Fig. 3A), even though there is still at that time enough sucrose to feed on. Indeed, orally-infected flies appear to display a marked reduction of their glycogen stores only on the fourth day of the infection (data not shown). Furthermore, we note that fat stores are progressively replenished within three days when sucrose is added to the infection vials on a daily basis. As a basis for comparison, flies systemically infected with *Mycobacterium marinum*, which induces wasting, undergo this degree of loss only after seven days, when they are about to succumb to the infection (Dionne et al, 2006). Interestingly, the important depletion of fat stores we have observed in our oral infection model may be linked to the bacterial infection *per se* and not to a possible deprivation of amino acids, as preliminary data indicate that flies starved for amino acids (feeding on sucrose only) for 24 hours only lose 40% of such reserves. As this effect is observed rapidly even though there is still enough sucrose, it suggests that it is a distinct phenomenon from what occurs when flies are totally deprived of food. Thus, it will be interesting to determine the physiological basis for this effect. We note that *S. xylosus* strains secrete an extracellular lipase (Bertoldo et al, 2011; Kolling et al, 2010; Rosenstein & Gotz, 2000) that could be involved in fat reserve depletion.

In larvae, it is known that starvation leads to a depletion of triglycerides stores in the fat body, partially through the Brummer lipase (Gronke et al, 2005). Released lipids are then metabolized in oenocytes, which perform a function similar to mammalian hepatocytes (Gutierrez et al, 2007). We did not find any indication of a significant increase in *Brummer* expression in wild-type starved adult flies (data not shown). Thus, lipid mobilization might be performed by another lipase in starved adults, lipase 3 for instance. As regards the Toll pathway, it has recently been reported that its activation by an immune challenge leads to an attenuation of insulin signaling, leading for instance to the nuclear localisation of the FOXO transcription factor, with a concomitant mild decrease in fat stores (DiAngelo et al, 2009). Here, we observe that there is a much reduced decrease of fat stores upon starvation in *MyD88* mutants as compared to wild-type flies (Fig. 4B and C). This finding is consistent with the hypothesis that the utilization of triglycerides during starvation may be mediated by the Toll pathway. Indeed, a mild FOXO-mediated increase of AMP expression has been reported in starved second instar larvae, which is much less intense than that occuring during the systemic immune response. Thus, one might envision that Toll pathway activation would lead to the nuclear localisation of FOXO, which in turn would drive AMP, especially *Drosomycin*, expression. However, no significant induction of *Drosomycin* or other AMPs has been observed after 24 hour of nutrient deprivation of adult flies (Buch et al, 2008), a result we confirmed as regards *Drosomycin* (data not shown). Thus, we have no indication that there is indeed an activation of the Toll pathway during the first 24 hours of starvation in adults, at least one that would yield an activation of the systemic immune response. As *Drosomycin* expression might not be a relevant read-out of Toll pathway activation under these circumstances, we therefore privileged a genetic approach and asked whether another Toll pathway gene, *Dif* (Rutschmann et al, 2000), was also involved in the fat loss that we observe 24 hours after the beginning of nutrient deprivation. We found that *Dif* mutants were

dying from starvation almost at the same rate as *MyD88* flies. However, because *Dif* mutants have been generated in another genetic background, we included nonmutagenized flies as controls in this experiment and found that they were resisting starvation even better than *Dif* mutants (data not shown). Thus, while these experiments suggest that *Dif* may not be involved in that phenomenon, we cannot formally rule out a role for other genes of the Toll pathway, for instance *Dorsal*, which encodes the other transcription factor regulated by the Toll pathway that is mostly used during development. We note that *Dorsal* and *Dif* are functionally partially redundant as regards the immune response in larvae, but not in adults (Manfruelli et al, 1999; Meng et al, 1999; Rutschmann et al, 2000). Another possibility is that *MyD88* may have a Toll-independent function activated during starvation. This hypothesis is supported by the discovery of a recessive suppressor of *MyD88*, which suppresses only the starvation but not the developmental or immune phenotypes of *MyD88* (Charatsi et al, 2003; Tauszig-Delamasure et al, 2002). The analysis of the suppressor mutation that we have identified by the insertion of a wild-type *MyD88* transposon on the X chromosome will likely shed some light on this issue. One important conclusion of this study is that *MyD88* may play a third role beyond those described in development and innate immunity.

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FIGURES LEGENDS

Fig. 1: *S. xylosus* is a virulent pathogen in the septic injury model

(A) Flies were either pricked with the concentrated bacterial pellet obtained from overnight culture or bacteria were first diluted in sterile PBS to OD 6 (OD_{600}) prior to pricking. Flies were then transferred to 25°C and survival was monitored on daily basis. Note that the three survival curves with the concentrated bacteria are indistinguishable from each other. This represents one experiment out of three. (B) The induction of Toll pathway was monitored using a Drosomycin-GFP reporter fly line 48 hours post infection (OD_{600} 6); Ml: *Micrococcus luteus*. n=3. (C) Ten bacteria diluted in PBS were injected in flies, with or without prior injection of latex beads (LXB). The hemolymph was extracted and plated, thus allowing the measurement of the bacterial titer. The results shown represent one out of two experiments.

Fig. 2: Ingested *S. xylosus* appears to kill *MyD88* flies slower than wild-type flies in a process that does not involve a systemic infection

(A-B) *S. xylosus* overnight culture diluted in sucrose (50mM) to final OD of 0.4 (OD_{600} 0.4) was orally fed to wild-type and *key* (IMD pathway mutant) flies (A) and survival monitored. (B) Same as (A), except that the Toll pathway mutant *MyD88* is compared to wild-type flies. (C-E): A few *S. xylosus* bacteria cross the intestinal barrier but are unlikely to cause the death of their host. Hemolymph was collected and plated to determine the bacterial titer without (C) or with (E) prior latex beads injection. The data show the mean of three experiments; error bars represent standard error. The ablation of phagocytic activity by latex bead pretreatement did not influence the survival rate of flies that had ingested *S. xylosus* (D). (F) The DUOX-mediated ROS response in the gut is likely not responsible for the death of flies that have ingested *S. xylosus*, as the addition of N-acetylcyteine to the bacterial solution did not alter the survival of infected flies. All experiments have been repeated twice.

Fig. 3 Flies feeding on *S. xylosus* likely die from starvation

(A) Loss of fat reserves (reference is day 0) were measured on flies that had ingested *S. xylosus*, either with the daily addition of 200 µl of water or 100mM sucrose solution. Statistical analysis was performed for each day by comparing the water to the sucrose treatment (p-values; D1: p=0.5 n=6 (ns), D2: p=0.1 n=6 (ns), D3: p=0.0002 n=9 (**), D4: p<0.0001 n=9 (***). (B) The sucrose concentration on the filters the flies feed on was measured under the water or sucrose regimens. Statistical analysis was performed by comparing the measured concentration to the initial one (D0); p-values for *water supplementation*: D1: p=0.58, D2: p=0.67, D3: p=0.2, D4: p=0.003 (**); p-values for *sucrose supplementation*: D1: p=0.009 (*), D2: p=0.195, D3: p=0.67, D4: p=0.48; n=9 for all data series. (C) Survival of flies that had ingested *S. xylosus* and that were either under the *water regimen* (plain lines) or *the sucrose regimen* (dashed lines). Flies under the *sucrose* regimen did not succumb to ingested *S. xylosus*. A statistical analysis was performed to compare the effect of distinct genotypes with wild-type flies, all under the *water regimen* (D). LT50 values (time it takes for 50% of the flies to die) were determined and averaged (p-values: *MyD88* flies: p=0.002 n=7 (**); 2xUAS-XChr (*UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*; MyD88/MyD88*) flies: p=0.6 n=7 (ns); 2xUAS-2ndChr (*MyD88, UAS-MyD88*$^{+}$*/MyD88, UAS-MyD88*$^{+}$) flies: p=0.02 n=6 (*)). Error bars represent standard error in all panels.

Fig. 4 Wild-type *MyD88* facilitates starvation-induced lipid consumption

(A) *MyD88* flies endure starvation better than wild-type flies, a phenomenon that can be suppressed by the insertion at an unknown locus on the X chromosome of a transgene carrying a wild-type copy of *MyD88*, whereas the insertion of the same transgene at another site on the second chromosome did not rescue the *MyD88* starvation resistance phenotype.

LT50s values were calculated for each survival experiment performed under food deprivation and values were compared with the wild-type A5001 flies (p-values, *MyD88* flies: p<0.0001 n=49 (***), 2xUAS-XChr-NP1 (*UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*; MyD88/MyD88, NP1-Gal4*) flies: p=0.4 n=15 (ns), 2xUAS-XChr (*UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*; MyD88/MyD88*) flies: 0.1 n=18 (ns), 2xUAS-2ndChr (*MyD88, UAS-MyD88*$^{+}$*/MyD88, UAS-MyD88*$^{+}$) flies: p=0.0006; n=9 (**)). There is no difference in the LT50 of flies homozygous for the X-insertion whether the transgene is expressed in the gut under the NP1 driver or not. (B) Loss of fat contents was measured in male flies of different genotypes that had been starved for 24 hours (left panel). Fat reserves were relatively well preserved in starved *MyD88* mutant flies as compared to that of wild-type flies, while there was not statistical difference between fat losses by 1xUAS-X/YChr-NP1 (*UAS-MyD88*$^{+}$*/Y; MyD88/MyD88, NP1-Gal4*) and wild-type flies (p-values: *MyD88* flies p=0.0001 n=9 (***); 1xUAS-X/YChr-NP1 flies: p=0.1 n=8 (ns)). Loss of glycogen reserves were also measured in starved male flies (right panel) and statistically analysed in the same way as in case of fat contents (p-values: *MyD88* flies p=0.4 n=5 (ns); 1xUAS/Y-XChr-NP1 flies p=0.96 n=5 (ns)). (C) Fat reserves are depleted in wild-type but not in *MyD88 flies.* Fat bodies from flies starved for 24 hours were dissected, mounted in glycerol, and observed by light microscopy without any further treatment. This experiment has been performed twice with at least 10 flies dissected each time. Error bars represent standard errors in all panels.

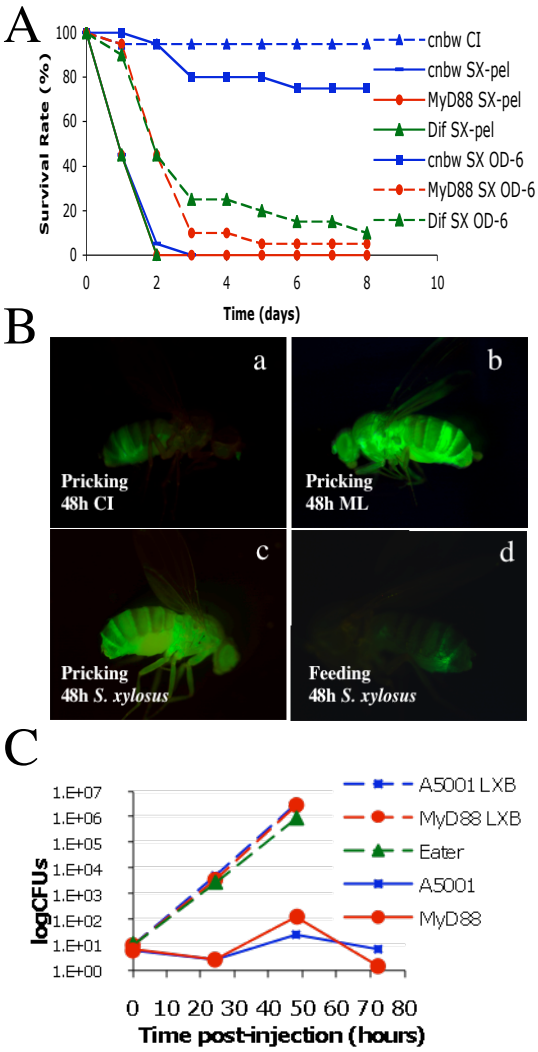
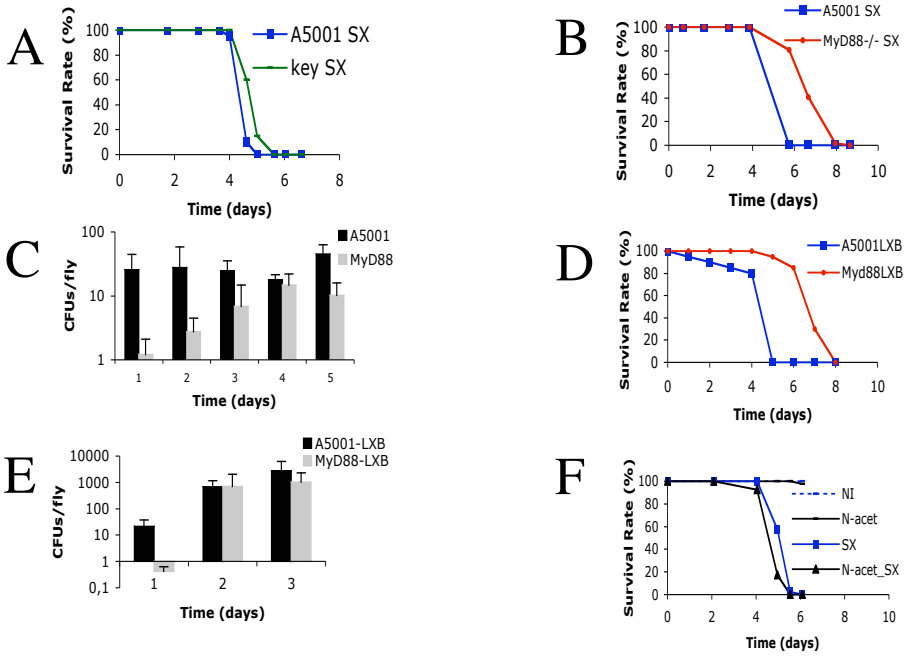

A
Survival Rate (%)
100
80
60
40
20
0
0
2
4
6
8
10
Time (days)
cnbw CI
cnbw SX-pel
MyD88 SX-pel
Dif SX-pel
cnbw SX OD-6
MyD88 SX OD-6
Dif SX OD-6
B
a
Pricking
48h CI
b
Pricking
48h ML
c
Pricking
48h S. xylosus
d
Feeding
48h S. xylosus
C
logCFUs
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0 10 20 30 40 50 60 70 80
Time post-injection (hours)
A5001 LXB
MyD88 LXB
Eater
A5001
MyD88

