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Etude des relations hôte-pathogène dans des modèles d'infection intestinales de *Drosophila melanogaster*

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Abbreviations

3-oxo-C_{12}-HSL	3-oxo-dodecanoyl-homoserine lactone
AMP	anti-microbial peptide
C_4-HSL	butanoyl-homoserine lactone
CF	cystic fibrosis
DAP	diaminopimelic acid
DIAP2	*Drosophila* inhibitor of apoptosis 2
Dif	Dorsal related immunity factor
Dome	Domeless
Dscam	Down syndrome cell adhesion molecule
DUOX	dual oxidase
EB	enteroblast
EC	enterocyte
EMS	ethyl methanesulfonate
Erk	extracellular signal-regulated kinase
GlcNAc	N-acetylglucosamine
GNBP	gram-negative binding protein
GPCR	G-protein coupled receptor
Gprk	G-protein-coupled receptor kinase
hFAF1	human Fas associated factor 1
Hop	Hopscotch
IAP	inhibitor of apoptosis
IBM	IAP-binding motif
IMD	Immune deficiency
IP3	1,4,5-triphosphate
IRAK	IL-1R associated kinase
ISC	intestinal stem cell
Key	Kenny
LPS	lipopolysaccharide
LRR	leucine-rich repeats
Lys	lysine
MAMP	microbe associated molecular pattern
MAPK	mitogen-activated protein kinase

ModSP	modular serine protease
MurNAc	N-acetylmuramic acid
N-acyl-HSL	N-acylhomoserine lactone
NADPH	nicotineamide adenine dinucleotide phosphate
ORF	open reading frame
PGN	peptidoglycan
PGRPs	PGN-recognition proteins
PLCβ	phospholipase C-β
PO	phenoloxidase
PPAE	Prophenoloxidase activating enzyme
PQS	Pseudomonas Quinolone Signal
ProPO	prophenoloxidase
PRRs	pattern recognition receptors
Psh	Persephone
Pvf	PDGF- and VEGF-related factor
Pvr	PDGF- and VEGF-receptor related
QS	quorum sensing
ROS	reactive oxygen species
ROS	reactive oxygen species
SPE	Spätzle-processing-enzyme
Spz	Spätzle
Tep	Thioester-containing protein
TLR	Toll-like receptor
Tot	Turandot
TPSS	two partner secretion system
upd	unpaired
WntD	wnt inhibitor of Dorsal
βGRP	β-glucan recognition proteins

1 Introduction

1.1 Overview

Multicellular organisms have to face life-challenging infection by a variety of microbes over and over. Therefore, throughout the evolution, the animal and plant phyla developed powerful mechanisms to fight invading microorganisms. Being able to sense different microbes and to induce appropriate defenses, which means having a potent immune system is a key advantage to host survival. These basic defense mechanisms appeared early in the evolution of multicellular organisms and are referred to as innate immunity. The innate immune system involves germ-line encoded receptors that are able to recognize infectious non-self particles and subsequently trigger the expression of effectors that target the microorganisms. Later in evolution, in the ancestors of cartilaginous fish, another arm of immunity appeared: adaptive immunity. It is restricted to vertebrates and displays a second line of defense in addition to the innate immune system. The adaptive immune system relies on the generation of a complex repertoire of immune receptors in lymphocytes. This huge variety of receptors is generated by somatic gene rearrangement. Innate immunity reactions trigger the adaptive immune response and orient the effector mechanisms of this response (Fearon 1997; Janeway *et al.* 2002). In addition to fighting invading microorganism, it is essential for the host to be able to deal with damage caused by the microbes.

Pathogenic microorganisms have coevolved with their hosts, always developing novel strategies to overcome the defense mechanisms of multicellular organisms. The first barriers microbes face are physical, like skin or cuticle and barrier epithelia in, for example, respiratory or digestive organs. After having overcome these barriers, they have to withstand the attack of the immune system to successfully infect the host. For this purpose they have developed sophisticated strategies and weaponry. To date, we by far do not understand all interactions between host and pathogen that lead to infectious diseases.

Therefore, the goal of my PhD was to use the strength of genetics to better understand host-pathogen interactions between the genetic model organism *Drosophila melanogaster* and two Gram(-) bacteria, *Serratia marcescens* and *Pseudomonas aeruginosa*, in an oral infection model.

1.2 *Drosophila melanogaster*

The fruit fly as a model organism has several advantages. It is very small and easy to maintain. The short life cycle and its high number of offspring allow to obtain high numbers of flies and permit fast genetic manipulation. Furthermore, a century of working with *Drosophila* generated many powerful genetic tools. The genome of *Drosophila* has been fully sequenced (Adams *et al.* 2000) and large collections of mutant and transgenic lines are accessible. The yeast UAS-GAL4 system is widely used in *Drosophila* to generate transgenic lines, in which transgene expression can be induced in a spatio-temporally controlled manner (Brand *et al.* 1993). In addition, saturation mutagenesis is achievable by using several techniques. Chemical mutagenesis by feeding ethyl methanesulfonate (EMS) to the flies, for example, creates point mutations (Jenkins 1967). Transposon-mediated mutagenesis, which leads to a disruption or deregulation of gene expression (Rubin *et al.* 1982), is another possibility. A method to downregulate gene expression in an inducible manner is to combine the UAS-GAL4 system with RNA interference (RNAi), by expressing a hairpin dsRNA construct, targeting the gene of interest, under the control of a GAL4 promoter (Kennerdell *et al.* 2000). In addition to diverse genetic tools such as balancer chromosomes, this palette of mutagenesis techniques renders *Drosophila* a powerful genetic model.

As 80% of extant organisms *Drosophila* is highly resistant to microbial infection, even though it has no adaptive immune system and therefore completely relies on the innate immune response. This makes the immune system of the fly easier to study since the adaptive arm of the immune system cannot mask the phenotypic effects of mutations in genes implicated in innate immunity. Therefore it is easier to correlate a genetic mutation to a phenotype. The immune system of the fly consists of several mechanisms. After wounding, several proteolytic cascades are activated, of which one leads to the deposition of melanin at the wound site and the production of cytotoxic reactive oxygen species (ROS) that antagonize invading microorganisms (Nappi *et al.* 1993). Those microbes are also dealt with by hemocytes, which are capable of phagocyting invaders (Braun *et al.* 1998). Injury as well as the presence of microbes in the hemocoel leads to the systemic induction of antimicrobial gene expression in the fat body, a functional equivalent of the mammalian liver. Sensing of wounding or invading microbes triggers many genes including those coding for antimicrobial peptides (AMPs) via, amongst others, two NF-κB pathways, the Toll and

the Immune deficiency (IMD) pathway. AMPs are secreted into the hemolymph, where they counteract the infection (reviewed in (Ferrandon *et al.* 2007; Lemaitre *et al.* 2007), Figure 1). The epithelia of the fly provide the first barrier against microorganisms both at the physical and chemical level by secreting AMPs and ROS (Ferrandon *et al.* 1998; Ha *et al.* 2005a). In the following I shall introduce the systemic, cellular, and epithelial immune responses of the fly in more detail.

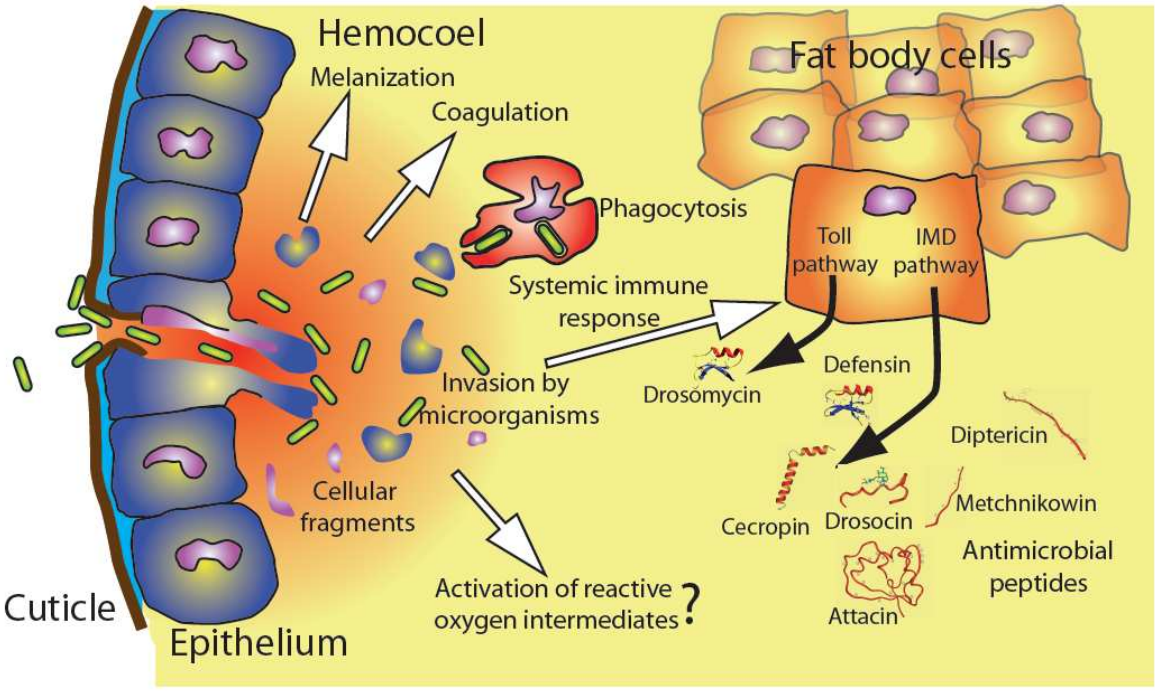

Figure 1: **The *Drosophila* systemic immune reaction**. A septic wound triggers the systemic immune reaction of the fly. The melanization and coagulation cascades are activated to trap pathogens and close the wound. Invading microbes are phagocytosed by hemocytes. In addition, the production of reactive oxygen intermediates might be triggered to fight microorganisms. A systemic infection, as well as wounding to some extent, induces the IMD and Toll pathway-dependent production of antimicrobial peptides (AMPs) by the fat body and their secretion into the hemolymph. From Limmer *et al.* (see Annex)

Systemic response

Recognition of microbes

Since pathogenic microorganisms are very diverse, any living organism requires several defense mechanisms to be able to fight efficiently different invaders. In order to raise an appropriate reaction, the immune system needs the ability to distinguish between distinct classes of microbes. The immune system of *Drosophila* has developed several sensing mechanisms to differentiate between Gram(+) bacteria,

Gram(-) bacteria and fungi or yeasts. These different sensors are being introduced below.

Recognition of Gram(-) bacteria via DAP-type Peptidoglycan (PGN)

The cell wall of Gram(-) bacteria contains lipopolysaccharide (LPS) that is highly immunogenic in mammals (Beutler *et al.* 2003). Interestingly, LPS does not activate the immune system of *Drosophila* (Kaneko *et al.* 2004). The component of the Gram(-) cell wall that is sensed in *Drosophila* is *meso*-diaminopimelic acid (DAP)-type PGN, which is thought to be released during cell growth and division (Leulier *et al.* 2003; Kaneko *et al.* 2004). PGN consists of glycan chains, built of β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), that are cross-linked by short peptide stems (Figure 2). These peptides are diversified depending on the bacterial species (Schleifer *et al.* 1972). In the case of Gram(-) bacteria and some Gram(+) bacilli, the third amino acid in the peptide stem is *meso*-diaminopemilic acid. The PGN of most Gram(+) bacteria in contrast contains L-Lysine (Lys-type PGN). *Drosophila* is able to discriminate between these two types of PGN using pattern recognition receptors (PRR) of the family of PGN-recognition proteins (PGRPs). The *Drosophila* genome encodes 13 PGRP genes that are spliced into at least 17 different PGRP proteins (Werner *et al.* 2000). All members of the family contain a PGRP domain, which is related to the bacteriophage type II amidases. Some PGRP proteins have kept the enzymatic activity (catalytic PGRPs) (Mellroth *et al.* 2003; Mellroth *et al.* 2006). The detection of Gram(-) bacteria is mediated by two noncatalytic members of the family, PGRP-LC and PGRP-LE (Figure 3). After binding to DAP-type PGN they activate the Immune deficiency (IMD) pathway (see below) (Choe *et al.* 2002; Gottar *et al.* 2002; Ramet *et al.* 2002b; Takehana *et al.* 2002). PGRP-LC exists in three isoforms (a, x and y), which result from differential splicing (Werner *et al.* 2000). Short PGN fragments such as tracheal cytotoxin (TCT; Figure 2), which are released during bacterial cell division and growth, are sensed by heterodimers of PGRP-LCx and PGRP-LCa (Mellroth *et al.* 2005). The structure of the PGRP domain of PGRP-LE and the TCT complex suggests that the binding of the ligand to PGRP-LE mediates PGRP-LC/LE polymerization (Lim *et al.* 2006). This would result in an activation of the IMD pathway and the subsequent production of antimicrobial effectors, that by destroying bacteria trigger an additional release of large PGN fragments that can be sensed by PGRP-LCx homodimers (Mellroth *et al.* 2005). PGRP-LE can act extracellularly (here as a naturally truncated form,

containing only the PGRP domain) in synergy with PGRP-LC (Takehana *et al.* 2004) or in the cytoplasm, independently of PGRP-LC, to detect intracellular pathogens. In this case PGRP-LE also induces, apart from the IMD pathway, autophagy to inhibit bacterial growth (Yano *et al.* 2008). The overexpression of PGRP-LE triggers in addition to the IMD pathway also the prophenoloxidase (proPO) cascade, which leads to melanization (Takehana *et al.* 2002). To avoid a detrimental long-term activation of the IMD pathway, catalytic PGRPs (PGRP-SC1, PGRP-SB1 and PGRP-LB) digest the PGN and thereby remove its immunostimulatory properties (Mellroth *et al.* 2003; Zaidman-Remy *et al.* 2006). PGRP-LF, a non-catalytic PGRP, also seems to play an inhibitory role in immunity. PGRP-LF is a transmembrane receptor, but its intracellular tail contains just 23 amino acids. It has two PGRP domains in its extracellular part, which display high affinity for DAP-type PGN and low affinity to Lys-type PGN. Cultured cells depleted for PGRP-LF show infection-independent activation of the IMD pathway (Persson *et al.* 2007). In addition, infection-induced *Drosomycin* expression is strongly downregulated in PGRP-LF overexpressing flies, rendering the flies susceptible to Gram(-) bacterial infection (Maillet *et al.* 2008).

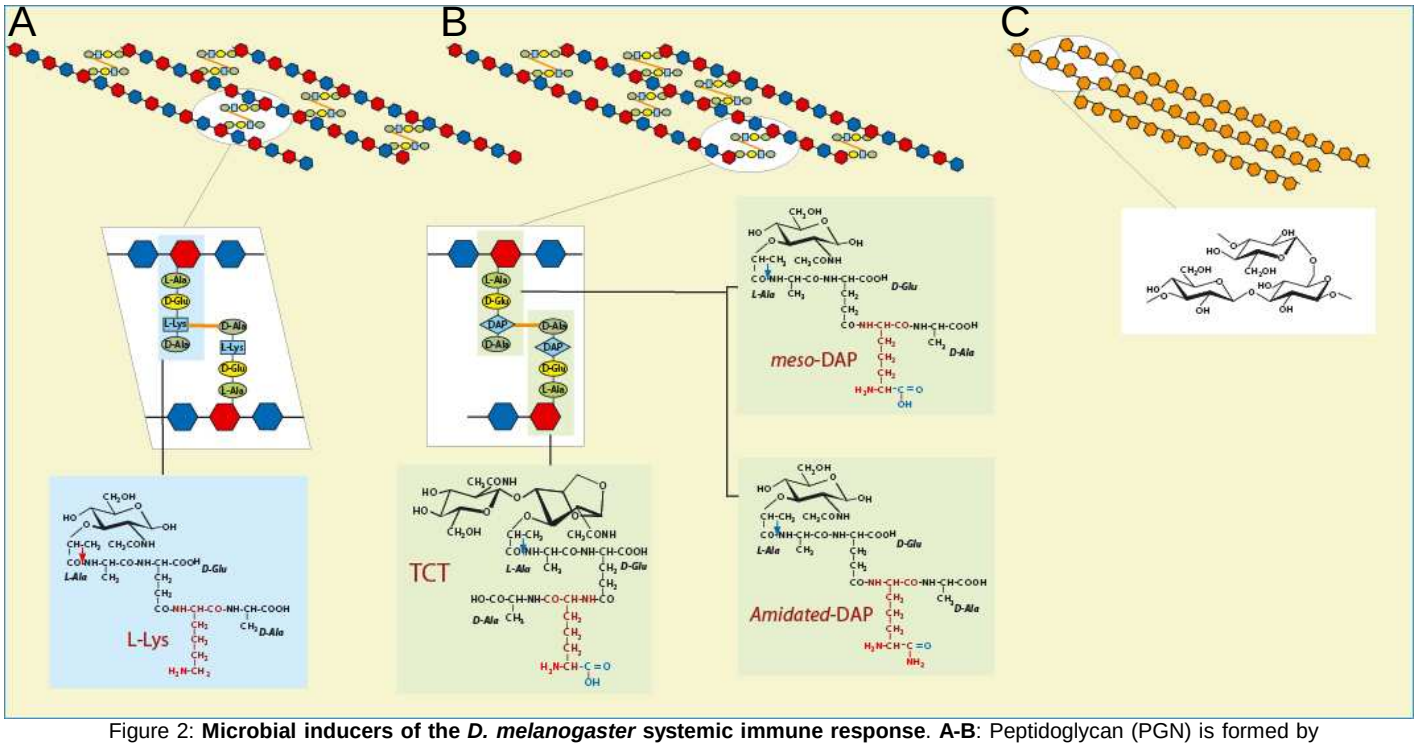

Figure 2: **Microbial inducers of the *D. melanogaster* systemic immune response**. **A-B**: Peptidoglycan (PGN) is formed by chains of repeated disacharidic subunits consisting of N-acetylglucosamine (blue hexagons) linked to N-acetylmuramic acid (red hexagons) through a β(1-4) bond. The lactyl groups of muramic acid are substituted with stem peptides containing usually four alternating L- and D-amino acids. The stem peptides from distinct chains can be cross-linked (white box), either directly or through short peptides (for clarity, the noncross-linked peptide stems attached to each muramic-acid residue have not been drawn). The identity of the third amino acid of the stem is either a L-Lys (**A**, red characters) or a diaminopimelic acid (DAP; **B** , red characters), which can be further amidated, thus decreasing the number of negatively charged oxygen atoms (shown with blue characters). Note, however, that not all PGN contain a Lys or a DAP residue and that the nature of the stem and of the peptidic bridge varies depending on the bacterial species considered (Schleifer *et al.* 1972). The minimum structure that activates the Toll pathway consists of two disaccharides cross-linked by the peptide stems (Filipe *et al.* 2005). The shortest structure detected by the PGN-recognition protein-LC (PGRP-LC)–PGRP-LE system is similar to tracheal cytotoxin (TCT, **B**), except that it lacks the fourth amino acid of the stem (Stenbak *et al.* 2004). One specific feature of TCT is the presence of an internal (1, 6) anhydro bond that specifically interacts with PGRP-LC–PGRP-LE. This anhydro bond is also present at the end of peptidoglycan chains. The cleavage site of the N-acetyl-muramy-L-Alanine amidases is indicated by red (PGRP-SC1) and blue (PGRP-SC1, PGRP-LB and PGRP-SB) arrows respectively. **C**: β(1,3) glucan chains (orange hexagons represent the glucose subunit), cross-linked by interchain β(1,6) bonds are a constituent of the cell wall of most fungi. Attempts to define a minimal activating structure have failed so far as laminarins (short soluble β(1,3)-glucan chains) do not consistently induce the Toll pathway. However, the N-terminal domain of Gram-negative binding protein 3 (GNBP3) does bind curdlan beads (16-20mers of insoluble β(1,3)-glucan) *in vitro* (Kim *et al.* 2000; Gottar *et al.* 2006; Mishima *et al.* 2009). From (Ferrandon *et al.* 2007).

Recognition of Gram(+) bacteria via Lys-type Peptidoglycan (PGN)

The cell wall of Gram(+) bacteria contains high amounts of Lys-type PGN (Figure 2). As DAP-type PGN, Lys-type PGN is sensed by PGRP family members, here PGRP-SA and possibly PGRP-SD. In addition GNBP1, a Gram-negative binding protein (GNBP; this family of proteins is also known as β-glucan recognition proteins (βGRP); Figure 3), helps in recognition of Lys-type PGN. PGRP-SA and GNBP1 bind Lys-type PGN (Chang *et al.* 2004; Wang *et al.* 2006). GNBP1 cooperates with PGRP-SA in sensing Gram(+) bacteria and activating the Toll pathway (see below) (Gobert *et al.* 2003). The glucanase domain of GNBP1 is predicted to be catalytically inert, nevertheless a muramidase-like activity and cleavage of Lys-type PGN chains have been reported *in vitro* (Wang *et al.* 2006). It has been suggested that GNBP1 processes PGN into short di- or tetrameric muropeptides that are then presented to PGRP-SA (Wang *et al.* 2006). PGRP-SD cooperates with PGRP-SA in the detection of some Gram(+) bacteria (Bischoff *et al.* 2004) to activate the Toll pathway (Figure 3). PGRP-SC1B, a catalytic PGRP, is able to degrade Lys-type PGN, thereby having a negative effect on Toll pathway activation (Mellroth *et al.* 2003).