Fig. 1


A
Survival Rate (%)
Time (days)
A5001 SX
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B
Survival Rate (%)
Time (days)
A5001 SX
MyD88-/- SX
C
CFUs/fly
Time (days)
A5001
MyD88
D
Survival Rate (%)
Time (days)
A5001LXB
Myd88LXB
E
CFUs/fly
Time (days)
A5001-LXB
MyD88-LXB
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Survival Rate (%)
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NI
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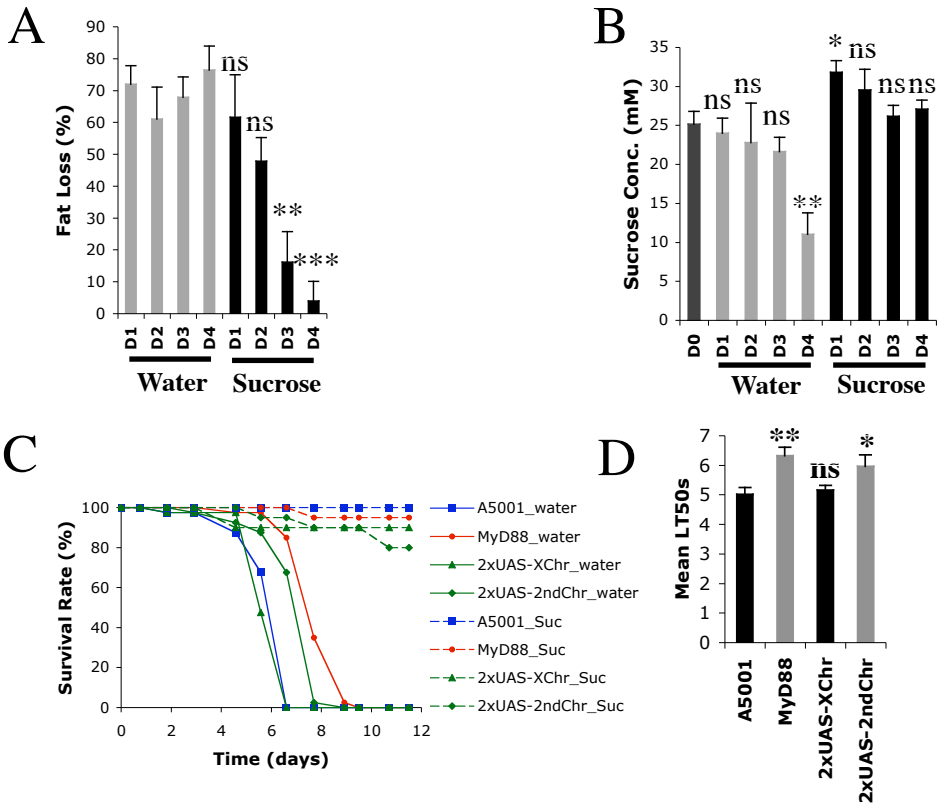
Fig. 2


A
Fat Loss (%)
90
80
70
60
50
40
30
20
10
0
ns
ns
**
***
D1
D2
D3
D4
D1
D2
D3
D4
Water
Sucrose
B
Sucrose Conc. (mM)
35
30
25
20
15
10
5
0
ns
ns
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**
*
ns
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D0
D1
D2
D3
D4
D1
D2
D3
D4
Water
Sucrose
C
Survival Rate (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (days)
A5001_water
MyD88_water
2xUAS-XChr_water
2xUAS-2ndChr_water
A5001_Suc
MyD88_Suc
2xUAS-XChr_Suc
2xUAS-2ndChr_Suc
D
Mean LT50s
7
6
5
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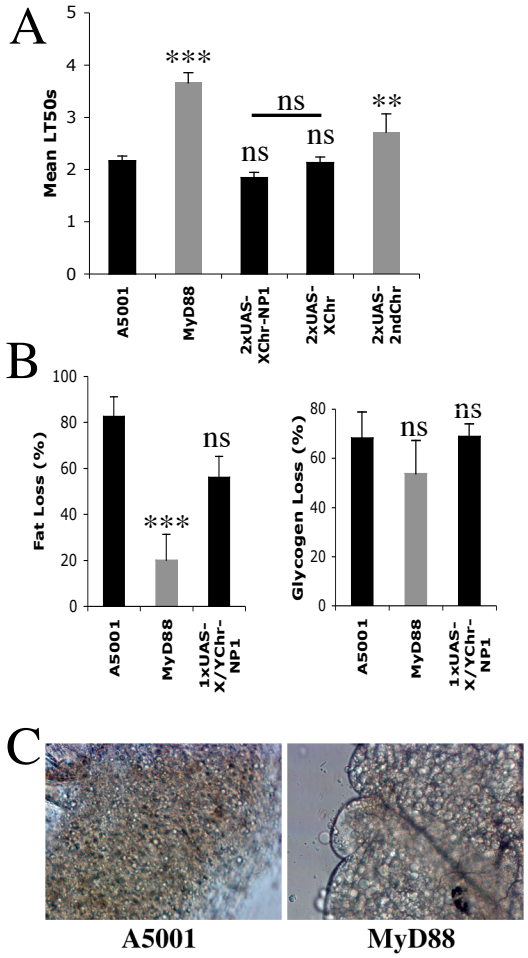
A5001
MyD88
2xUAS-XChr
2xUAS-2ndChr

Fig. 3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Mean LT50s
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MyD88
2xUAS-XChr-NP1
2xUAS-XChr
2xUAS-2ndChr
B
Fat Loss (%)
100
80
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A5001
MyD88
1xUAS-X/YChr-NP1
Glycogen Loss (%)
80
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A5001
MyD88

Fig. 4

Supplementary Data

A negative role of *MyD88* in the resistance to starvation as revealed in an intestinal infection of *Drosophila melanogaster* with the Gram-positive bacterium *Staphylococcus xylosus*

AYYAZ A., P. GIAMMARINARO, S. LIEGEOIS, and D. FERRANDON

Supplementary Materials and methods

Fly strains

The transgenic fly strains 10XSTAT-GFP (*stat92*E-GFP) (Bach et al, 2007), Hml-Gal4 [the hml-Gal4 line is actually hmlΔ-Gal4] (Sinenko & Mathey-Prevot, 2004) and MyD88_CL Excision (clean excision line for *MyD88* mutation) (Tauszig-Delamasure et al, 2002) were reared at 25°C on standard corneal-agar medium.

Construction of ROS biosensor *E. coli* Cat-GFP strain

The *catG* promoter was amplified from *Escherichia coli* using primers 5′cgggatcctcaggcggatttgctta3′ and 5′cgtctagacaatgtgctcccctcta3′ and cloned in the plasmid pTAC4598 (Atlung et al, 2002) by placing the promoter of *catE* in front of the GFP reporter gene, using BamHI and XbaI restriction sites. The ligated plasmid was used to transform DH5α strain of *E. coli*.

Microscopy

Gut dissections, staining of brush boarder using FITC-labelled phalloidin, observations by confocal microscopy and staining of β-galactosidase activity in infected guts were performed as previously described (Nehme et al, 2007).

N-acetylcysteine treatment

Flies were kept overnight at 25°C on sucrose solution (50mM) containing N-acetylcysteine (20mM). The next day the ROS biosensor *E. coli* Cat-GFP strain (OD 0.1 diluted from overnight culture grown in LB at 37°C) was added to the sucrose solution plus N-acetylcysteine solution. Flies were then transferred in the vials containing filters and incubated at 25°C for three hours. Dissected guts were mounted in 80% glycerol solution and observed under fluorescent microscope.

Survival experiments for bacterial toxins

An overnight bacterial culture (grown at 37°C) was diluted to OD 1 in LB before heat killing for 1 hour at 95°C. These heat-killed bacteria were then diluted ten times in 50mM sucrose solution and added to the absorbent filter pads in medium tubes. Flies were next transferred and shifted to 25°C. For another batch of flies, PBS washed bacteria were diluted ten times in sucrose solution to a final OD of 0.1 and added to the filter pads in medium vials. Flies were either transferred directly in the vials or bacteria on filter pads were first killed by UV treatment (120,000 μJoules for five minutes, three times), before starting the survival experiment. For yet another batch of flies, supernatant from overnight bacterial culture was filtered twice before diluting ten times in sucrose solution, added to the filter pads before transferring the flies in the vials for survival experiment. Survival data are collected daily.

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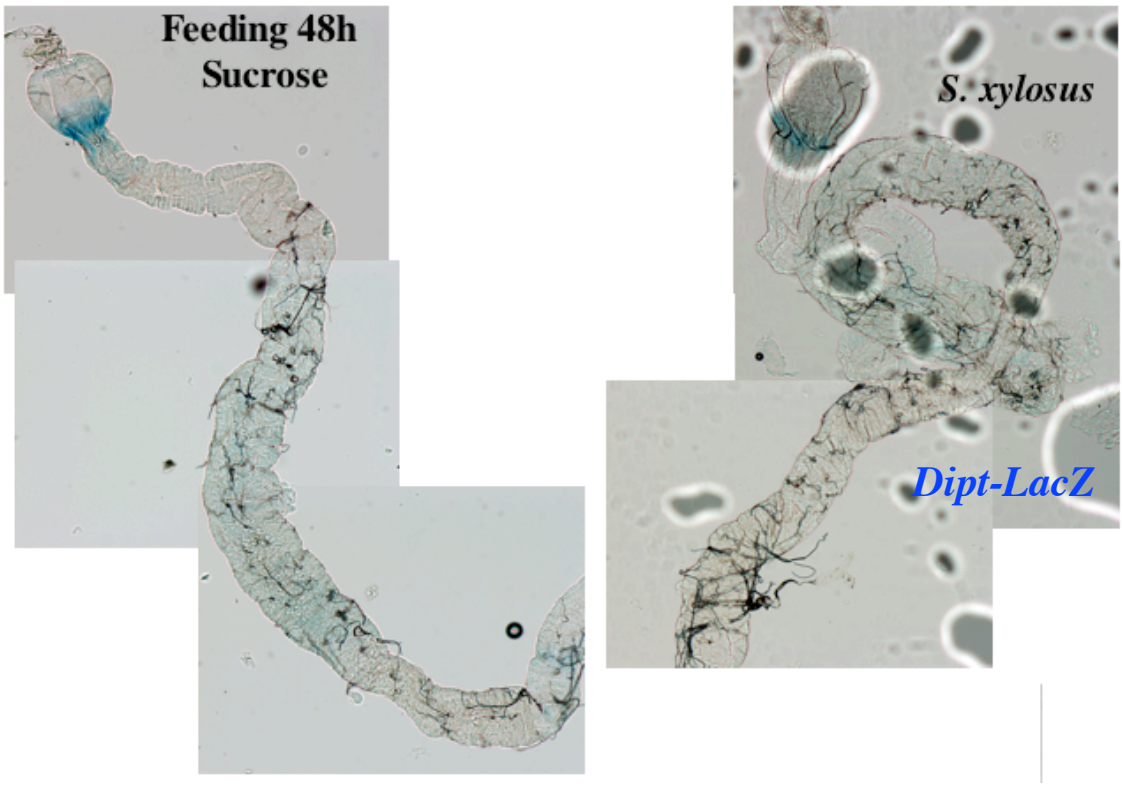


Fig. S1 : *S. xylosus* does not induce IMD pathway (Diptericin-LacZ)

Digestive tracts of *cn bw* flies fed on infection solution containing *S. xylosus* (OD_{600} 0.4) were dissected 48 hour post-infection, fixed with 4% PFA and stained for X-gal activity.

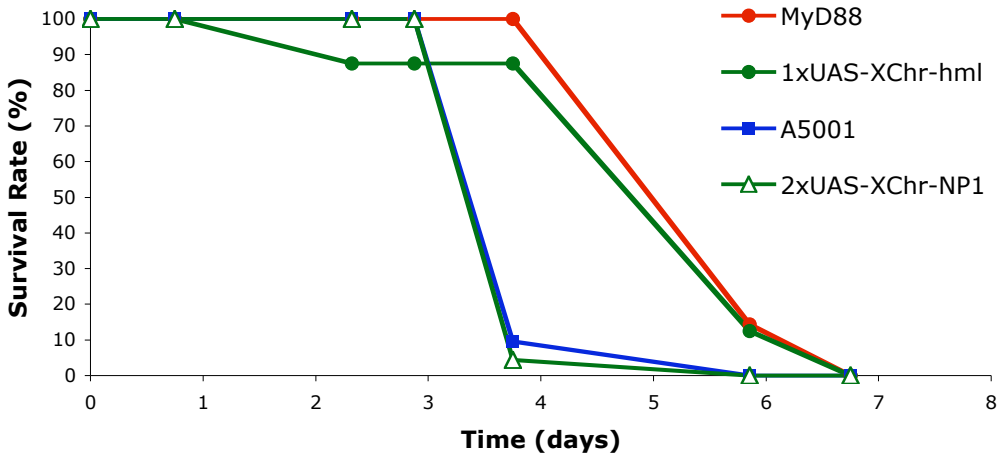


Fig. S2 : Apparent tissue-specific reversion of the *MyD88 S. xylosus* resistance phenotype by the *MyD88*$^{+}$ transgene

Flies were orally fed on 50mM sucrose solution infected with *S. xylosus* (OD_{600} 0.4) and transferred at 25°C. Survival data was noted on daily basis. Tissue specific overexpression of the *MyD88*$^{+}$ transgene was achieved in *MyD88* mutants by NP1 (midgut-specific) and hml-Gal4 (hemocytic-specific) drivers to construction 2xUAS-XChr-NP1 (*UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*; MyD88/MyD88, NP1-Gal4*) and 1xUAS-XChr-hml (*UAS-MyD88*$^{+}$*/+; MyD88/MyD88; hml-Gal4*) fly lines, respectively.