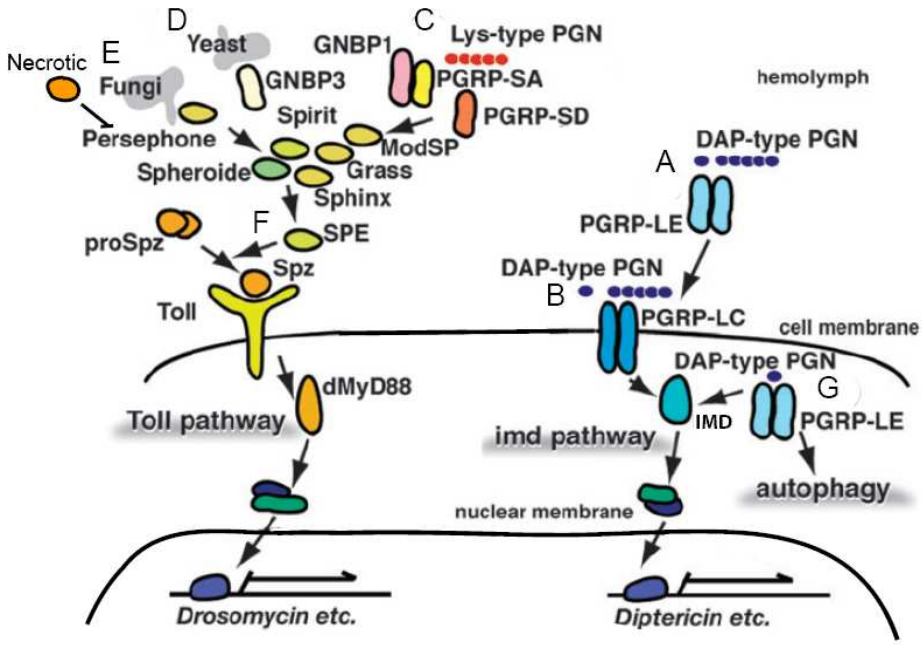

Figure 3: **Recognition of microorganisms and induction of innate immune responses in *D. melanogaster*. A**: A constitutive hemolymph protein, PGRP-LE, binds to monomeric and polymeric DAP-type peptidoglycans (PGNs), which are components of many Gram(-) and some Gram(+) bacteria and is involved in activating the secondary humoral response, the IMD pathway-dependent induction of antibacterial peptides. **B**: Membrane-bound PGRP-LC is also required for monomeric and polymeric DAP-type PGN-mediated activation of the IMD pathway. **C**: PGRP-SA in the hemolymph is involved in the recognition of Gram(+) bacteria with Lys-type PGN and is required for activation of the Toll pathway in cooperation with GNBP1. PGRP-SD has some redundant functions with PGRP-SA and GNBP1. **D**: GNBP3 is involved in yeast-mediated activation of the Toll pathway. **E**: The fungal protease PR1 cleaves ProPersephone into active Persephone **F**: The activation of the Toll pathway is mediated by its active ligand, Spätzle (SPZ), cleaved from proSPZ by the serine protease cascades, including the SPZ-processing-enzyme (SPE), Spheroide, Spirit, Sphinx, Persephone, ModSP and Grass. The Serpin Necrotic negatively regulates Persephone. **G**: In addition to the extracellular functions, PGRP-LE induces antimicrobial peptides through the IMD pathway as well as autophagy in the cytoplasm. Signaling via the Toll or IMD pathway activates NF-κB and the subsequent transcription of genes for antimicrobial peptides, such as *Drosomycin* and *Diptericin*, and other effector genes. Modified after (Kurata 2010).

Recognition of fungi

Like Gram(+) bacterial infection, fungal infection triggers the activation of the Toll pathway (Lemaitre *et al.* 1997). Fungi are sensed by two different means. First, GNBP3 binds β-(1,3)-glucans (Figure 2), a component of the fungal cell wall, and subsequently activates the protease cascade that leads to the cleavage of the Toll-ligand Spätzle (SPZ) (see below; Figure 3) (Mishima *et al.* 2009). In addition GNBP3

is able to activate the Phenoloxidase pathway to induce melanization, which is thought to be an important defense against fungal infection (Matskevich *et al.* 2010). Considering the distinct binding properties of GNBP1 and GNBP3, the GNBP family is, as the PGRP family, crucial for discrimination between different classes of pathogens.

Second, a fungal protease (PR1) used by the entomopathogenic fungus *Beauveria bassiana* to digest the cuticle of the fly as well as Gram(+) bacterial proteases, cleave the *Drosophila* zymogen Persephone (PSH) into an active protease (Ligoxygakis *et al.* 2002; Gottar *et al.* 2006; El Chamy *et al.* 2008) (Figure 3). PSH subsequently triggers the proteolytic cascade that leads to Toll pathway activation.

Signal transduction

Activation of the Toll pathway

The *Drosophila* genome encodes a family of Toll receptors (Toll, 18-wheeler and Toll3-9). Toll9 resembles mammalian TLRs and might be a classical pattern recognition receptor (PRR). Unlike mammalian TLRs Toll itself is a cytokine receptor that is activated upon binding of the cytokine SPZ. An immune function has just been shown for Toll itself, but not for the other eight members of the family. They are involved in developmental processes during embryogenesis and possibly later in development (Eldon *et al.* 1994; Tauszig *et al.* 2000; Kambris *et al.* 2002; Gay *et al.* 2007). Toll has an extracytoplasmic domain with numerous leucine-rich repeats (LRR). The intracytoplasmic domain is homologous to the intracytoplasmic signaling domain of the mammalian interleukin-1 receptor and of all mammalian TLRs (referred to as TIR domain (Hashimoto *et al.* 1988)).

Toll is activated by binding to the active form of the cytokine SPZ (the SPZ family comprises 6 members). SPZ, structurally related to neurotrophins, is synthesized as an inactive dimeric precursor (proSPZ) and secreted into the hemolymph (Weber *et al.* 2003; Hu *et al.* 2004; Irving *et al.* 2005). ProSPZ is the target protein processed by proteolytic cascades that are activated by PRGP-SA, PGRP-SD, GNBP1 or GNBP3, in the case of the modular serine protease ModSP cascade, or fungal and bacterial proteases, in the case of the cascade activated by PSH (Figure 3). At one point, downstream of ModSP and PSH, the two cascades merge and lead to the activation of SPZ by SPZ-processing-enzyme (SPE) (Jang *et al.* 2006). These two cascades,

which are distinct from the cascade required for activation of Toll during development (Lemaitre *et al.* 1996), comprise in addition to ModSP and PSH several serine proteases: Spirit, Spheroide, Sphinx, Grass, and probably other unidentified serine proteases (Ligoxygakis *et al.* 2002; Kambris *et al.* 2006; El Chamy *et al.* 2008; Buchon *et al.* 2009c). *spheroide* and *sphinx* encode serine protease homologs with an inactive catalytic site (Kambris *et al.* 2006). The exact order and interactions of all these proteases and pseudo-proteases remain to be established.

The Toll pathway

The amino-terminal cleavage of SPZ by SPE leads to the binding of a dimer of its carboxy-terminal fragment to Toll. SPZ binding at the amino-terminal end of Toll induces the formation of a 2:2-complex (2xSPZ, 2xToll) and thereby triggers the activation of the downstream signaling cascade (Mizuguchi *et al.* 1998; Weber *et al.* 2003; Weber *et al.* 2005; Gangloff *et al.* 2008). The dimerized TIR domains interact with a platform of three death domain-containing proteins, dMyD88, Tube and Pelle (Figure 4) (Lemaitre *et al.* 1996; Tauszig-Delamasure *et al.* 2002). dMyd88 is homologous to mammalian MyD88 and interacts through its TIR domain with the TIR domain of Toll. The association with Tube is mediated by the death domains of the two proteins. The death domain of Tube is bifunctional, which allows the interaction with a second death domain, the one of Pelle (Sun *et al.* 2004). Pelle is a member of the IL-1R associated kinase family (IRAK) of serine-threonine kinases, the substrate of which remains unknown.

By a still uncharacterized mechanism Cactus (the *Drosophila* homolog of I-κB) is phosphorylated and undergoes K48 ubiquitination, which leads to its degradation by the proteasome (Belvin *et al.* 1995; Fernandez *et al.* 2001). Hence, Dorsal and/or Dorsal related immunity factor (DIF) are released and translocate to the nucleus where they bind κB-response elements and activate the expression of target genes (Bergmann *et al.* 1996; Reach *et al.* 1996) (Figure 4). One of their target genes is *Drosomycin*, which is widely used as a reporter gene for Toll activation. DIF is sufficient to mediate the Toll response in adults while DIF and Dorsal seem to play redundant roles in the control of *Drosomycin* expression at the larval stage (Manfruelli *et al.* 1999; Meng *et al.* 1999; Rutschmann *et al.* 2000).

Recently, G-protein-coupled receptor kinase (Gprk)2, CG15737/Toll pathway activation mediating protein, and U-shaped have been reported to be required for normal *Drosomycin* response *in vivo*. Interaction studies using *Drosophila* S2 cells

suggest that Gprk2 interacts with Cactus, but is not required for Cactus degradation (Valanne *et al.* 2010). Furthermore it has been shown that the endosomal proteins Myopic (MOP) and Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) are required for the activation of the Toll pathway. This indicates an important role of endocytosis for Toll signaling (Huang *et al.* 2010), which is paralleled in TRIF-dependent TLR4 signaling (Kagan *et al.* 2008).