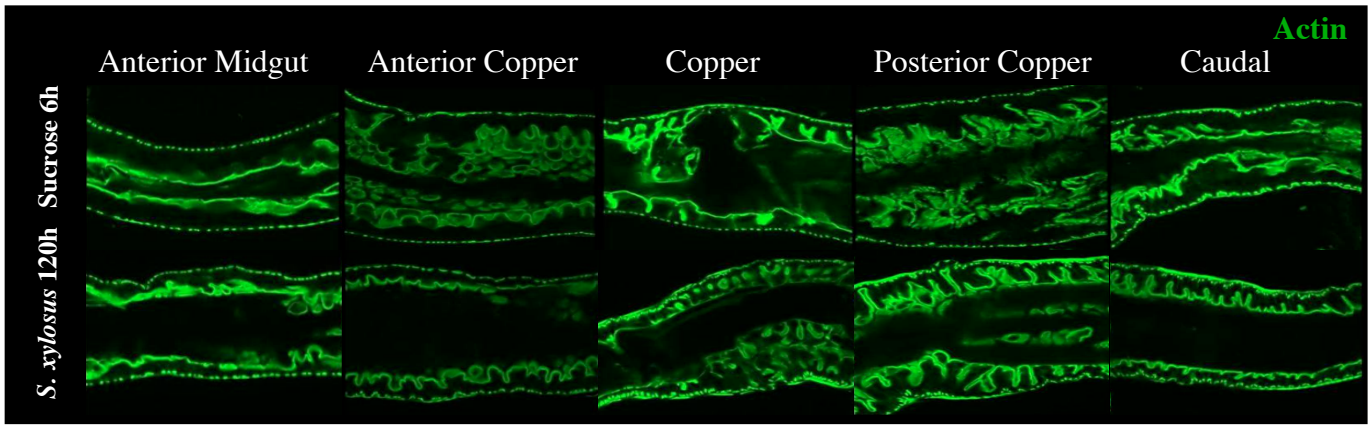


Fig. S3 : Midgut morphology intact even 5 days post-infection

Infected guts were dissected 5 days post-infection, fixed for 30 minutes in 4% FPA and stained using a FITC-labelled Phalloidin stain. The mounted guts were observed using confocal microscopy.

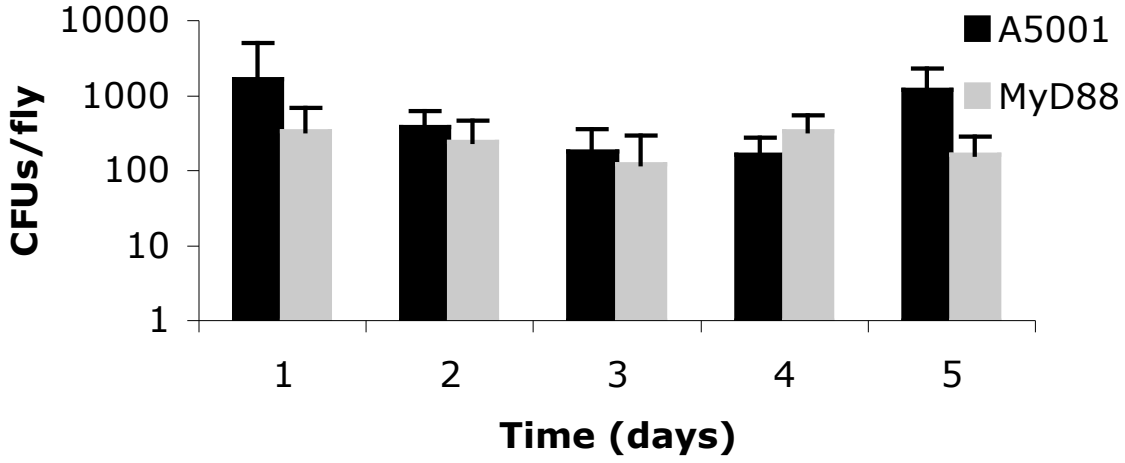
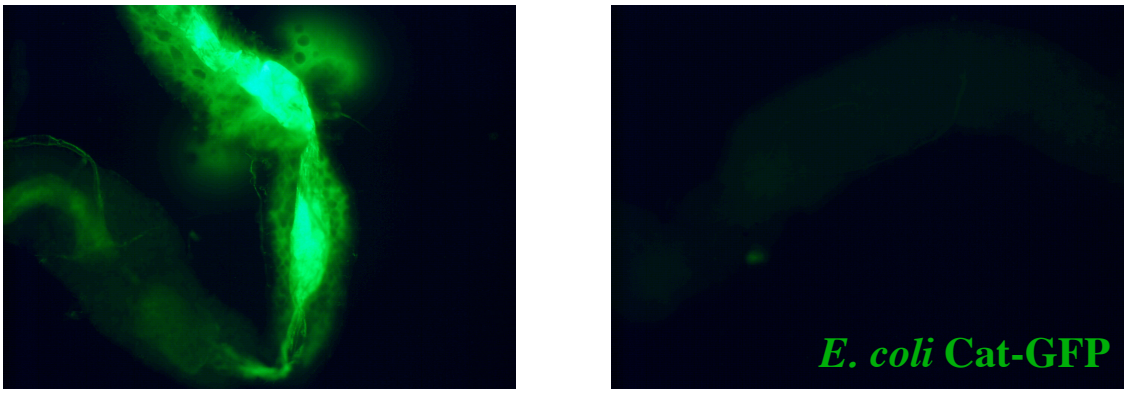


Fig. S4 : Time course of the *S. xylosus* in the gut

Guts of infected flies were dissected in sterile PBS in batches of ten, crushed and spread on media plates containing Streptomycin (100µg/mL).



Sucrose **N-acetylcysteine**

Fig. S5 : N-acetylcysteine treatment neutralizes the ROS response in the fly gut

Flies were kept overnight on sucrose solution (50mM) with or without N-acetylcysteine (20mM). Next day, flies were allowed to feed for 3 hours on a sucrose solution containing the ROS biosensor *E. coli* Cat-GFP strain with or without N-acetylcysteine. Dissected guts were mounted in 80% glycerol solution and observed by fluorescence microscopy.

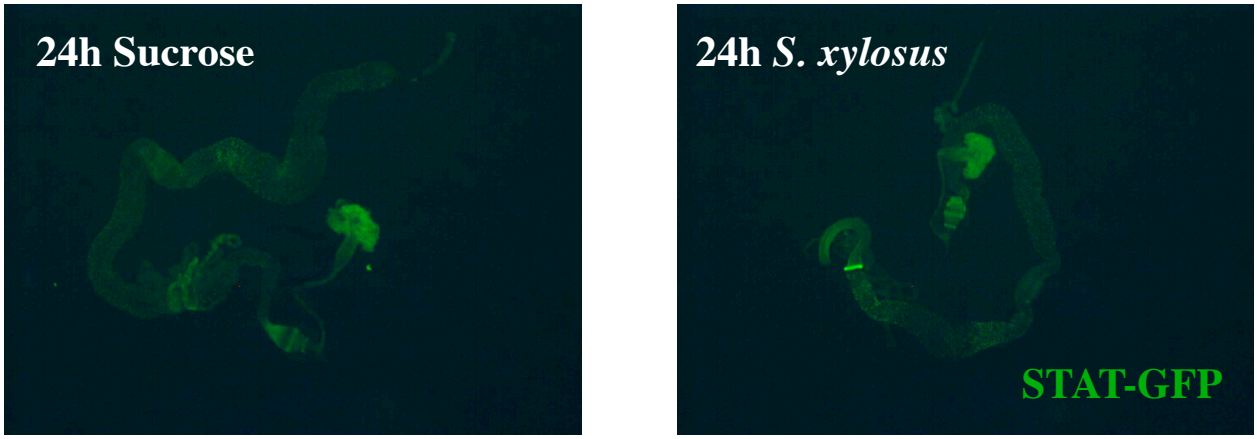


Fig. S6 : JAK/STAT pathway was not induced 24 hours post-infection

Guts of orally infected JAK/STAT pathway reporter fly strain, 10XSTAT-GFP were dissected 24 hours post-infection, fixed in 4% PFA for 15 minutes and observed by fluorescence microscopy. Fluorescence is mostly observed in the crop and the proventriculous

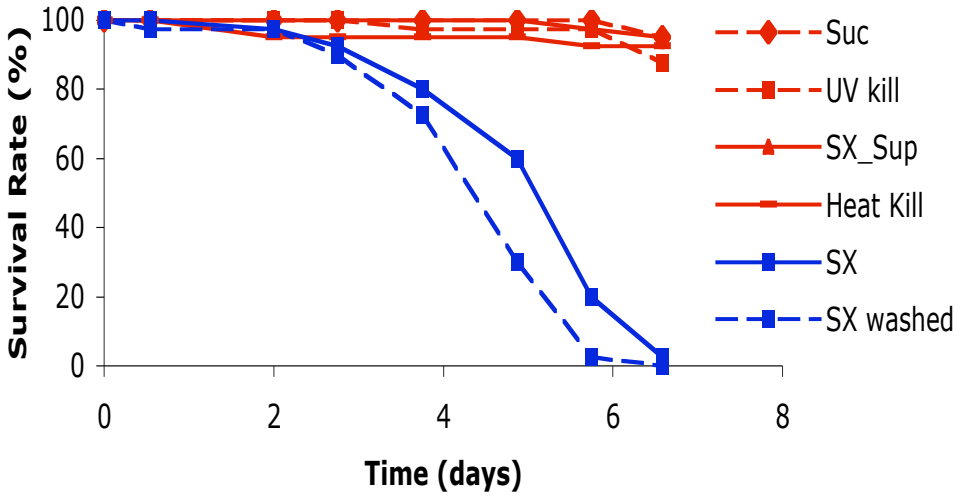


Fig. S7 : Flies do not appear to succumb as a result of exposition to a bacterial toxin

Wild-type flies A5001 were fed on sucrose solution (50 mM) containing: i) the filtered supernatant from overnight culture of *S. xylosus*, ii) heat-killed bacteria, iii) UV light-killed bacteria, or live bacteria iv) with or v) without a prior washing with PBS (see supplementary materials and methods).

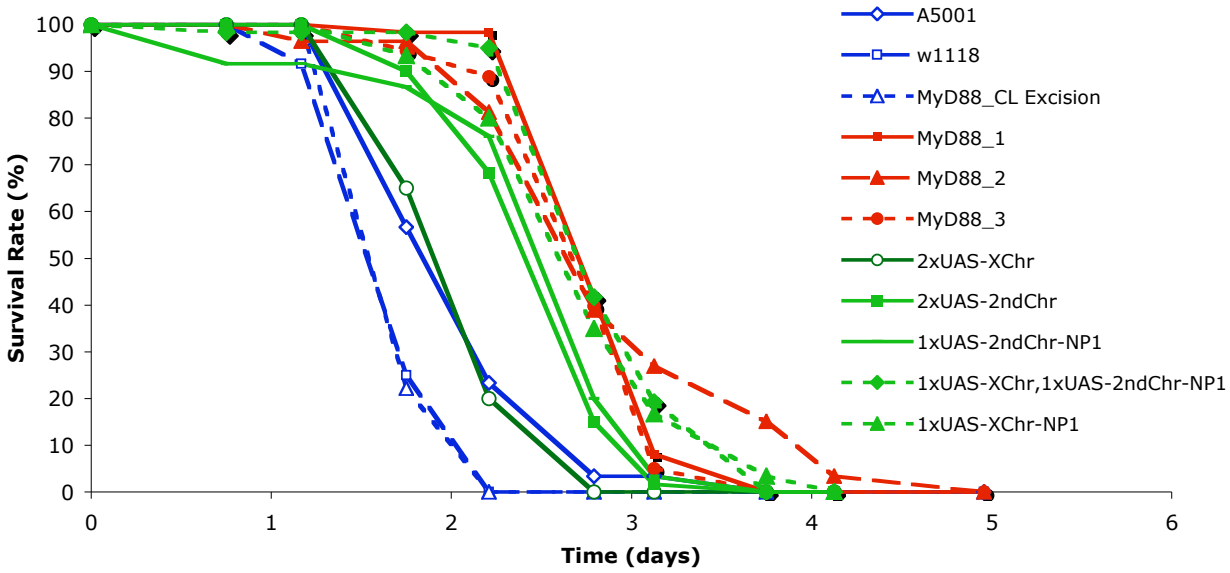


Fig. S8 : The insertion of the *MyD88*$^{+}$ transgene on the X predicts a recessive suppressor of the *MyD88* starvation phenotype

All fly types were kept on sterile water and survival experiment was performed at 25°C. *w1118* is another wild-type fly line while the transposon inserted at the *MyD88* locus has been excised in MyD88_CL Excision flies (Tauszig-Delamasure et al, 2002). We did not find any interline difference between three genetically similar *MyD88* mutant fly lines (1-3) collected from several investigators in the laboratory. Exact genotypes of the mutant strains tested are described as follows:

2xUAS-XChr: *UAS-MyD88*$^{+}$*/UAS-MyD88*$^{+}$*;MyD88/MyD88*

2xUAS-2ndChr: *w; MyD88,UAS-MyD88*$^{+}$*/MyD88, UAS-MyD88*$^{+}$

1xUAS-2ndChr-NP1: *w; MyD88,UAS-MyD88*$^{+}$*/MyD88,NP1*

1xUAS-XChr,1xUAS-2ndChr-NP1: *UAS-MyD88*$^{+}$*/+;MyD88,UAS-MyD88*$^{+}$*/MyD88,NP1*

1xUAS-XChr-NP1: *UAS-MyD88*$^{+}$*/+; MyD88 /MyD88,NP1*

5.2. Role of *MyD88* in nutrient metabolism during starvation

The Toll pathway plays a critical role in *Drosophila* during early embryogenesis as well as in innate immune response against microbes. We have uncovered a novel role for the major cytoplasmic adaptor of the Toll pathway, MyD88, in resistance to starvation in adult flies. We have shown that the *MyD88* mutants survived better than wild-type controls in a food starvation assay. Moreover, a significant loss of lipid reserves was observed after one day of food starvation in the wild-type controls as compared to *MyD88* mutant flies.

Although we did not find any evidence of Toll pathway activation in starved flies as monitored *Drosomycin* expression, we cannot rule out that the Toll pathway might be activated to trigger the target genes other than those involved in immune response or development. These findings raised other questions such as the biological basis of the starvation resistance that we observed in *MyD88* mutant flies. A part of this question relates as to how nutrient metabolism is changed in starved *MyD88* flies. Since the Toll pathway is one of the major antimicrobial immune defense, it is worth asking whether it influences the composition of the microbiota and how the microbiota evolves during starvation. To answer these questions and understand the underpinning biological mechanisms I performed further experiments, which are described below.