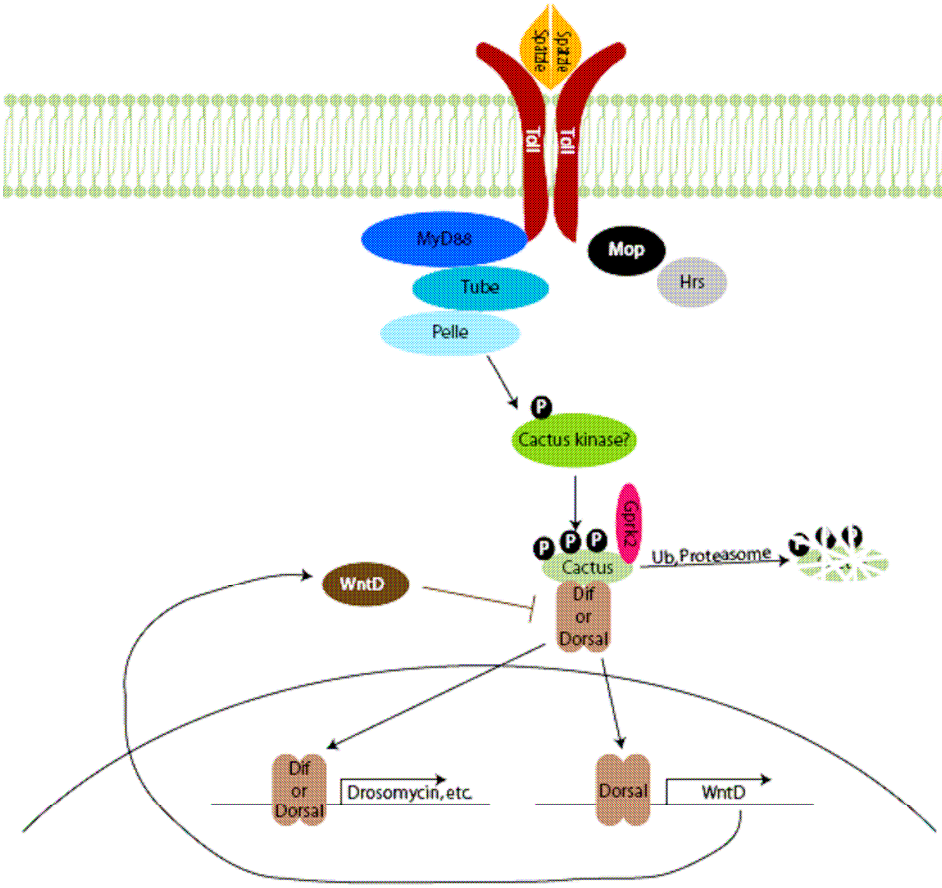

Figure 4: **Schematic overview of the Toll pathway.** After dimerization Toll recruits MyD88, Tube and Pelle. Through an unknown process Cactus is phosphorylated, which leads to its degradation by the proteasome. After Cactus degradation DIF and/or Dorsal are released and translocate to the nucleus, where they activate effector gene transcription. Dorsal activates, amongst others, the production of WntD, which, in a negative feedback loop, might inhibit DIF and Dorsal translocation to the nucleus. Gprk2 has been shown to interact with Cactus. Its exact function remained to be investigated. In addition, the endosomal proteins Myopic (MOP) and Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) have been shown to be implicated, suggesting a role of endocytosis during Toll signaling. Modified after (Aggarwal *et al.* 2008b).

Negative regulation of the Toll pathway

PSH, a serine protease that is part of a cascade that leads to cleavage of SPZ, is inhibited by Necrotic, a serine protease inhibitor of the serpin family (Figure 3). Lack of Necrotic leads to constitutive activation of the Toll pathway in a PSH-dependent manner (Levashina *et al.* 1999; Ligoxygakis *et al.* 2002). In addition to this regulation of PSH by Necrotic and the cleavage of Lys-type PGN by PGRP-SC1B (see above), the Toll pathway may be repressed by an intracellular feedback loop. Activation of the Toll pathway triggers the expression of WntD (wnt inhibitor of Dorsal). WntD is capable of blocking the nuclear translocation of Dorsal in *cactus* mutants during development, therefore acting downstream of or in parallel to Cactus. WntD regulates the Toll pathway in embryonic patterning and possibly also in the context of immunity (Figure 4) (Ganguly *et al.* 2005; Gordon *et al.* 2005; Gordon *et al.* 2008).

The IMD pathway

After binding to PGN, the PGRP-receptors dimerize or multimerize, which is crucial for signaling via their N-terminal domains (Choe *et al.* 2005), who share a conserved motif in PGRP-LE and PGRP-LC (Kaneko *et al.* 2006). This motif is weakly homologous to the RHIM motif, found in proteins critical for the TRIF-dependent pathway in mammalian TLR signaling (Sun *et al.* 2002; Meylan *et al.* 2004). Following receptor activation, IMD, FADD and the caspase-8 like protein DREDD are recruited, which leads to the DREDD-dependent cleavage of IMD (Paquette *et al.* 2010) (Figure 5). Upon cleavage, an inhibitor of apoptosis (IAP)-binding motif (IBM) is exposed, which then interacts with the BIR domains of DIAP2 (*Drosophila* inhibitor of apoptosis 2). Subsequently IMD is K63-ubiquitinated, an event that has been suggested to play a critical role in IMD signaling (Gesellchen *et al.* 2005; Kleino *et al.* 2005; Zhou *et al.* 2005; Leulier *et al.* 2006; Huh *et al.* 2007). It is thought that DIAP2 functions as the E3-ligase for K63-ubiquitination in the IMD pathway. In addition the E2 complex of Bendless (Ubc13 homolog), Effete (Ubc5 homologue) and Uev1a appears to be involved in IMD ubiquitination (Zhou *et al.* 2005; Paquette *et al.* 2010) (Figure 5). IMD-linked K63-polyubiquitin chains are likely to serve as scaffolds to recruit the kinases Tak1 (MAPKKK) and IKKβ (Ird5 in *Drosophila*). Both include regulatory subunits with highly conserved K63-polyubiquitin binding domains. *Drosophila* TAB2, which complexes with TAK1, and IKKγ (Kenny (KEY) in *Drosophila*) are predicted as well to contain K63-polyubiquitin-binding domains (Kleino *et al.* 2005; Zhou *et al.*

2005; Ea *et al.* 2006; Zhuang *et al.* 2006). Therefore, it is likely that IMD-linked K63-polyubiquitin chains recruit the TAK1/TAB2 and the IKK complex. Activation of the TAK1/TAB2 complex leads to the activation of the JUN N-terminal kinase (JNK) pathway and NF-κB/Relish (Silverman *et al.* 2003)(Figure 5). The JNK pathway (see below) is activated by TAK1-mediated signaling to Hemipterous, the *Drosophila* MKK7/JNKK homolog (Sluss *et al.* 1996; Holland *et al.* 1997; Chen *et al.* 2002), which then phosphorylates Basket (dJNK) that in turn activates AP-1 (Figure 5). Besides its role in differentiation, stress response, apoptosis, and directed cell movement (Huang *et al.* 2004; Varfolomeev *et al.* 2004; Dhanasekaran *et al.* 2008), JNK signaling is an element of vertebrate and invertebrate innate immunity. IMD-dependent JNK signaling has been linked to the up-regulation of wound repair and stress response genes (Boutros *et al.* 2002; Silverman *et al.* 2003).

The activated IKK complex can directly phosphorylate Relish, a bipartite protein similar to mammalian NF-κB precursors p100 and p105. It contains a N-terminal Rel homology domain and an inhibitory I-κB domain. Relish is likely cleaved by DREDD, independently of its phosphorylation by the IMD pathway. Subsequently, its N-terminal domain is translocated to the nucleus, where it activates immune genes. This activation is phosphorylation-dependent (Stoven *et al.* 2000; Stoven *et al.* 2003). Amongst the induced genes is the AMP gene *Diptericin*, the expression of which is widely used as readout for IMD pathway activation.

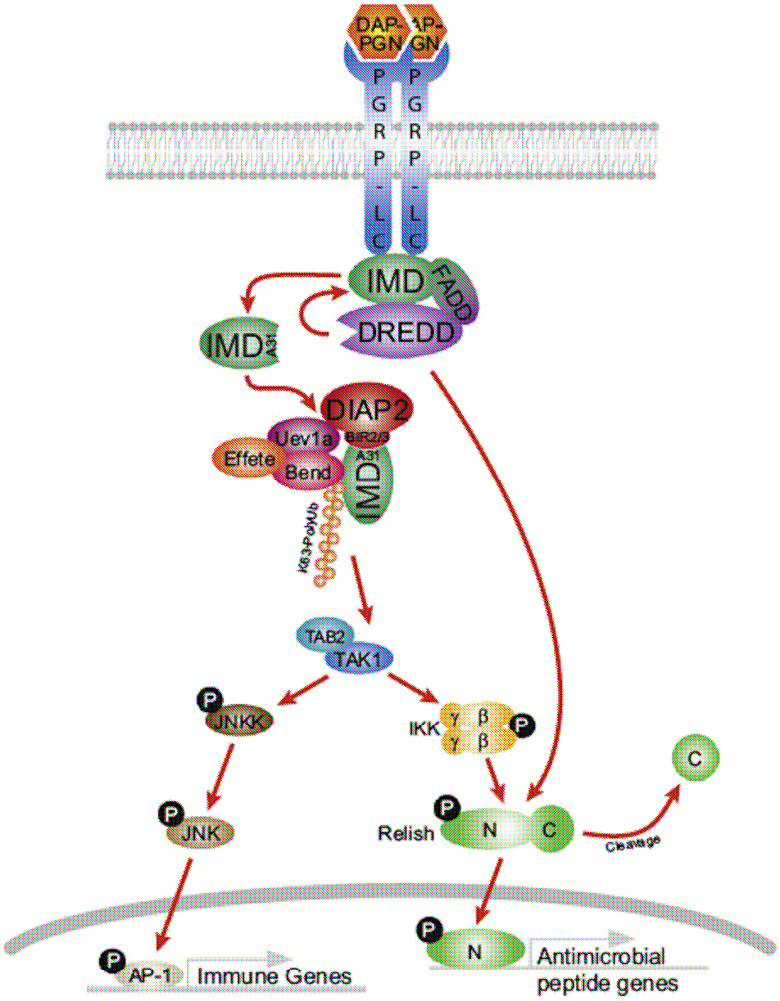

Figure 5: **Schematic overview of the IMD pathway.** DAP-type PGN binding causes di- or multimerization of PGRP receptors. This likely recruits the adaptor proteins IMD, FADD, and the caspase DREDD. Once in proximity, DREDD cleaves IMD, generating an exposed neo-N-terminal A31 residue. This neo-N terminus then binds the E3-ligase DIAP2 via its BIR2/3 domains. In conjunction with the E2-ubiquitin-conjugating enzymes UEV1a, Bendless (Ubc13), and Effete (Ubc5), IMD is then K63 polyubiquitinated. These polyubiquitin chains then induce the activation of downstream kinases, ultimately leading to the phosphorylation and activation of Relish and the induction the expression of downstream targets such as AMP genes. From (Paquette *et al.* 2010).

Negative regulation of the IMD pathway

In addition to the direct or indirect negative regulation by several PGRPs (see above), the IMD pathway seems to be inhibited by an E3 protein known as DNR1. DNR1 overexpression in flies leads to a blockade of IMD signaling and renders the flies susceptible to Gram(-) bacterial infections (Guntermann *et al.* 2009). In keeping with this, DNR1-RNAi expression in adult flies leads to *Diptericin* transcription in the absence of infection (Guntermann *et al.* 2009). DNR1 is thought to bind to DREDD and to target it for proteasome-mediated degradation. Immune stimulation of *Drosophila* S2 cells stabilizes DNR1 in an IMD-dependent manner, leading to a negative feedback loop (Foley *et al.* 2004; Guntermann *et al.* 2009) (Figure 6).