Starvation studies in *Drosophila* have been mostly performed on larvae, a developmental stage during which significant growth takes place. Nutrient deprivation has many consequences on the metabolism of *Drosophila*. The insulin pathway and the target of rapamycin (TOR) pathway are downregulated upon starvation. The activity of TOR, the major intra-cellular nutrient sensing module, is regulated either directly by nutrient availability (Colombani et al., 2003) or indirectly by the insulin pathway through at least two negative regulators of TOR (Figure 1.9**)**. One of the major downstream targets of both the insulin and the TOR pathways is the eukaryotic initiation factor 4E binding protein (4E-BP), a repressor of translation. On the one hand, downregulation of TOR under starved conditions leads to the dephosphorylation of 4E-BP. This prevents the translational machinery in ribosomes from synthesizing nascent polypeptides. On the other hand, as a result of starvation,

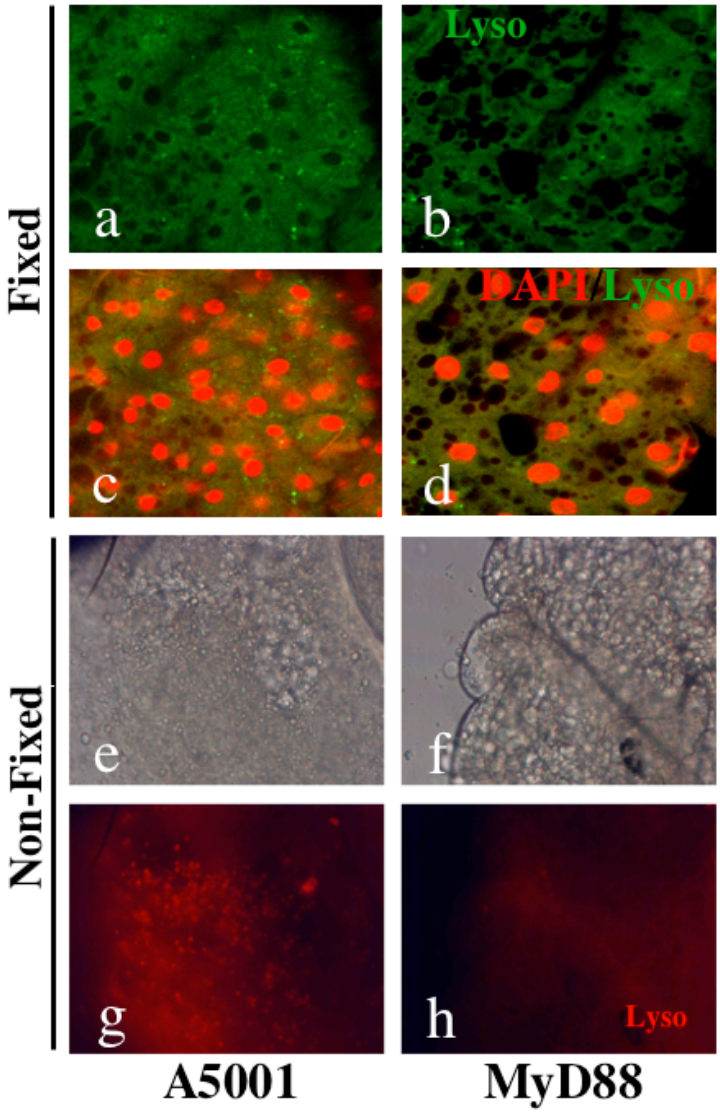


Figure 5.1: **Autophagy and lipid droplets in starved wild type and *MyD88* mutant flies.**

Wild type (A5001) and *MyD88* mutant flies were starved for 24 hours at 25−C. (**a-d**) Dissected fat body and other tissues were treated with Lysotracker from Sigma (100nM) (green) for 10 minutes, fixed for 15 minutes in 4% PFA (para-formaldehyde), mounted with Vectashield containing DAPI stain (red), and observed under confocal microscope. Black holes represent lipid droplets, n=2. (**e-h)** Lysotracker stained tissues (red) were mounted with glycerol (80%) and observed under fluorescent microscope, n=3.

the intra-cellular insulin pathway serine/threonine protein kinase Akt dephosphorylates Forkhead head protein (dFOXO). This leads to the nuclear localization of FOXO. 4E-BP is a transcriptional target of FOXO that, thus, becomes overexpressed (Teleman et al., 2005). In the absence of TOR-mediated phosphorylation, the higher cytoplasmic concentration of 4E-BP further re-enforces the blockade of protein synthesis initiated by TOR activity. As a result, growth and development are halted in the starved organism and available energy resources are redirected towards biological processes indispensable for survival.

Autophagy, defined as the self-digestion of cellular components, is downstream of the conserved metabolic pathways and is activated upon starvation in a TOR-dependent process (Scott et al., 2004). Activated TOR inhibits the first step of autophagy controlled by Atg1 (Figure 1.9). It has been shown in mice that fat stored in lipid droplets is digested by autophagolysosomes (Singh et al., 2009). Lipids are degraded into simple fatty acids and transported to the mitochondrion for the generation of energy. Moreover, 4E-BP reportedly mediates lipid catabolism in starved *Drosophila* (Teleman et al., 2005). These observations predict a link between the consumption of lipids, autophagy and conserved nutrient pathways under starvation stress.

5.2.1. Autophagy does not appear to be induced in *MyD88* mutants starved for a day

I first hypothesized that the reduced consumption of lipid reserves that we observed in starved *MyD88* mutant flies might be a consequence of a decreased autophagic activity. Adult female flies were dissected after one day of food starvation. The dissected fat body were then stained with Lyso-tracker (Berry and Baehrecke, 2007) and were either directly observed by fluorescent microscopy (Figure 5.1e-h) or were fixed prior to observation by confocal microscopy (Figure 5.1a-d). Lysotracker monitors the acidic compartment of phagosomes or autophagosomes. Using both techniques, I did not observe a significant accumulation of the stain in vesicles of the fat body lobules dissected from *MyD88* starved mutant flies (Figure 5.1). Such vesicles were numerous in starved wild type controls (Figure 5.1). Furthermore, lipid droplets were more numerous and bigger in fat body lobules dissected from starved *MyD88* mutant flies, both in fixed and non-fixed tissues. This observation is

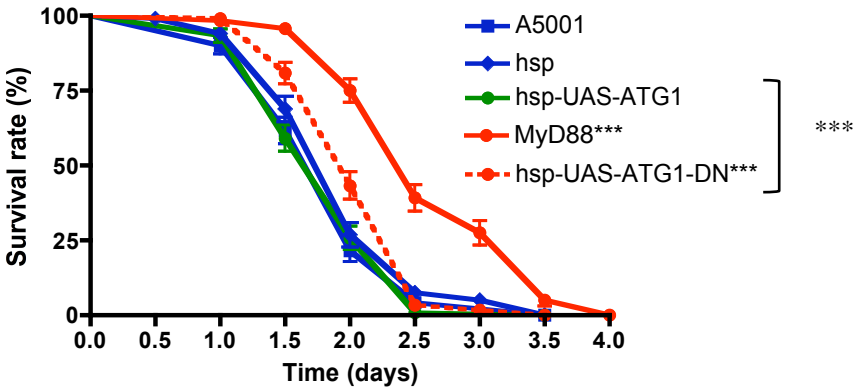


Figure 5.2: **Autophagy blockade slightly improves survival during starvation**
Autophagy was temporary induced or blocked in adult females by over-expressing UAS-ATG1 or UAS-ATG1-DN (dominant negative), respectively, using an ubiquitous, heat-shock induced hsp-Gal4 driver. 3-6 days old flies were heat-shocked and transferred to sterile water at 25°C. Data was noted twice per day. All survival curves were compared with A5001 (p values: hsp: $p = 0.2$ (ns), hsp-UAS-ATG1: $p = 0.998$ (ns), MyD88: $p < 0.0001$ (***), hsp-UAS-ATG-DN: $p = 0.0007$ (***); n=6 for all analyses). Hsp-UAS-ATG1-DN were also statistically different from hsp-UAS-ATG1 ($p = 0.0002$ (***)). Error bars represent standard error.

consistent with earlier experiments in which fat loss appeared to be less important in starved *MyD88* mutant flies as compared to wild type controls (Figure 4C, Ayyaz et al., *submitted* (Chapter 5.1)).

Before drawing a firm conclusion, however, some additional control experiments are required. First, we should use another marker for the formation of autophagic vesicles. At the onset of autophagy, phagosomes not only recruit circulating cytoplasmic proteins but the expression of autophagy-specific genes is induced. One such protein, ATG8, can be either stained for immunohistofluorescence microscopy or or used on western blots. Alternatively, a tagged form of an autophagy-related fusion protein, huLC3-GFP, should be specifically overexpressed in the fat body of adults and observed for localization of the reporter to distinct sub-cellular vesicles characteristic of autophagy. This reporter should also be analyzed in a *MyD88* starvation context.

A second line of evidence should be provided by genetic analysis. Autophagy can be induced by overexpressing ATG1 or conversely inhibited by expressing a dominant-negative transgenic construct $ATG1^{DN}$. I thus submitted such flies to starvation (Figure 5.2), using an ubiquitous *hsp*-GAL4 driver, which is strongly induced by heat shocks. As shown in Figure 5.2, I did not observe a higher death rate of wild-type flies in which the UAS-ATG1 transgene was overexpressed. One interpretation may be that autophagy is already fully activated by starvation. Interestingly, I observed that wild-type flies in which autophagy was somewhat prevented by the overexpression of $ATG1^{DN}$ resisted significantly better to starvation. However, this effect was mild. One possibility is that the dominant-negative construct is not fully blocking the activity of endogenous ATG1. Another parameter may be the fact that starvation already starts during heat-shocks. As a consequence of starvation is a shut-off of protein synthesis, it is possible that the $ATG1^{DN}$ transcript is not efficiently translated. Of note, I have not yet checked the efficiency of the treatment on those transgenic lines using lysotracker staining. Thus, these experiments are only suggestive at this stage. However, the critical experiment to ask whether the *MyD88* starvation resistance phenotype is mediated by an absence of the induction of autophagy during the first day of starvation still needs to be performed. Namely, I should attempt to induce autophagy in starved *MyD88* mutants, for instance by forcing the expression of the *UAS-ATG1* transgene.

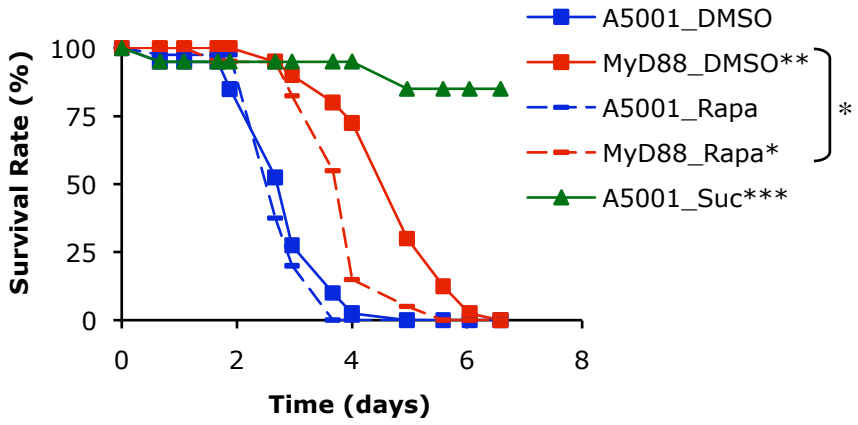


Figure 5.3: **Rapamycin to starved wild type and *MyD88* mutant adult flies.**

Flies were fed on sterile water containing either DMSO (2.5%) only or also containing rapamycin (Sigma, 50μM). For statistical analysis LT50s were calculated from each survival curve and compared with A5001 feeding on DMSO (p values: MyD88_DMSO: p = 0.008 (**), A5001_Rapa: p = 0.18 (ns), MyD88_Rap: p = 0.02 (*), A5001_Suc ; n=4 for all analysis) Since A5001 flies fed on sucrose solution do not die until the end of experiment, we can not calculate LT50 for their survival curve that forced us to use Log Rank test (in Prims) to compare A5001_DMSO and A5001_Suc (p < 0.0001 n=2 (***)). *MyD88* mutant flies feeding on Rapamycin were significantly different from A5001_DMSO as well as MyD88_DMSO (p=0.04 n=4 (*)) indicating a partially reversed phenoype by overexpression of autophagy.

Of note, the *MyD88* flies homozygous for the suppressor on the X chromosome displayed a somewhat intermediate phenotype in these assays. Since we observe a full reversion of the starvation phenotype, this suggests that autophagy is contributing to the phenotype but that other processes are likely involved as well.

5.2.2. TOR (target of rapamycin) apparently plays a role to mediate *MyD88* phenotype

Since the induction of autophagy is negatively regulated by TOR, I expect that the blockade of TOR in *MyD88* mutant flies should rescue the *MyD88* survival phenotype. Therefore, I treated flies with rapamycin, a TOR inhibitor (which actually gave the name to the protein). This treatment partially reversed the *MyD88* starvation resistance phenotype (Figure 5.3). Again, no effect of rapamycin treatment was observed in wild type flies, consistent with a possible physiological inhibition of TOR during starvation. This observation suggests that TOR acts downstream of *MyD88*. In other words, $MyD88^+$ activity might be required for the repression of TOR activity following starvation.

It will be necessary to check that TOR activity is indeed not inhibited in starved *MyD88* flies, for instance by monitoring the phosphorylation status of S6 kinase and 4E-BP on western blots of adult fat body (carcass of the abdomen). Complementary experiments with RNAi lines targeting the TOR control pathway should be also performed both in a wild-type and in a *MyD88* mutant background to confirm this hypothesis. Thus, inactivating TOR in *MyD88*-starved mutants should trigger autophagy and reverse its starvation resistance phenotype. By testing different levels of the TOR regulatory pathway (Figure 1.9), there might be a possibility to map the step regulated by MyD88 (amino acid sensing, ATP/ADP ratio [AMPK], insulin pathway).