A homolog of the human Fas associated factor 1 (hFAF1), Caspar, is another negative regulator of IMD signaling. hFAF1 associates with various components of the TNF/NF-κB pathway, namely FAS, FADD, caspase-8 and NF-κB (Chu *et al.* 1995; Ryu *et al.* 2003; Park *et al.* 2004b). *caspar* mutant flies show infection-

independent constitutive expression of *Diptericin*, whereas Caspar overexpression inhibits AMP gene induction (Kim *et al.* 2006). It is hypothesized that Caspar blocks Relish cleavage by interfering with DREDD (Figure 6).

Another negative regulator of the IMD pathway is SKPA, a homologue of human Skp1 protein, which is a subunit of the SCF-E3 ubiquitin ligase that targets substrates for K48-ubiquitination and degradation by the 26S proteasome. Flies that have an EMS-induced mutation in the *skpA* gene have been found to constitutively induce IMD signaling in absence of infection.The same phenotype occurs in other mutants that effect the *Drosophila* SCF components, *slimb* and *dCullin1*. In cell culture, RNAi-mediated downregulation of *skpA* or *slimb* leads to an accumulation of both (full-length and cleaved) forms of Relish. Therefore it is thought that SKPA, Slimb and dCullin1 regulate the IMD pathway by controlling the stability of Relish (Figure 6) (Khush *et al.* 2002).

In addition, the overexpression of PIRK (poor IMD response upon knock-in, also known as PIMS or RUDRA), a cytoplasmic protein, reduces IMD pathway activation after Gram(-) bacterial infection. In keeping with this result, RNAi-mediated downregulation of *pirk* leads to the hyperactivation of the IMD pathway after infection. In addition, *pirk* expression is rapidly upregulated after infection in a Relish-dependent manner. PIRK is believed to downregulate the IMD pathway via a negative feedback loop acting at the level of PGRP-LC by interrupting the signaling complex (Aggarwal *et al.* 2008a; Kleino *et al.* 2008; Lhocine *et al.* 2008) (Figure 6).

Another regulatory mechanism has been found by Thevenon *et al.* (Thevenon *et al.* 2009). The *Drosophila* ubiquitin-specific protease, USP36, acts as a negative regulator of IMD ubiquitination. USP36 is capable of removing K63-ubiquitin chains from IMD, thereby promoting K48-mediated polyubiquitination and the degradation of IMD. Animals in which USP36 is overexpressed exhibit decreased levels of IMD ubiquitination and reduced pathway activity (Figure 6).

Moreover, IMD-induced JNK-dependent and Relish-dependent signaling cross-regulate each other. On the one hand, Relish regulates in cell culture the IMD-dependent JNK activation by inducing certain genes that lead to the degradation of TAK1 (Park *et al.* 2004a). On the other hand, the JNK branch of the IMD-pathway seams to induce the formation of a repressor complex that inhibits AMP genes. This involves the well-established JNK-target dAP-1 (Davis 1999; Kim *et al.* 2005). AP-1 works together with the *Drosophila* STAT protein STAT92E, which is expressed upon

IMD dependent JAK/STAT activation (Agaisse *et al.* 2003). AP1 and STAT92E bind the promoter regions of several Relish-dependent AMP genes, with the help of the HMG protein Dsp1. Thereafter, they recruit the histone deacetylase dHDAC1 to form a repressosome complex, which is inhibiting the transcription of effector genes (Kim *et al.* 2005; Kim *et al.* 2007) (Figure 6). In contrast, the JNK pathway has also been reported to be required for AMP expression (Delaney *et al.* 2006). Thus, the exact role of the JNK pathway remains to be established *in vivo*. In addition, it has been suggested that the PVR (PDGF- and VEGF-receptor related) ligands PVF2 (PDGF- and VEGF-related factor 2) and PVF3 are activated in a JNK-dependent manner upon IMD induction. PVR is thought to signal via dERK to negatively regulate the IMD pathway, by dampening the TAK1-dependent phosphorylation of JNK and Relish (Bond *et al.* 2009).

As the preceding remarks show, the IMD pathway is very tightly regulated. A proper balance of the level of IMD activation is very important, since it has been shown that both hyperactivation and hypoactivation of the IMD pathway can be detrimental to the fly (Kim *et al.* 2006; Zaidman-Remy *et al.* 2006; Kim *et al.* 2007; Ryu *et al.* 2008).

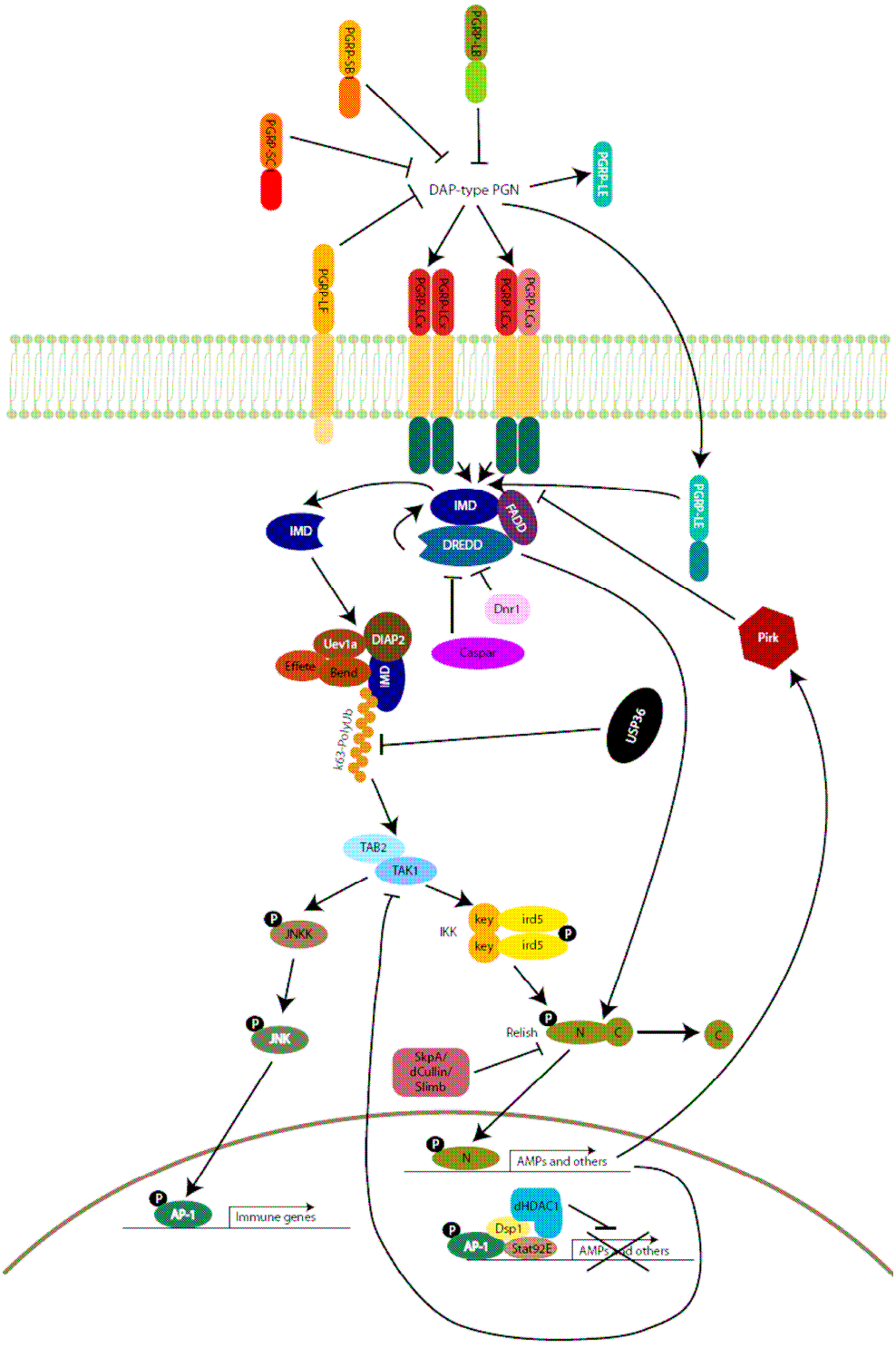

Figure 6**: Negative regulation of the IMD pathway.** The IMD pathway is very tightly regulated. Different catalytic and noncatalytic PGRPs negatively regulate the concentration of immunogenic PGN. PIRK acts at the level of PGRP-LC/IMD. The DREDD caspase is negatively regulated by DRN1 and Caspar. USP36 removes the K63-polyubiquitin chains from IMD, thereby promoting K48-mediated polyubiquitination and degradation of IMD. SKPA, Slimb, and dCullin1 regulate the IMD pathway by controlling the stability of Relish. Moreover IMD-induced JNK-dependent and Relish-dependent signaling cross-regulate each other. On the one hand, Relish regulates the IMD-dependent JNK activation by inducing some genes that lead to the degradation of TAK1. On the other hand, JNK-dependent formation of a repressosome blocks the transcription of Relish target genes. Modified from (Aggarwal *et al.* 2008b) and (Paquette *et al.* 2010).

The JAK/STAT pathway

The evolutionary conserved Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway has been implicated in mammalian immunity (reviewed in (Trinchieri 2003)), where it mediates cytokine signaling downstream of cytokine receptors. In *Drosophila*, the Unpaired (UPD) cytokines (UPD, UPD2 and UPD3) activate JAK/STAT signaling upon binding to the receptor Domeless (Dome). This leads to receptor dimerization and the cross-activation of the receptor-associated JAK kinase (Hopscotch (Hop)). This JAK kinase phosphorylates tyrosine-residues in the cytoplasmic tail of the receptor, which then function as docking sites for cytoplasmic STAT proteins (STAT92E). JAK phosphorylates the STAT proteins, which dimerize and translocate to the nucleus where they induce target gene expression (Figure 7). The JAK/STAT pathway is autoregulatory by inducing positive and negative regulators (Arbouzova *et al.* 2006b). As concerns positive regulation, the transcription of STAT92E is induced by JAK/STAT signaling (Xi *et al.* 2003). Several negative regulators have been reported. Suppressors of cytokine signaling 36E (SOCS36E) is a potent suppressor of JAK/STAT signaling, the expression of which is triggered in a negative feedback loop by the JAK/STAT pathway (Callus *et al.* 2002; Karsten *et al.* 2002) (Figure 7). SOCS44A, even though it is not regulated by the pathway, can inhibit its activity (Rawlings *et al.* 2004). Protein inhibitors of activated STAT (PIAS) are known to suppress the pathway by binding to STATs and thereby target them for degradation via SUMOlation (Kotaja *et al.* 2002; Ungureanu *et al.* 2003; Wormald *et al.* 2004). *Drosophila* PIAS has been shown to physically interact with STAT92E and to suppress JAK/STAT signaling (Betz *et al.* 2001) (Figure 7). In addition a truncated form of STAT92E has been shown to negatively regulate JAK/STAT signaling (Henriksen *et al.* 2002). Ken and Barbie (KEN), a member of the family of BTB/POZ domain containing transcriptional repressors, specifically downregulates some JAK/STAT target genes (Arbouzova *et al.* 2006a). It has been suggested that KEN recruits NURF (nucleosome remodeling factor) to this end. Activated STAT92E can overcome this KEN and NURF dependent repression of promoters. It enters the nucleus, binds target promoters and, in addition to recruiting co-activators, displaces KEN and NURF (Kwon *et al.* 2008) (Figure 7). A phosphatase, PTP61F, negatively regulates the pathway, most likely at the level of STAT92E (Müller *et al.* 2005) (Figure 7). Genome-wide screens in *Drosophila* cells that were performed by (Müller *et al.* 2005) and (Baeg *et al.* 2005) pointed to several

more putative regulators of JAK/STAT signaling, such as the negative regulator PP1α96A.