5.2.3. Role of the Insulin pathway

TOR activity is directly regulated by the availability of nutrients, for instance amino acids (see Figure 1.9 and (Colombani et al., 2003)). However, the insulin pathway also regulates TOR activity by shutting down it negative regulators when induced. One of the main readouts of the downregulation of insulin pathway is to

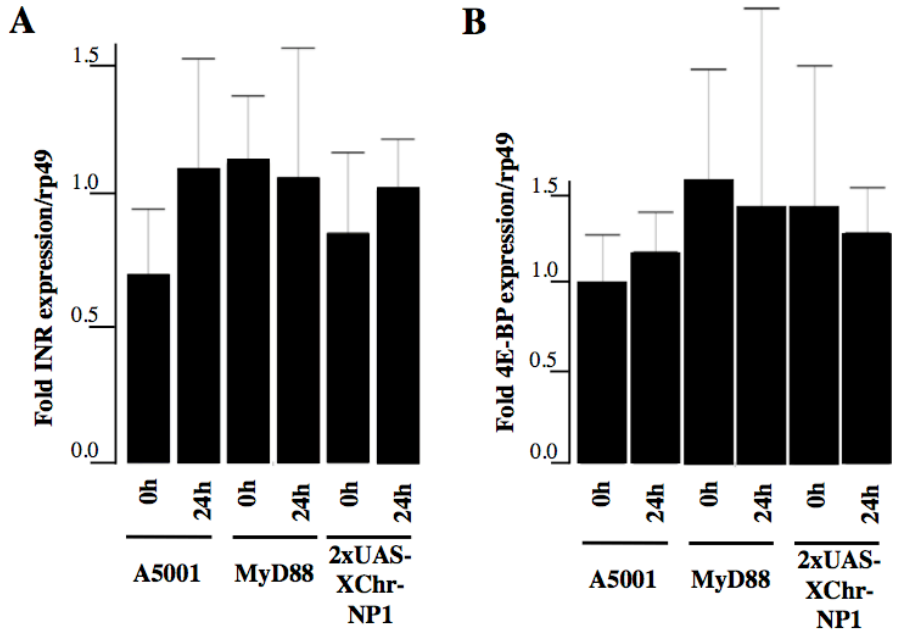


Figure 5.4: **Expression of FOXO targets: INR and 4E-BP, during starvation.**

RNA from flies starved for 0 and 24 hours. One-way ANOVA (Prism) showed insignificant difference among different values, (**A**) INR RNA fold expression: $p > 0.05$ n=5 (ns), (**A**) 4E-BP RNA fold expression: $p > 0.05$ n=13 (ns). Where n represents biologically independent replicates in each of six groups and error bars represent standard error in both panels. The graphs are representative of two experiments for INR and three experiments for 4E-BP.

check for the nuclear localization of FOXO, which is inhibited by insulin signaling (see above). I therefore observed the subcellular distribution of FOXO by immunohistochemistry. After 24 hours of starvation of *MyD88* mutant and wild type flies, I did not detect a nuclear localization of FOXO in dissected tissues (fat body and midgut) in any of the fly lines tested. At this stage, I cannot discount the possibility of a technical problem and should test the FOXO-antiserum on starved larvae, a condition in which nuclear FOXO has been observed (Wang et al., 2011).

As an alternative strategy, I monitored by Q-RT-PCR the expression of the *4E-BP* and *insulin receptor* (INR) genes, which are direct transcriptional targets of FOXO, both in *MyD88* mutant and wild type flies, under starvation or nonstarvation conditions. I did not observe any significant changes in the expression of these genes, although there was some important variability between biological replicates (Figure 5.4).

Thus, I have not so far obtained any evidence for an induction of the insulin pathway after 24 hours of starvation. I however cannot exclude that this time point is too late for such studies. Thus, a time course experiment should be performed to monitor the activity of the insulin pathway. Also, a complementary approach would be to genetically manipulate the insulin pathway in *MyD88* mutants. If the insulin pathway were working downstream of MyD88, then we expect to observe a reversion of the effects in survival experiments and as regards the consumption of lipid reserves upon starvation.

5.2.4. Lipid metabolism in starved gut

Neural Lazarillo (*NLaz*) and *Lipase 3* (*Lip3*) encode proteins that play critical role in lipid metabolism (Hull-Thompson et al., 2009; Maynard et al., 2010). They are among the most significantly upregulated genes in starved larvae (Zinke et al., 2002). The RNA levels of *NLaz* and *Lip3* are widely used as markers for metabolic stress and starvation symptoms in *Drosophila*. I therefore analysed by Q-RT-PCR the expression of NLaz and Lip3 in wild type, *MyD88*, and the phenotypic revertant fly line (suppressor of the *MyD88* starvation phenotype), 2xUAS-XChr-NP1, upon one day and two days of food starvation. No induction of *NLaz* was observed in any of the starved fly lines (Figure 5.5A). *Lip3* expression, however, was induced in wild types

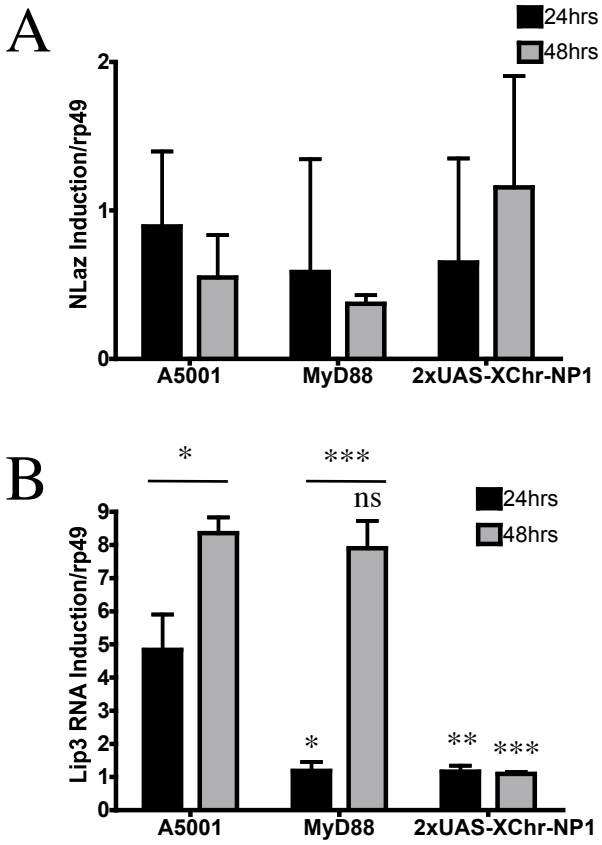


Figure 5.5: **RNA Expression of Lip3 and NLaz upon starvation.**

RNA was extracted from whole fly extracts, 0, 24 and 48 hours post-starvation. To calculate gene induction, the RNA expression for each gene after 24 and 48 hours of starvation was divided by that in non-starved flies (0 hour). One-way ANOVA (Prism software) was used to statistically analyze different values. (A) Induction of NLaz was not observed in any starved fly line ($p > 0.05$ n=5 (ns). (B) Lip3 induction was observed in wild type A5001 flies and used as standard to analyze Lip3 induction in other fly lines: (p values for 24 hours of starvation: A5001 vs MyD88: $p < 0.05$ n=5 (*), A5001 vs 2xUAS-XChr-NP1: $p < 0.01$ n=5 (**); p values for 48 hours of starvation: A5001 vs MyD88: $p > 0.05$ n=4 (ns), A5001 vs 2xUAS-XChr-NP1: $p < 0.001$ n=2 (***)). Lip3 induction at 24 and 48 hours in the same fly lines was also compared (A5001 at 24h vs 48h: $p < 0.05$ n= 4 (*); MyD88 at 24h vs 48h: $p < 0.001$ n=5 (***)). Error bars represent standard error in both panels.

flies starved for one day and this expression was further upregulated after two days of starvation (Figure 5.5B). Strikingly, no induction of *Lip3* was observed in *MyD88* mutant flies unless they were starved for 48 hours. This is consistent with the hypothesis that the consumption of lipid stores is delayed in *MyD88* mutant flies under starvation conditions. Thus, in *MyD88* mutants, two major mechanisms that have been shown to be involved in the catabolism of lipid droplets, namely autophagy and lipase3 digestion are impaired.

Interestingly, the revertant fly strain did not show any induction of *Lip3* at any stage of starvation. This suggests that the wild-type version of the suppressor gene is required for the induction of *Lip3* (the suppressor transposon insertion mutation is not in the *Lip3* gene as this gene is on the third chromosome). Interestingly, the suppressor fly strain dies as rapidly as wild-type flies, thus indicating that the delayed induction of *Lip3* in *MyD88* flies is not at the origin of the starvation resistance phenotype. As the Brummer lipase is not induced by starvation in adult flies, this suggests that fat loss is either mediated by yet another lipase, or alternatively, is only mediated by autophagic degradation.

5.2.5. The gut microbiota appears to mediate the *MyD88* starvation resistance phenotype

Recently, a link has been made between specific components of the microbiota and larval growth and developmental timing under nutrient-poor conditions (Shin et al., 2011; Storelli et al., 2011). Interestingly, one study established that the beneficial effect of one strain of *Lactobacillus plantarum* is mediated through the TOR pathway. I therefore asked whether the microbiota might play a role in the differential starvation susceptibility I have observed between wild-type and *MyD88* flies. While ideally this question should be tested using axenic fly strains, a first set of experiments was attempted using antibiotics treatment to clear the microbiota. A cocktail of four antibiotics that efficiently eradicates the microbiota has been developed in our Research Unit by Dr. Hidehiro Fukuyama. Of note, our strains tested Wolbachia-negative so that any observed effect will not be caused by the loss of this endosymbiont. As shown in Figure 5.6, this treatment reverted the *Myd88* starvation phenotype, with antibiotics-treated *Myd88* males succumbing even faster

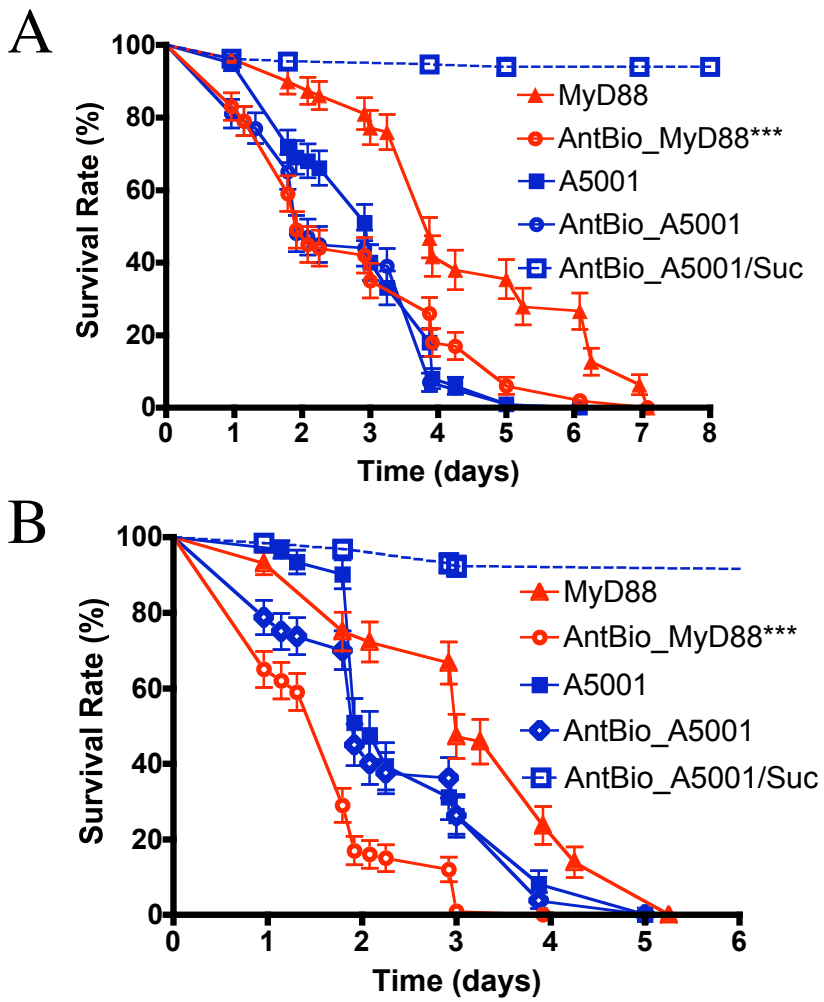


Figure 5.6: **Starvation experiment using axenic flies.**

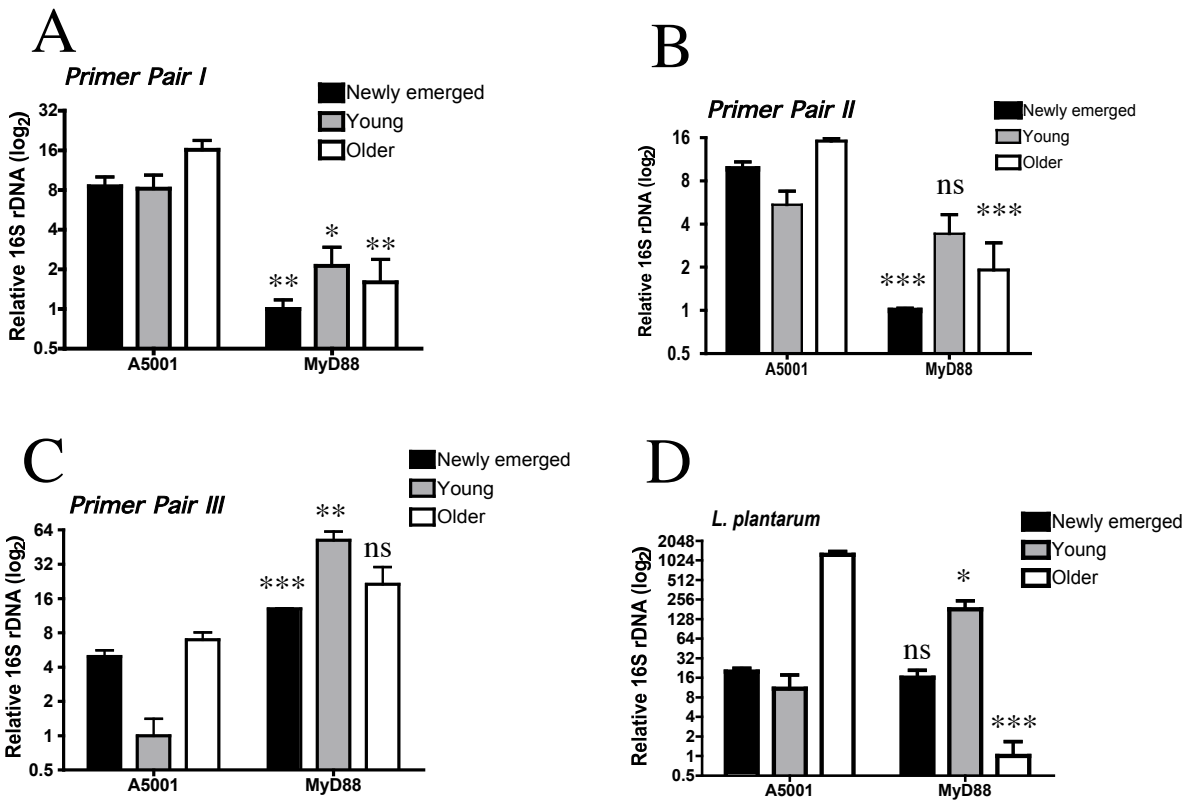
Wild type (A5001) and *MyD88* mutant female (A) and male (B) flies were kept on sucrose solution containing a mix of 4 antibiotics (Vancomycin: 50μg/mL, Neomycin: 100μg/mL, Metronidazole: 100μg/mL and Ampicilin: 100μg/mL), for 4 days at 18°C. Then they were shifted to Ampicilin (100μg/mL) containing sucrose solution (50mM) for overnight. Next day flies were sifted to sterile water containing only Ampicilin (100μg/mL) and starvation experiment was performed at 18°C. Effect of antibiotics was monitored by analyzing treated and non-treated flies (p values for females: MyD88: $p < 0.0001$ (***), A5001: $p > 0.05$ (ns); p values for males: MyD88: $p < 0.0001$ (***), A5001: $p > 0.05$ (ns); while n=5 for all data sets). Error bars represent standard error in both panels.

to starvation than wild-type males. These data suggest that the microbiota plays an important role in the starvation resistance of *MyD88* flies.