In addition to the canonical JAK/STAT pathway another function of STAT92E has been proposed (reviewed in (Brown *et al.* 2008; Li 2008)). A portion of the unphosphorylated STAT92E is localized to the nucleus where it is associated with HP1 on heterochromatin (Shi *et al.* 2008) (Figure 7). This heterochromatin-associated STAT92E is essential for maintaining HP1 localisation and heterochromatin stability. Activation of the JAK/STAT pathway and subsequent phosphorylation of STAT92E causes STAT92E scattering away from the heterochromatin, which leads to heterochromatin destabilization (Shi *et al.* 2008). Whether STAT92E activation always leads to heterochromatin destabilization in all settings remains to be established. This effect of STA92E activation might be dependent of the intensity of the signal. Possibly, low levels of JAK/STAT activation stimulate expression of target genes without having an effect on heterochromatin, whereas high levels of activation would result in global epigenetic changes by heterochromatin disruption (Shi *et al.* 2008).

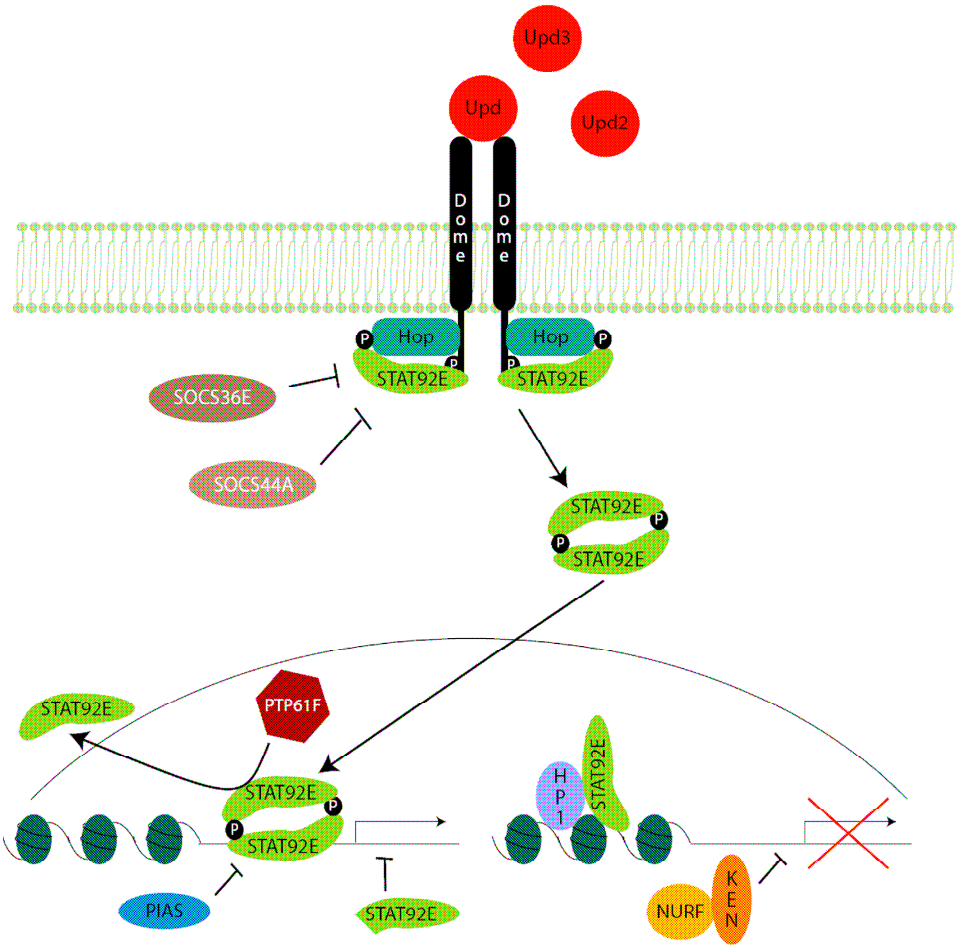

Figure 7: **The JAK/STAT pathway.** The core components of JAK/STAT signaling in *Drosophila* are the three ligands (Upd, Upd2, Upd3), the receptor Domeless (Dome), the Janus kinase Hopscotch (Hop) and STAT92E (the only *Drosophila* STAT). The pathway positively regulates itself by triggering the expression of STAT92E and is negatively regulated by two SOCS proteins, PIAS and the phosphatase PTP61F. Some target genes of the pathway are suppressed by a complex of KEN and NURF and by a truncated form of STAT92E. A part of the unposphorylated STAT92E is localized to the nucleus where it associates with HP1 and heterochromatin. This association is crucial for heterochromatin stability. Activation of the JAK/STAT pathway and subsequent phosphorylation of STAT92E disrupts STAT92E/HP1 complexes and leads to heterochromatin destabilization and epigenetic changes. However, it remains unclear whether JAK/STAT activation always has those effects or if the outcome is dependent on the strength of the signal. Modified from (Brown *et al.* 2008).

JAK/STAT signaling is regulating cell proliferation, differentiation, stress response, survival and migration (Ekengren *et al.* 2001b; Hou *et al.* 2002; Arbouzova *et al.* 2006b). Gene expression profiles identified several immune response genes as regulated by the JAK/STAT pathway, namely the *Tep1* and the *turandot (tot)* genes (reviewed in (Agaisse *et al.* 2004)). The regulation of these genes is rather complex, with an impact of the IMD as well as the MAPK (mitogen-activated protein kinase)

pathways (Brun *et al.* 2006). It has been proposed that UPD3 is produced in hemocytes upon septic injury and induces JAK/STAT signaling in the fat body of the fly. This suggests a role of JAK/STAT in response to tissue damage (Agaisse *et al.* 2003). JAK/STAT deficient flies do not show any susceptibility to bacterial or fungal infection and express normal AMP levels. In contrast, those flies are susceptible to viral infections (Dostert *et al.* 2005). Overall, the potentially multiple roles of JAK/STAT signaling in *Drosophila* immunity has not been clearly established yet.

Immune effectors

Antimicrobial peptides

AMP genes are highly induced upon infection in a Toll- and/or IMD-dependent manner in the fat body. AMPs are small molecules that are secreted into the hemolymph, where they exercise their antimicrobial functions. Seven structurally diverse families of AMPs have been found: Diptericins (2 genes), Drosocin, and Attacins (4 genes) are effective against Gram(-) bacteria. Defensins (2 genes) counteract mostly Gram(+) bacterial infection. Drosomycins (7 genes) and Metchnikowin have antifungal properties, and some Cecropins (4 genes) have been shown to act against both bacteria and some fungi. Most insect AMPs are membrane active, but their exact mode of action is still under investigation. AMPs can reach concentrations between 1µM (Defensin) and 100µM (Drosomycin) in the hemolymph of immune challenged flies (reviewed in (Imler *et al.* 2005; Zhang *et al.* 2009)). *DiptericinA* and *Drosomycin1* are widely used as read-outs for IMD and Toll pathway activation respectively.

Tep proteins

The TEPs (Thioester-containing proteins) are a family of proteins with significant similarities to the complement C3/α2-macroglobulin superfamily. While TEP1-5 contain the canonical thioester-motif, TEP6 (Mcr) lacks it. Tep1-4 and Tep6 (Mcr) have been shown to be expressed in *Drosophila*, while Tep5 has not been shown to be expressed. Some *Tep* genes (Tep1-4) have are strongly activated in the fat body upon immune challenge (Lagueux *et al.* 2000). The proteins contain a signal peptide, which indicates that they are secreted into the hemolymph. TEP proteins have been suggested to function as opsonins to facilitate phagocytosis (see below). For *Anopheles gambiae* TEP1 an opsonin function has been documented. It is also

involved in *Plasmodium* killing (Blandin *et al.* 2004). The importance of *Drosophila* TEPs for phagocytosis has been investigated in S2 cells (Stroschein-Stevenson *et al.* 2006). TEP2 seems to be required for efficient phagocytosis of *E. coli*, TEP3 to help phagocytosis of Gram(+) bacteria, and TEP6 (Mcr) for binding and internalization of *C. albicans*. However, the *in vivo* function of TEPs remains to be established in *Drosophila*.

Other effectors

Many other proteins have also been reported to be activated by an immune challenge (De Gregorio *et al.* 2001; Irving *et al.* 2001; De Gregorio *et al.* 2002). Some of them are implicated in the regulation of the systemic immune response, while others are thought to participate in distinct defense mechanisms (*e.g.* melanization or clotting). Another group includes putative immune effectors. This group comprises members of the DIM (*Drosophila* immune molecule) and the Tot families. These are small peptides secreted into the hemolymph (Uttenweiler-Joseph *et al.* 1998; Ekengren *et al.* 2001a; Ekengren *et al.* 2001b; Levy *et al.* 2004). In addition, a catalase, transferin, and an iron transporter gene are upregulated upon infection, pointing to a role of ROS (reactive oxygen species) and iron sequestration in host defense (Yoshiga *et al.* 1999; De Gregorio *et al.* 2001). A ROS response has been shown to play a role in the local immune response of barrier epithelia (see below), while an implication of ROS in the systemic immune response remains to be established.

Local immune responses

Epithelia are the first point of contact between microbes and the host. They already physically form a border between the outside, microbe-rich world, and the internal milieu of the organism. In addition, those epithelia, like *e.g.* tracheal and intestinal epithelia, are also able to launch an important immune response. (Ferrandon *et al.* 1998; Tzou *et al.* 2000; Onfelt Tingvall *et al.* 2001). In the following, I focus on the immune response in the gut epithelium, which is the first line of defense against ingested pathogens.