5.2.6. *MyD88* mutant flies harbor different microbiota

Because one major role of *MyD88* is in regulating the systemic immune response and because the immune system plays a key role in setting the tolerance threshold to microbiota, which may in some cases differentially affect distinct members of this microbial community, I set out to measure the amount of microbiota present in these fly strains using whole flies for a first step. Thus, I expect to measure both the microbiota present on the cuticle as well as that on barrier epithelia, including the gut (Ren et al., 2007). As it has been shown that the microbiota increases as a function of age, I have tested three periods, namely i) *newly emerged* flies, which were collected from 0-1 day, ii) *younger* flies (3-8 days), and iii) *older* flies (20-25 days on a medium that contains fresh yeast on vials changed every three days). Of note, age is relative and the *older* flies are relatively young when compared to the lifespan of flies (around 60 days for males at 25°C under our conditions).

Next, I examined the relative amount of microbiota in whole fly extracts by qPCR. The original idea was to use a pair of universal primers to quantify the *Drosophila* microbiota. However, upon a careful analysis of the published literature, I realized that there is no "ideal" universal primer pair that would amplify all bacterial DNA independently of its origin. Therefore, I decided to test three different universal primer pairs, referred as *primer pair I* (Nadkarni et al., 2002), *II* (Maeda et al., 2003), and *III* (Tseng et al., 2003) thereafter. They amplify conserved 16S rRNA protein encoding gene in DNA of different bacteria species, referred as Microbes-(1-3), respectively. The spectrum of bacterial specificity targeted by the three primer pairs has been described on the basis of number of species from each bacterial phylum that show a perfect DNA sequence match with both the forward and the reverse primers (Figure 5.7 and reviewed in (Horz et al., 2005)). Briefly, the *primer pair I* can detect more than 75 species from the phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteriodetes, but no species from Spirochetes and Chlamydiae. *Primer pair II* and *III*, on the other hand, can amplify *16S rDNA* from a large number of species of Proteobacteria and Actinobacteria but fewer Firmicutes and Bacteroidetes species (50-75). *Primer pair III* is the only probe in the three primer



E

	Primer Pair I (Microbes-1)	***(rimer Pair II*** (Microbes-2)	***Primer Pair III*** (Microbes-3)
Proteobacteria	**++++**	**++++**	**++++**
Actinobacteria	**++++**	**++++**	**++++**
Firmicutes	**++++**	**+++**	**+++**
Spirochetes	**-**	**++**	**+++**
Chlamydiae	**-**	**-**	**++++**
Bacteroidetes	**++++**	**+++**	**+++**

- < 5; + = 5-25; ++ = 25-50; +++ = 50-75; ++++ >75 (Horz et al., 2005)

Figure 5.7: **Young and old wild-type and *MyD88* mutant flies harbor varied microbiota**

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pairs that can specifically detect species from the phylum Chlamydiae (79 species). Numerous bacterial species from the phylum Spirochetes (57 species) are also detected by the primer *pair III*. *Primer pair II*, however, also amplifies the *16S rDNA* from 44 species belonging to the phylum Spirochetes. Flies used in this study were reared at 25°C in standard corneal-agar medium.

Flies showed differences in the amount of commensal bacteria depending on their age and genotype. Bacteria specifically amplified by *primer pair I* dwelled in newly emerged wild types (Figure 5.7A). These species stayed associated with wild type fly lines during the young age. *MyD88* mutant flies, however, contained significantly lower number of these species throughout their adulthood. Similarly, a lower titer of microbiota specifically amplified by the *primer pair II* was observed in *MyD88* mutant flies during *newly emerged* and *older* age but no significant difference was found between *young MyD88* mutant and wild type flies (Figure 5.7B). In contrast to the results obtained from *primer pairs I* and *II*, *newly emerged* and *young MyD88* mutant flies harbored a significantly higher titer of bacterial populations specifically amplified by the *primer pair III* (Figure 5.7C).

One may draw two conclusions from these results. First, there was a significant shift in the microbial populations associated with MyD88 mutant flies as compared to wild type controls, indicating that the two fly strains contained different microbial populations. Second, *MyD88* mutant flies may not increase their microbial burden as they age, in contrast to wild type controls. Of note, the *MyD88* starvation resistance phenotype tends to decrease in older flies.

To further verify these results I selected flies strictly from 4 $\pm$ 1 days old stocks (*young*) keeping other experimental settings same as above. Additionally, I included in the study the suppressor fly strain, 2xUAS-XChr, which is as sensitive to food deprivation as wild-type flies. Moreover, I monitored the stability of bacterial populations in these three fly lines after 24 hours of starvation as well. First, the data on unstarved *young* flies confirm those I had made above for all three primer pairs. Interestingly, the 2xUAS-XChr strain behaved as the *MyD88* strain, thus establishing that the microbiota differences observed in flies prior to starvation are not explaining the differences in starvation resistance. The second notable difference was that whereas wild-type A5001 flies maintained quantitatively their microbiota upon 24 hours of starvation (independently of the primer pairs used), both *MyD88* and the

Figure 5.7: **Young and old wild-type and *MyD88* mutant flies harbor varied microbiota**

Bacterial titer was monitored by Q-PCR by amplifying *16S rRNA* gene in whole fly DNA extracts of wild-type A5001 and *MyD88* mutant flies at three different ages. Quantification of the bacterial titer is relative to each other. Values obtained with each primer pair for *MyD88* were statistically compared with those of corresponding control values observed in wild type flies using unpaired t-test (Prism). (A) Universal microbes-1 were amplified by *primer pair I*: (p values: Newly emerged: p=0.003(**), Young: p=00.3(*), Older: p=0.002(**)), (B) microbes-2 by *primer pair II:* (p values: Newly emerged: p=0.0003(***), Young: p=0.3(ns), Older: p<0.0001(***)), (C) microbes-3 by *primer pair III*: (p values: Newly emerged: p<0.0001(***), Young: p=0.002(**), Older: p=0.16(nsç) and (D) *L. plantarum* using primer pair that specifically amplified this bacterial species: (p values: Newly emerged: p=0.49(ns), Young: p=0.036(*), Older: p=0.0002(***); n=4 for all panels except for (A) where for Newly emerged n=4 while for Young and Older n=3). Error bars represent standard error in each panel.

(E) Bacterial specificity of the universal *primer pairs* (*I-III*) to amplify microbes-(1-3), respectively, is shown on the basis of number of species from each bacterial phylum that show a perfect match with both the forward and the reverse 16S rDNA primers.

2xUAS-XChr strain exhibited a marked quantitative decrease of their microbiota, as monitired using the three primer pairs. Again, this phenotype is thus unlikely to account for the difference between starvation resistance observed in these strains.

5.2.7. *Lactobacillus plantarum* dwells in younger *MyD88* flies

As mentioned above, two bacterial species, namely *Acetobacter pomorum* and *L. plantarum* inhabit the midgut of *D. melanogaster* and regulate its growth, development, and metabolism under nutrient-poor conditions. I therefore decided to check whether our fly strains differed in terms of titer for these bacterial species. Two primer pairs were used in this study that amplify 16S rRNA encoding gene specifically from the DNA of *A. pomorum* or *L. plantarum* (Wong et al., 2011). Two sets of experiments were designed as described above. In the first set of experiments, wild type and *MyD88* mutant flies were taken from three different age groups (Figure 5.7), while in the second set of experiments, 4 $\pm$ 1 days old wild type, *MyD88* mutant, and 2xUAS-XChr fly strains were used to quantify the two bacterial species (Figure 5.9).

A. pomorum was not found in any of the fly lines used in both sets of experiments. *L. plantarum*, however, was found in all fly strains at low titers, when compared with the results obtained from universal primer pairs that measure the bulk of the associated microbiota. I observed variations in the titer of *L. plantarum* depending on the age and genotype of the fly lines. Freshly emerged *MyD88* mutant and wild type flies displayed a similar titre of *L. plantarum* (Figure 5.7 D). I found a significantly higher titer of *L. plantarum* in the *young MyD88* mutants as compared to the wild type controls, which however was not confirmed (Figure 5.9). *Older MyD88* mutants, however, lost the bacterium while highly significant numbers of the bacterium were observed in *older* wild type controls. Taken together the titer of *L. plantarum* varied in *MyD88* mutants and wild type flies as a function of age, which might be associated to the starvation phenotype observed in *MyD88* mutants.

Interestingly, important variations in the *L. plantarum* titer was observed upon food deprivation. First, *young* flies had a quantitatively similar titer, whether wild-type, *MyD88*, or 2xUAS-XChr flies (Figure 5.9). Wild type flies lost almost entirely *L. plantarum* after a day of starvation while there was a drastic decrease of the titer in

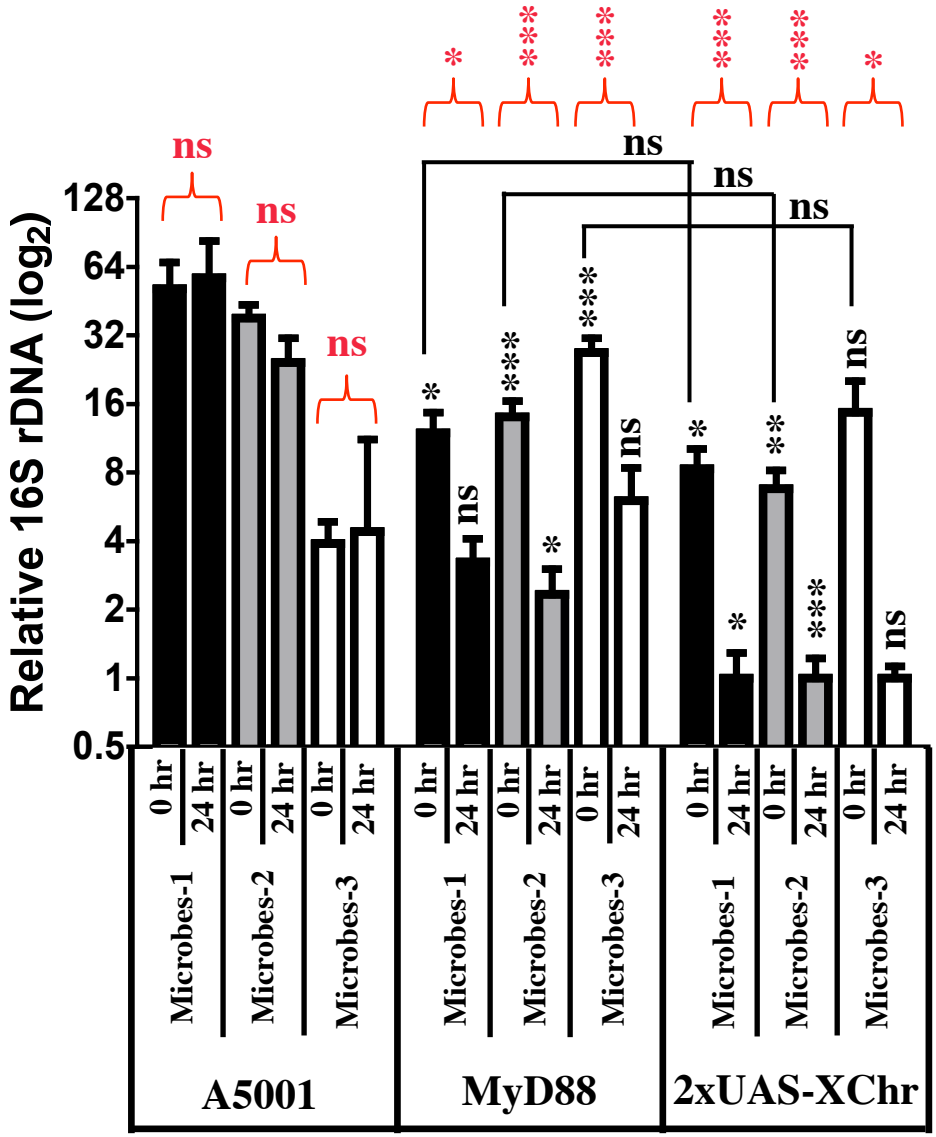

Figure 5.8: **Effect of starvation on universal microbiota**

Q-PCR was used to quantify universal microbes-(1-3) three *primer pairs* (*I-III*) that amplified *16S rRNA* gene in whole fly DNA extracts of wild types A5001, *MyD88* mutants and the revertant strain. The quantification is shown relative to each other. For statistic analyses, each value was compared with the corresponding value of wild type controls (shown with *black* notations) using student t-test of the software Prism. The microbial burden before and after starvation was also compared in each fly strain (shown in *red*). Criterium for significance: p>0.05 (ns), 0.05>p>0.01 (*), 0.01>p>0.001 (**), and p<0.001 (***), n=12 for each data set except for: *microbe-1*: 2xUAS-XChr-0hr: n=8, A5001-24hr: n=10, MyD88-24hr: n=9, 2xUAS-XChr-24hr: n=11; *microbes-2*: A5001-24hr: n=8, My88-24hr and 2xUAS-XChr-24hr: n=11; mirobes-3: A5001-24hr: n=11). Error bars represent standard error.

2xUAS-XChr flies. *MyD88* mutants, however, still retained significantly higher numbers of the bacterium as compared to the wild type controls after 24 hours of starvation. Thus, the *MyD88* starvation resistance phenotype may be the consequence of the ability of *MyD88* flies to retain *L. plantarum* in higher numbers during starvation.