Physical barrier and hostile environment in the midgut

The intestinal epithelium is a monolayer that is mainly composed of enterocytes interspersed with hormone producing enteroendocrine cells and intestinal stem cells (ISCs). Octoploid enterocytes are the absorptive cells of some regions of the intestine, whereas they secrete digestive enzymes in other parts of the midgut. The adult midgut (Figure 8) undergoes constant renewal with a turnover of approximately one week. ISCs are located near the basal membrane and are close to the underlying circular muscles, which are important for stem cell maintenance (Lin *et al.* 2010). ISCs give rise to immature enteroblasts (EB) that subsequently differentiate into mature enterocytes or enteroendocrine cells (reviewed in (Charroux *et al.* 2010), Figure 9). A layer of longitudinal muscles is located basally with respect to the circular muscles. On the luminal (apical) side, the gut epithelium is lined by a chitinoproteinaceous layer known as the peritrophic matrix. This matrix restrains microorganisms in the gut lumen (Shanbhag *et al.* 2009). Therefore microorganisms invading the body cavity of the fly, have to overcome the peritrophic matrix and the epithelium itself. In addition, since the gut is a digestive organ, it contains an acidic region (copper cell region, Figure 8) and the gut cells constantly secret enzymes, such as proteases, catalytic PGRPs and lysozymes that degrade the bacterial cell wall. Besides these antibacterial agents, the gut epithelium can launch inducible weapons such as AMPs and ROS.

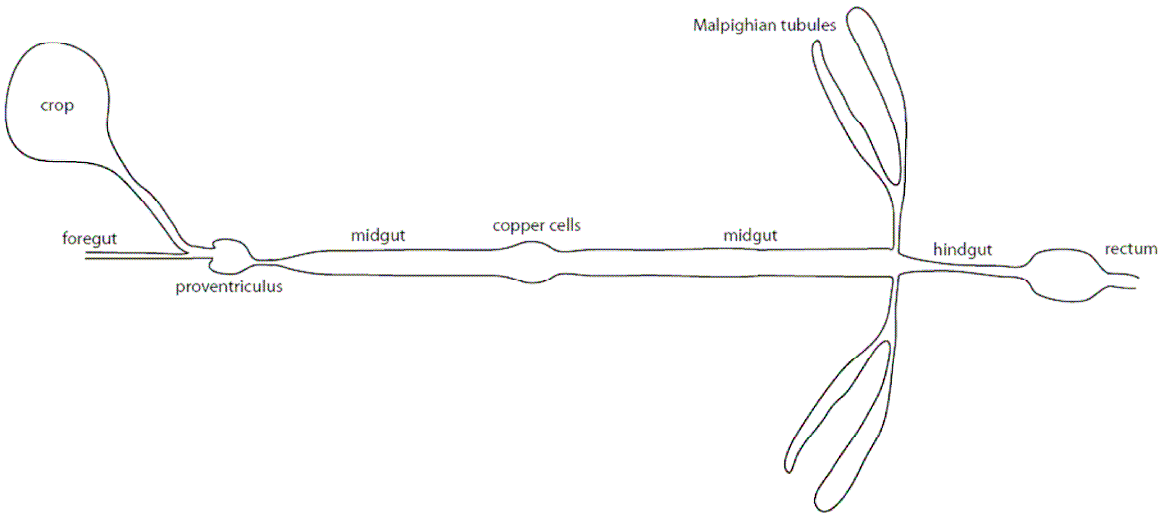

Figure 8: **Scheme of the Drosophila gut**.

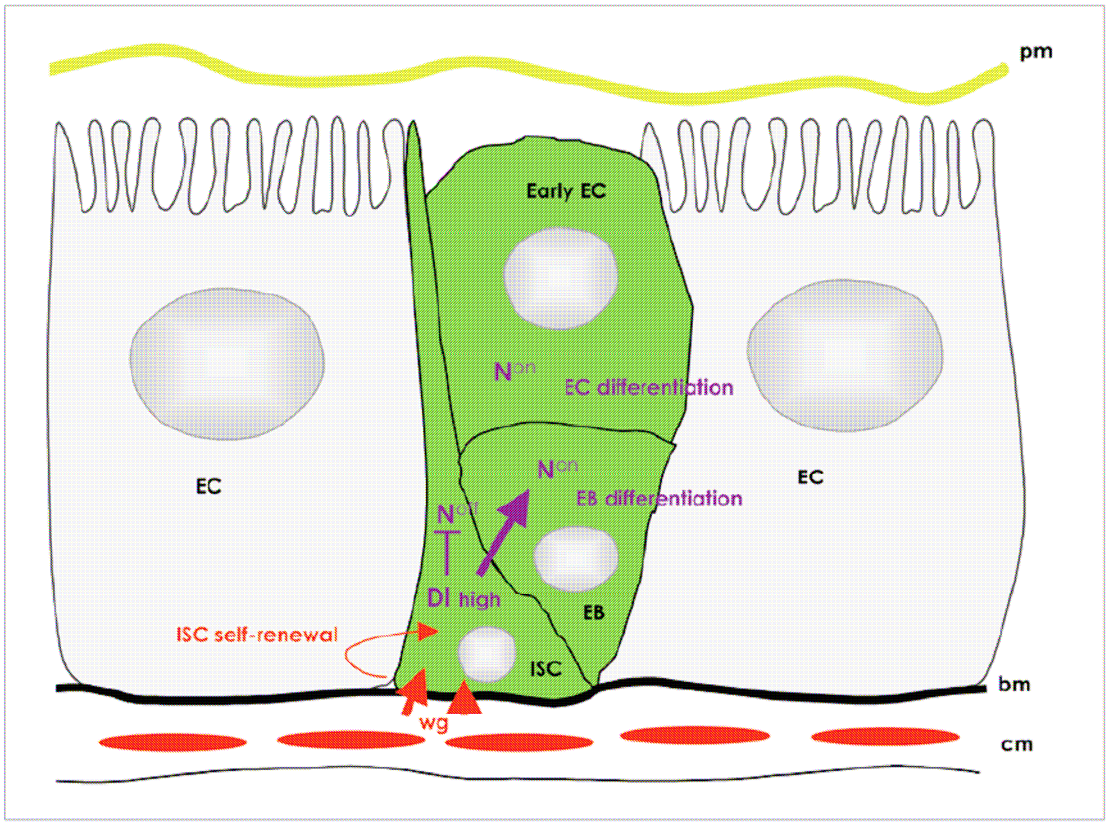

Figure 9: **Structure of the *Drosophila* midgut epithelium**. The basally located intestinal stem cells (ISCs) receive a wingless (wg) signal from the circular muscles that induces self-renewal and keeps their "stemness". ISCs contain high levels of the Notch (N) ligand Delta (Dl). After division, one cell keeps high Dl levels and remains a stem cell, while the other cell detaches from the basal membrane, loses Dl and becomes an enteroblast (Lin *et al.* 2010). EC: enterocyte, EB: enteroblast, cm: circular muscles, bm: basal membrane, pm: peritrophic matrix. From (Charroux *et al.* 2010).

AMP expression

In contrast to AMP gene expression in the fat body, AMP production in the gut is completely independent of Toll signaling. AMP gene expression is regulated by Relish and Caudal. The question that always comes up in intestinal immunity is: how is the epithelium able to mount an efficient immune response against invading pathogens, while tolerating commensal bacteria in the gut lumen? In absence of infection, gut commensals release low quantities of PGN, which activate IMD signaling via PGRP-LC (Figure 10). Interestingly, the nuclear localization of Relish is not sufficient to drive the expression of AMPs. Binding sites for the Caudal DNA-binding repressor present in the regulatory regions of IMD AMP target genes prevent their expression in the absence of infection to maintain the commensal microbiota (Ryu *et al.* 2008). Yet, not all IMD regulated genes are under Caudal transcriptional

control. The transcription of the effectors of IMD induced negative feedback loops, like catalytic PGRPs, USP36 and PIRK (see above), is not repressed by Caudal. Since these genes are only under Relish control, they are constantly expressed and diminish PGN-induced signaling. In contrast, in an infectious context, high levels of PGN are present. These high concentrations increase pathway induction to overcome the negative regulation of the IMD pathway and trigger AMP production (Figure 10). As this also leads to a higher expression of the negative regulators, the system can come back to normal homeostasis very fast. Even though Caudal expression is restricted to the posterior region, a local AMP response can be triggered throughout the whole midgut. This suggests that the intensity of IMD-dependent AMP production is differentially regulated in different parts of the midgut (reviewed in (Charroux *et al.* 2010), see also Annex).

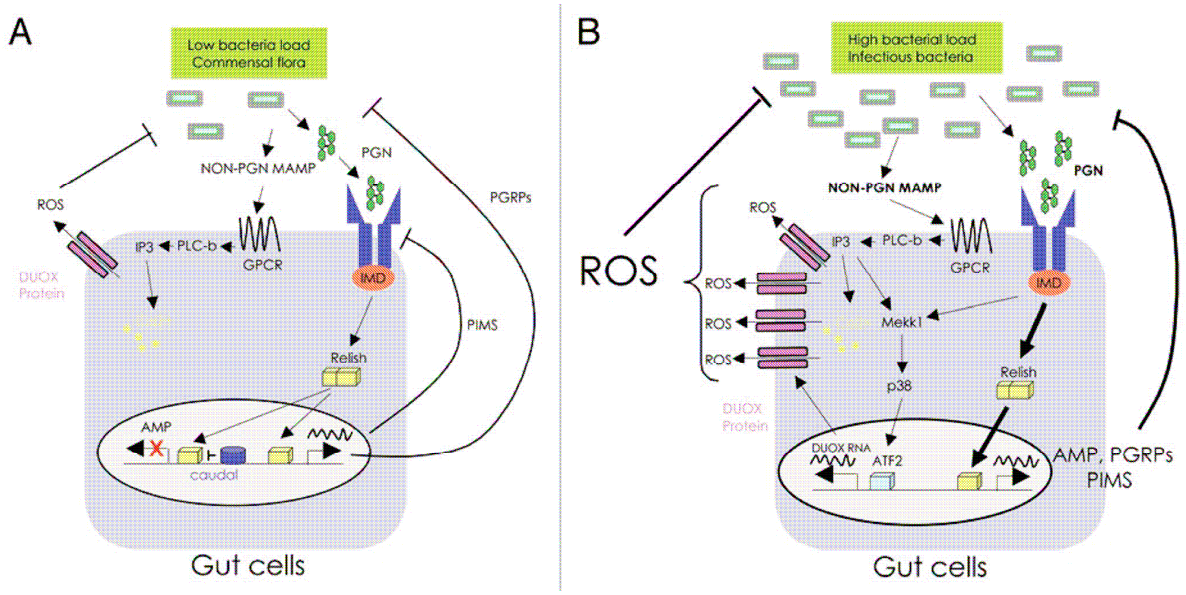

Figure 10: **Control of gut AMP and ROS production in the presence of commensal bacteria and increased microbial burden. A**: Caudal inhibits expression of IMD-dependent AMP gene transcription. **B**: In the presence of high microbial burden and therefore high concentrations of microbe associated molecular patterns (MAMPs), IMD signaling is strongly induced leading to high nuclear Relish levels that are proposed to be able to overcome Caudal repression and allow AMP gene transcription. In addition, DUOX gene transcription is upregulated by the PLC-β and the IMD pathway. High concentration of DUOX leads to a strong production of ROS required to fight infectious microbes. From (Charroux *et al.* 2010).