5.2.8. Yeast killed *MyD88* mutant flies faster upon starvation

Since I had observed an opposite trend in the shift of microbial load in *MyD88* mutants and wild type flies as a function of age, I wanted to check whether *older* age had a significant impact on the survival of flies when deprived of a food source. Contrary to my expectations, *MyD88* mutant flies kept on normal fly food containing live yeast, changed at three days intervals, lost their resistance to starvation (Figure 5.10A). Interestingly, older flies kept on the fly food medium without live yeast (also changed every three days) did not lose the starvation resistance phenotype, thus pointing out the role of yeast in the reversal of starvation resistance in *MyD88* mutant flies.

5.2.9. Longevity

Next I checked if there was a difference in the longevity between *MyD88* mutant and wild type flies kept on normal food containing live yeast. Wild type flies lived slightly but significantly longer than *MyD88* mutants (Figure 5.10B).

5.2.10. *Conclusions*

In this postface, I have presented data that suggest that starved wild type flies exhaust rapidly their fat reserves through TOR pathway -controlled lipophagy. Interestingly, the *MyD88* starvation phenotype is also linked to the microbiota as the phenotype disappears after antibiotic treatment. At a quantitative level, it appears that the microbiota composition differs between wild-type and *MyD88*. However, at this low level of resolution, the suppressor strain indicates that this coarse approach does not pinpoint the origin of the difference able to account for the starvation resistance of *MyD88*, as compared to wild-type AND 2xUAS-XChr suppressor line.

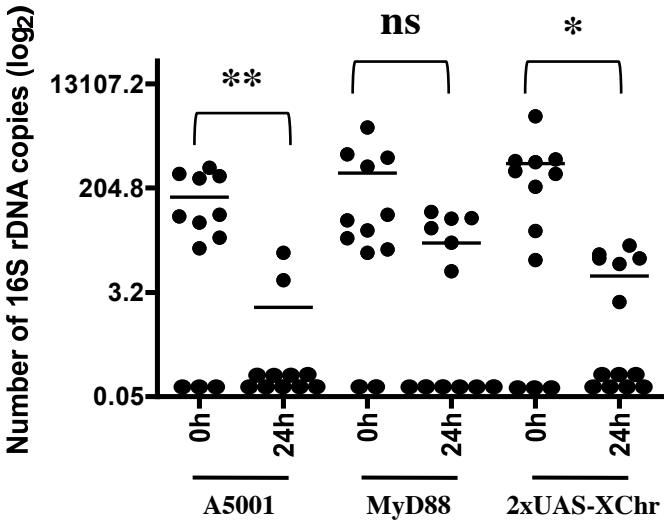

Figure 5.9: ***L. plantarum* before and after starvation**

Titer of *L. plantarum* was monitored by Q-PCR in the whole fly extracts. The quantification cycle (Cq) of 36 was considered equivalent to one copy of 16S rDNA and any value below this threshold was considered equivalent to zero number of copies. The quantity of *L. plantarum* 16S rDNA copies before (0 hours) and after (24 hours) starvation were compared in each fly strain, Mann-Whitney (non-parametric) test applied using the software Prims: (A5001: p=0.003 (**), MyD88: p=0.068 (ns), 2xUAS-XChr: p=0.019 (*); n=12 for each value). Medians are shown on the graph .

Possibly, this analysis might be more accurate if it were performed on dissected guts instead of whole flies. Indeed, there is also a microbiota growing on the cuticle (Ren et al., 2007) and possibly also in trachea and reproductive tract (Ferrandon et al., 1998; Tzou et al., 2000). Fortunately, the specific examination of *L. plantarum* titers yielded a unique insight in the potential basis for *MyD88* starvation resistance. Indeed, in larvae, *L. plantarum* enhances growth in a process that involves the TOR pathway when nutrient supplies are limited. Here, taking all of my results together, I propose the following synopsis. The critical observation is that *L. plantarum* appears to remain longer in starved *MyD88* flies as opposed to wild-type or 2xUAS-XChr suppressor control flies. The remaining *L. plantarum*, which we hypothesize would be remaining in the digestive tract, a supposition that needs to be verified, would still trigger the TOR pathway of *MyD88* flies to a level sufficiently high that it would inhibit starvation-induced autophagy and lipophagy. Thus, fat stores would be preserved longer and would thus be able provide the fly with energy later on during the starvation period, thus accounting for the enhanced resistance to starvation. To validate this hypothesis, we would need to perform additional experiments: i) check *L. plantarum* titers in dissected guts of *MyD88* and control flies ii) check that the TOR pathway is indeed activated for a longer period in starved *MyD88* flies, for instance by checking the phosphorylation status of some of its major target genes, *e.g.*, S6 kinase and 4E-BP iii) check that lipophagy is indeed involved by using LC3-GFP markers or lysotracker staining in the oenocytes iv) trigger autophagy in *MyD88* mutants by overexpressing *ATG1*. Another line of evidence would be provided by monoassociation studies between axenic *MyD88,* wild-type, and 2xUAS-XChr suppressor control flies and *L. plantarum*. Possibly, the difference between the genotypes would become less pronounced. A similar line of experiments would consist in homogenizing the microbiota of these different genotypes by growing *MyD88* and wild-type progeny in the same vials. Indeed, this approach has been very fruitful in mice (Elinav et al., 2011).

These findings raise two interesting questions. First, wild-type flies lose their fat stores very rapidly, within 24 hours, which is unexpected. As the fat body provides much more energy than glycogen stores, the question becomes how the wild-type flies expend their energy during the first 24 hours. A universal behavior of starved animals is to move around in search for a new source of food (Meunier et al., 2007;

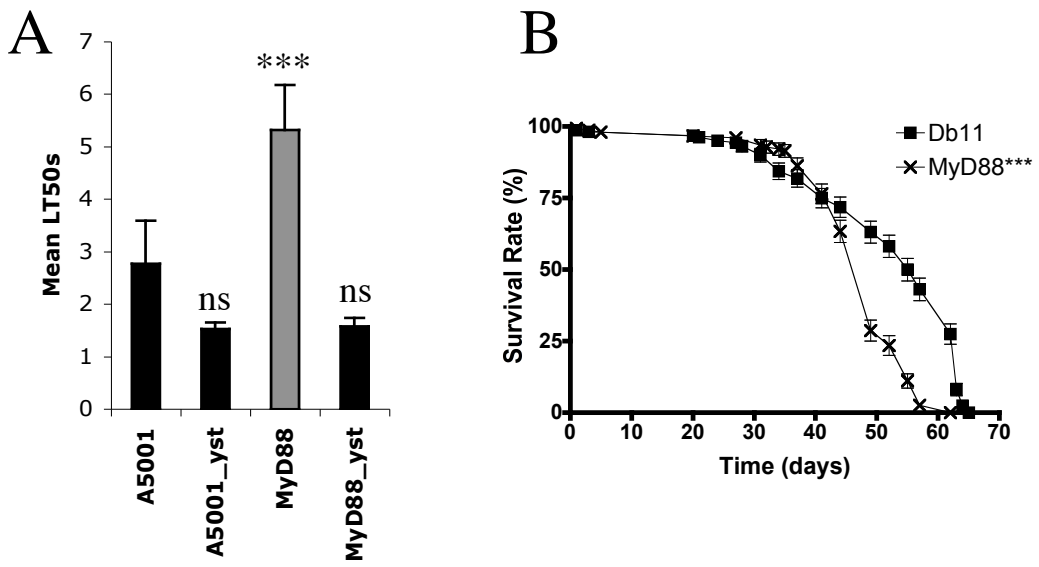

Figure 5.10: **Additional source of yeast affects survival of *MyD88* mutants**

(A) The wild type (A5001) or *MyD88* mutant flies were either kept with or without an addition of unmeasured quantity of yeast for 14 days before performing starvation experiment. Fresh live yeast was provided after each 2 days when tubes were changed. LT50 values (time it takes for half flies to die) were calculated using Prism software. Each LT50 value was than statistically compared with that of wild type flies kept on the food medium that did not receive live yeast (A5001_yeast: p=0.16 n=6 (ns), MyD88: p<0.0001 n=9 (***), MyD88_yeast: p=0.26 n=6 (ns)). (B) Longevity experiment was performed by rearing batches of 20 male flies at 25° C on standard fly food containing live yeast. Tubes were changed after each two days. Log rank test in Prism software was used to compare survivals of *MyD88* mutants and wild type flies (p<0.0001 n=8 (***)). Error bars represent standard deviations.

Nagata and Nagasawa, 2006). Adipokinetic hormone (AKH) is required for the enhanced locomotion of the starved *Drosophila* adults by mobilizing lipid and trehalose stores from the fat body (Isabel et al., 2005). We predict that *MyD88* flies will not display this behavior. This is experimentally difficult to test as one needs specific equipment to monitor locomotion (Inan et al., 2011). However, it might be easier to test this hypothesis in starved larvae as first step. If true, it will then become relevant to ask whether this is a behavior directly regulated by *MyD88* itself or whether this somehow involves *L. plantarum* and the activation of the TOR pathway.

The second question is that of the mechanism that favorizes the association of *MyD88* with *L. plantarum*, especially under starvation. It is an interesting question as the Toll pathway has not been reported to be activated in the digestive tract, although *Drosomycin* expression might not be an adequate read-out in this context. Attempts to detect a nuclear localization of DIF in the midgut epithelium were unsuccessful. Interestingly, *Myd88* is expressed at very high levels in salivary glands, about 10 fold more than in the fat body or ovaries (Chintapalli et al., 2007). Furthermore, it is the only gene of the intracellular Toll signaling cascade to have such a pattern of expression, Dorsal being also relatively highly expressed in salivary glands (similar levels as in ovaries). This observation reinforces the notion that *MyD88* might have Toll signaling-independent functions. Thus, the cloning of the suppressor gene will most likely reveal an interesting hint to decipher this novel function.

A puzzling observation is that *L. plantarum* is lost in *older* flies (Figure 5.7D). Also, the *MyD88* phenotype is not reproduced in *older* flies (Figure 5.10A), thus strengthening the correlation between *L. plantarum* association and starvation resistance. Of note, these *older* flies feed on vials that are changed every three days, with fresh yeast added. They are thus in a nutrient-rich environment in which *L. plantarum* may not provide an essential advantage to the flies and thus there might not be a selective pressure to maintain its association with flies. Strikingly, flies aged in the absence of fresh yeast were still starvation resistant. It remains to be established whether these still have maintained *L. plantarum*in their microbiota. An alternate view would have considered that ingested yeasts are killed in a *MyD88*-dependent process. This may however not be the case as the group of Won-Jae Lee has demonstrated that *S. cerevisiae* is likely killed by ROS produced by the DUOX

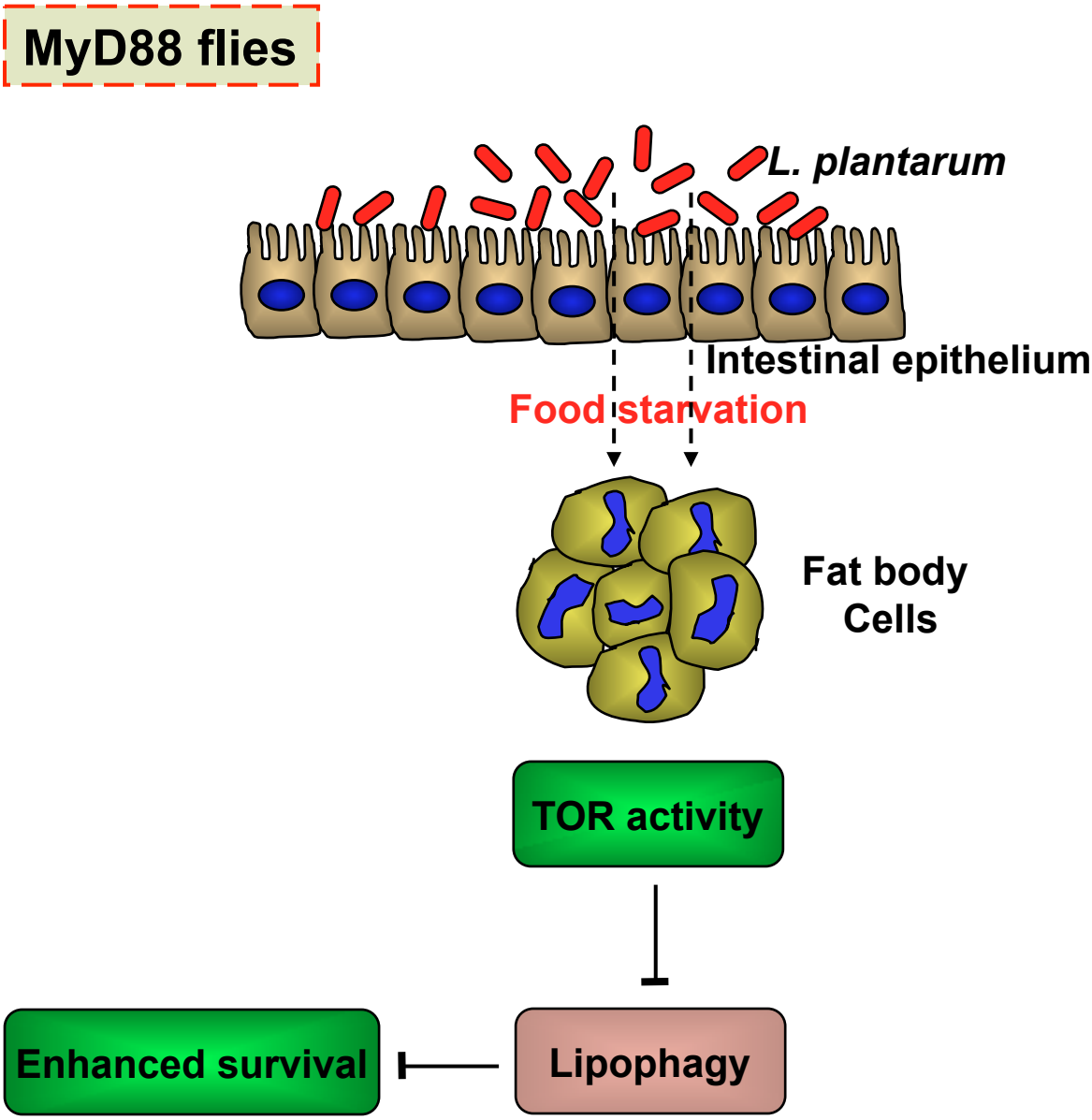

Figure 5.11 (a): **The working model**

The *MyD88* mutants retain *L. plantarum* better than the wild-type flies under starvation conditions. The *L. plantarum* in turn keeps TOR active in the fat body resulting in slow utilization of stored lipid reserves due to reduced lipophagy, thus enhancing the rate of survival of flies.

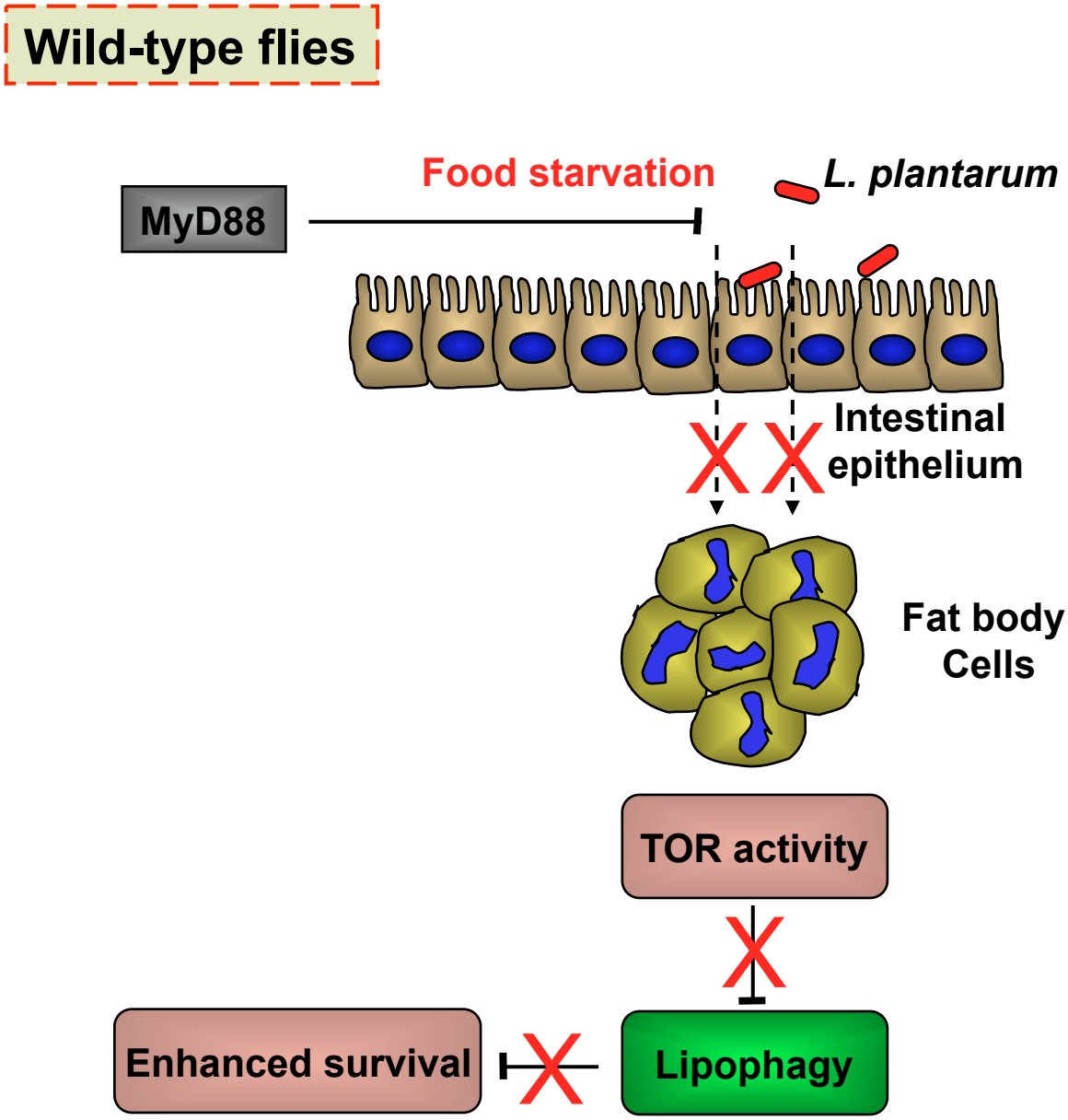

Figure 5.11 (b): **The working model**

The wild type flies remove *L. plantarum* from the gut during starvation, in a MyD88-dependant manner. The TOR activity is downregulated to the maximum potential resulting in faster utilization of food resources by lipophagy, thus leading to the rapid fly death.

enzyme, the activation of which appears to be independent of the Toll pathway (Ha et al., 2009).

In conclusion, this work exemplifies the complex interactions between the microbiota and the physiology of its host. The most original contribution may reside in the tentative attribution of a novel function to *MyD88* that is independent of its pivotal role in Toll signaling.

6. Conclusions

Assessment of the doctoral work

In this thesis, I started out by investigating the actual causes of the death of flies in an intestinal infection model first developed in the Boman laboratory thirty years ago (Flyg et al., 1980). Initially, the discovery that *S. marcescens* is able to cross the intestinal barrier suggested that it might cause the death of flies, given its high virulence in a septic injury model. However, the bacterial titer remained low throughout the infection (Nehme et al., 2007), unlike another intestinal infection model with *P. aeruginosa* (Limmer et al., 2011). Thus, death was unlikely to result from uncontrolled bacteremia. In the *cn bw* background, it was noted that the intestinal epithelium became thinner as the infection progressed (Nehme et al., 2007). However, these results appear to depend on the genetic background, possibly through the microbiota, as they were not observed in another genetic background, namely A5001 (Cronin et al., 2009; Thibault et al., 2004). While there were indeed signs of damage, it appeared that the homeostasis of the intestinal epithelium is maintained by multiple means, including the compensatory proliferation of intestinal stem cells. Here, I noted that flies that had been exposed to *S. marcescens* for five days were able to recover when they were placed on fresh medium, whether it contained *S. marcescens* or not. Interestingly, naive flies exposed to a bacterial medium on which a normal infection experiment had already been performed were killed much more rapidly. Thus, this led me and colleagues to perform additional experiments in which a source of carbon was added to the filters regularly. Taken together, these experiments demonstrate that flies actually succumb to starvation and not from direct bacterial damages. This was an unexpected result given the virulence of the bacterium. Thus, one might envision that flies and bacteria are competing for sucrose, the only available source of energy. Indeed, flies feeding under the same conditions on heat-killed *S. marcescens* did not succumb. As *S. marcescens* is able to resist many conditions, including those encountered in the gut, as judged by electron microscopy data, one possibility would have been that flies are unable to efficiently kill and digest bacteria. This is however not the case, as flies are unable to feed on *S. xylosus*, which appears to be killed in large quantities, even though its cell wall should be attacked by *Drosophila* lysozymes. Flies apparently are able to feed on yeasts, which are mostly killed by the DUOX-mediated ROS

response (Ha et al., 2009). It is not clear how flies are able to degrade the fungal cell wall as it lacks classical ß-glucanases (Bragatto et al., 2010; Pauchet et al., 2009), which however are found in mosquitoes.

As we had performed a whole screen to look for an impaired host defense against ingested *S. marcescens*, it was important to determine whether known "host defense" mutants are especially sensitive to starvation. I myself determined that *key* mutants are still sensitive to this infection under nonlimiting energy conditions. Of note, Dr Matthieu Lestradet (personal communication) did not find an obvious correlation between starvation sensitivity and many of the mutants that had been identified in the genome-wide screen. There is however an intriguing link between the energy status of the mutant flies and their susceptibility to infections. For instance, *key* mutants start dying at a time when there should still be some sucrose left on the filters. In contrast, they are dying much slower when sucrose is regularly added. One might think that bacteria may actually suffer from being exposed to sucrose concentrations that are to high, the so-called jam effect. My measurements have indicated that we never go above a 5% solution, even when sucrose is added. Also, *key* flies were dying more slowly when sucrose was added only every other day. Thus, a major theme worthy of investigation will be to determine how the nutritional status of the fly impinges on local immune responses and epithelial homeostatic processes. Of note, this effect does not appear to be manifest with regards to the cellular immune response. Clearly, this will require a thorough investigation of the exact role that *key* and other genes of the IMD pathway play in mucosal defenses. As shown in the latest part of my thesis, it will certainly be worth investigating the contribution of the microbiota during *S. marcescens* intestinal infections. Many years ago, competition experiments had been performed between fluorescently-labelled *S. marcescens* and *E. coli*: *S. marcescens* always won. However, *E. coli* is not a commensal of the *Drosophila* gut.

S. marcescens secretes many virulence factors. The exact role of each of them is not known and understanding the pathogenicity of this bacterium using the *Drosophila* model was one of the team major goals. A major limitation is the difficulty with which site-directed mutants are generated in this *S. marcescens* strain. Many of the techniques I attempted together with my previous supervisor, Dr. P. Giammarinaro, an experienced microbiologist, were unsuccessful. Thus, a screening

approach was a viable alternative, especially given the medium-throughput that is feasible in this organism. I thus undertook a couple of screens. The protease screen identified one locus, *lip*, as being required for the secretion of at least two proteases. Unfortunately, despite promising preliminary results, I did not find an enhanced sensitivity phenotype in the *eater* infection model. However, it would be worth investigating whether the permabilization of the peritrophic matrix I discovered is still taking place when flies are feeding on these mutants. It would also be interesting to determine whether ISC compensatory proliferation is affected with these bacterial mutants and whether these mutant bacteria invade the intestinal epithelium as efficiently as wild-type *S. marcescens*. Finally, the team is working on an early phase attack of the epithelium that is mediated by a secreted hemolysin. As this hemolysin has a short half-life of three seconds when secreted, it is likely that the peritrophic matrix limits the accessibility of this pore-forming toxin to enterocytes. I would thus expect that the early phase attack may be hindered in these protease secretion mutants as proteases are likely to particapte in the permabilization of this protective membrane. It would also be interesting to repeat this experiment when the peritrophic matrix is either removed biochemically by DTT treatment or genetically impaired (Kuraishi et al., 2011).

The screening of more than 1,300 mutants yielded rather disappointing results as only one mutant was finally retained, although it would certainly be worth retesting more extensively some of the mutant identified in the primary screen. One limitation of the bank is that transposon insertions are generated at random, and thus insertions in the same genes are possibly tested multiple times, in contrast to the ordered library generated in *P. aeruginosa* PA14 (Liberati et al., 2006). We found that *fliR* is required for: i) the efficient passage through the intestinal barrier; ii) for the invasion of enterocytes and cultured S2 cells; iii) for damaging enterocytes and thus triggering the compensatory proliferation of ISCs. I cannot exclude that this locus might also be required for exiting the enterocytes at the basal side. One possible way to test this possibility would be to make a rapid gentamicin treatment after *S. marcescens* uptake into cultured cells so as to kill all remaining extracellular bacteria. Then, one might remove the antibiotics and monitor if the extracellular count increases in a chase.

As FliR is required for secretion of proteins through the flagellar apparatus, it is at present unclear whether the phenotypes I have observed are due to a defective motility or some secreted virulence factor, which might not have been identified yet, besides phospholipase and nuclease. Thus, the conclusion that invasion is needed to cause epithelial damages remains tentative at this stage. Of note, a role for flagellin in these phenotypes would actually also account for them satisfactorily. The generation of additional mutants affecting the structure of flagellin would resolve this issue. For lack of time, I unfortunately could not tackle this problem.

I did not recover any mutants displaying a consistent increased virulence like that displayed in a septic injury model, although again it might be worth rechecking some of the initial mutants I isolated. The biological basis for this switch in virulence remains to be established and might be linked to swarming, which is likely to happen in the septic injury model (Dr Kwang Zin Lee and Dr Samuel Liegeois, personal communication). Actually, it is likely that *S. marcescens* in nature is not the only food that *Drosophila* flies ingest when feeding on decaying fruits and thus that only limited amounts are ingested. By being able to cross the digestive tract, it might be able to reach reproductive organs and thus be disseminated together with the fly progeny (Nehme et al., 2007). Thus, the bacterium may not be pathogenic in this context or its virulence might be altered in the specific conditions found in the digestive tract. It might become virulent when in the presence of a viral cofactor. For instance, *S. marcescens* is a bacterium retrieved in silkworms suffering from *flacherie*, a viral infection that was coexisting with Nosemosis, as discovered by Pasteur.

The *S. xylosus* model was started as a side-project by Dr. P. Giammarinaro. The question raised by intestinal infections by Gram-positive bacteria has rarely been tackled (Cox and Gilmore, 2007). We have not rigorously tested yet the hypothesis that *S. xylosus* is killed by the ROS response. This would entail monitoring the bacterial titer in the gut after N-acetylcysteine treatment or *duox* mutants. The one unexpected result was the higher resistance of *MyD88* to ingested *S. xylosus*. Again, the ultimate cause of death appears to be starvation. Interestingly, *S. xylosus*, in contrast to *L. plantarum*, appears to deplete rapidly fat stores. It will be thus interesting to determine whether this phenomenon requires the inhibition of the TOR pathway and lipophagy. Any interference with the gut microbiota should also be monitored. Testing a *S. xylosus* lipase mutant would also be interesting.

As regards the starvation resistance of *Myd88* mutants, I have now a model that can be experimentally tested, namely that *L. plantarum* prevents lipophagy by stimulating the TOR pathway. This is relatively straightforward. However, the more relevant question to my mind is how *MyD88* favorizes *L. plantarum* colonization and maintenance. The second issue is why the flies can survive longer by not triggering prematurely the consumption of energy stores. As discussed earlier, it is likely that this involves a behavioral change that would limit energy consumption. The identification of the exact molecular mechanisms involved promises to be highly stimulating.

In conclusion, my work has allowed the identification of the mechanisms that led to the death of flies that ingest bacteria, even though the explanation was rather far from the one originally envisioned. Nevertheless, these infection models remain relevant as mutant flies identified in screens generally remain sensitive to a bacterial challenge, even though it takes much longer to observe the fly's demise. My work, like that of several investigators (Lee, 2008, Leulier, PIMS, (Ryu et al., 2008; Shin et al., 2011; Storelli et al., 2011) points to the importance of the microbiota in several aspects of the biology of the flies. This is also the case in humans. My work is also relevant to an understanding of the homeostasis of the fly intestinal epithelium, which is also likely to be essential to understand the primary causes of genetic diseases such as ulcerative colitis and Crohn's disease.

7. Bibliography

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