ROS production

Low concentrations of ROS are constantly produced in the gut epithelium by the membrane-associated dual oxidase (DUOX), a member of the NADPH oxidase

family (Ha *et al.* 2005b). The gut microbiota triggers phospholipase C-β (PLCβ)-dependent production of 1,4,5-triphosphate (IP3) and subsequent mobilization of intracellular calcium, which enhances DUOX activity (Ha *et al.* 2009a). Since this activation requires a functional Gα protein (Gαq) it has been suggested, that an unidentified G-protein coupled receptor (GPCR) acts upstream of the pathway (Figure 10). The bacterial component that triggers the pathway remains unknown, but it is clear that it is distinct from PGN. This basal activity of DUOX is essential to control gut commensal homeostasis (Ha *et al.* 2005a). In case of high bacterial burden, basal ROS levels are not sufficient. In this case DUOX gene transcription is induced in a PGN-dependent (IMD pathway) and a PGN-independent manner. The two upstream pathways merge into a MEKK1-MEK3-p38-ATF2 pathway that activates *Duox* gene transcription (Figure 10) (Ha *et al.* 2009b). The higher DUOX level leads to an elevated ROS production that contributes in controlling the microbial load in the gut lumen. Notably, recent reports suggest that ROS, and the tissue damage they cause, are implicated in maintaining gut homeostasis after infection by regulating intestinal stem cell proliferation (Biteau *et al.* 2008; Amcheslavsky *et al.* 2009; Buchon *et al.* 2009a; Buchon *et al.* 2009b)(see also Annex).

Cellular immune response

In the *Drosophila* embryo, hemocytes that are derived from the procephalic mesoderm colonize the whole organism and remove apoptotic cells (Tepass *et al.* 1994; Franc *et al.* 1996; Franc *et al.* 1999). In larvae, hemocytes are produced in the lymph gland, a mesodermally derived organ closely associated with the dorsal blood vessel. The embryonic hemocytes represent the major circulating hemocytes in larvae. The lymph gland derived hemocytes do not enter circulation before metamorphosis in the absence of an infection. Upon the onset of metamorphosis the lymph gland releases a high number of hemocytes that play a crucial role in tissue remodeling. These hemocytes persist to the adult stage along with embryonic ones (reviewed in (Lemaitre *et al.* 2007)).

Drosophila larvae contain three types of hemocytes: plasmatocytes, crystal cells and lamellocytes (Lanot *et al.* 2001). In a healthy larva, plasmatocytes represent the largest group of hemocytes (90-95%), while crystal cells are just 5% of total hemocytes and lamellocytes are hardly found. Lamellocyte differentiation can be triggered by an immune challenge. Lamellocytes function in encapsulation and

thereby neutralize foreign “objects” too big to be phagocytosed, such as parasitoid wasp eggs. Crystal cells are nonphagocytic hemocytes involved in the melanization process. Mature crystal cells contain large amounts of a hemocyanin-related oxidoreductase, the prophenoloxidase (proPO), in crystallized form. They function as storage cells that upon activation disrupt and release their content into the hemolymph (reviewed in (Lemaitre *et al.* 2007)). Adult hemocytes have phagocytic properties (Elrod-Erickson *et al.* 2000). In adults, no hemocyte proliferation or differentiation into specialized cells, such as lamellocytes, has been reported. Therefore, the adult hemocyte population (1000-2000 cells/animal) is embryonic and larval-derived, and appears to be uniform (plasmatocytes) (Lanot *et al.* 2001; Holz *et al.* 2003).

Phagocytosis

Plasmatocytes are responsible for the recycling of apoptotic cells and killing invading microorganisms. They are able to phagocytose a variety of particles, from double-stranded RNA and ink particles to bacteria and yeasts. The phagocytic cell attaches to the particle, internalizes it through cytoskeleton modification, and, if possible, destroys it in the phagosome. Attachment to various particles involves a range of different receptors. These include the Drosophila homolog of Ced-1, Draper, the Nimrod proteins, and the EGF-domain protein Eater. An immune role has also been proposed for the IgSF-domain protein Dscam (Down syndrome cell adhesion molecule). *Drosophila* immune-competent cells are thought to have the potential to express more than 18,000 isoforms of Dscam (Pearson *et al.* 1995; Franc *et al.* 1996; Ramet *et al.* 2001; Manaka *et al.* 2004; Kocks *et al.* 2005; Watson *et al.* 2005; Garver *et al.* 2006; Ju *et al.* 2006; Kurucz *et al.* 2007).

Opsonins seem to play a role in helping plasmatocytes. Proteins like the TEP proteins (see above) and possibly secreted isoforms of Dscam are thought to bind foreign particles and thereby promote their phagocytosis.

Encapsulation

Encapsulation is a lamellocyte-mediated defense reaction against invading parasites in *Drosophila* larvae. Larvae encapsulate foreign structures, like wasp eggs or oil droplets that are detected by plasmatocytes (Russo *et al.* 1996). In the case of a wasp egg, the plasmatocytes bind to its chorion and induce a rapid and strong reaction in the lymph gland and in the sessile subepidermal hemocytes. This reaction

consists also of the fast differentiation of sessile subepidermal hemocytes into lamellocytes that encapsulate the egg at the first place (Markus *et al.* 2009). These lamellocytes are later accompanied by those derived from a strong proliferation of prohemocytes and massive differentiation in the lymph gland (Jung *et al.* 2005; Markus *et al.* 2009). They form a multilayered capsule around the invader, which is accompanied by a blackening of the capsule due to melanization (see below). The parasite inside the capsule may be eventually killed, likely by the local production of cytotoxic products such as ROS and intermediates of the melanization cascade (Nappi *et al.* 1995; Nappi *et al.* 2009). The molecular processes leading to encapsulation and parasite killing are virtually unknown (reviewed in (Lemaitre *et al.* 2007)).

Coagulation

Clotting is very important upon injury, to limit hemolymph loss and initiate wound healing. In addition it is crucial to rapidly form a barrier against infection by closing the wound, immobilizing bacteria and promoting their killing (Wang *et al.* 2010). In injured *Drosophila* larvae a clot is rapidly generated at the wounding site (Figure 11). This clot is composed of fibers, whose major component is hemolectin (Goto *et al.* 2003; Scherfer *et al.* 2004; Lesch *et al.* 2007), that trap hemocytes, and importantly, invading microorganisms. Subsequent steps in wound closure include melanization and tissue movements (Ramet *et al.* 2002a; Galko *et al.* 2004). Cross-linking of the fibers during clot formation is mediated by proteins such as transglutaminase and proPO (Karlsson *et al.* 2004; Scherfer *et al.* 2004; Scherfer *et al.* 2006; Lindgren *et al.* 2008; Wang *et al.* 2010).

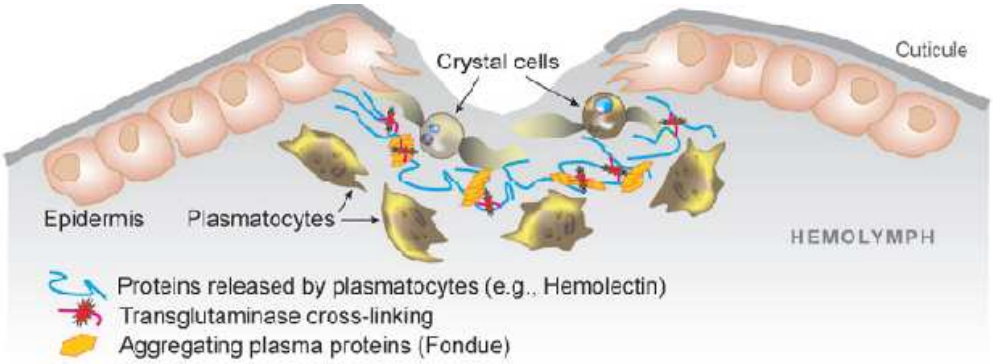

Figure 11: **Clot formation at a wounding site in larvae.** Upon injury, plasmatocytes release hemolectin and other proteins such as Fondue that form fibers that are subsequently cross-linked by proteins like Transglutaminase and proPO. ProPO is released by crystal cells. Hemocytes as well as bacteria or other foreign particles get trapped in the clot. From (Lemaitre *et al.* 2007).

Melanization

Melanization is involved in wound closure as well as in capsule formation. It consists in the *de novo* synthesis and deposition of melanin. It requires the activation of proPO. ProPO is enzymatically cleaved into the active phenoloxidase (PO) by Prophenoloxidase activating enzyme (PPAE). PPAE itself needs to be activated by an upstream serine protease cascade, which itself is activated by PRRs, such as GNBPs and PGRP-LE. Active PO catalyzes the oxidation of mono- and diphenols to orthoquinones, which polymerize nonenzymatically to melanin. In larvae, proPO stored in crystal cells is released and activated upon wounding or infection. The source of PO in adult flies remains unknown (reviewed in (Lemaitre *et al.* 2007)).

Other immune functions of hemocyte

Hemocytes are important storage cells. Larval crystal cells contain large amounts of crystallized proPO, needed for melanization. Plasmatocytes express immune molecules such as the clotting factor hemolectin or the Toll ligand SPZ (Goto *et al.* 2003; Irving *et al.* 2005; Shia *et al.* 2009). In addition, circulating plasmatocytes express many components of the extracellular matrix (Collagen IV, Peroxidasin, etc.) and may contribute to the formation of basal membranes (Fessler *et al.* 1994).

Septic injury has been shown to trigger IMD-dependent AMP expression in a subset of circulating hemocytes (Reichhart *et al.* 1992). Hemocytes are also believed to play an important role in signaling between immune responsive tissues. They are thought to signal to other tissues via the production of cytokines, such as UPD3 and SPZ (Agaisse *et al.* 2003; Charroux *et al.* 2009; Shia *et al.* 2009).

In addition, hemocytes have been reported to have an important function as surveillance system for damaged tissue. Hemocytes recognize sites of physical wounding as well as tumors and either engulf damaged cells or mount an immune reaction against the damaged tissue (Babcock *et al.* 2008; Pastor-Pareja *et al.* 2008).

After having introduced the different arms of the *Drosophila* immune response, I am in the following going to turn towards the bacterial pathogens that have been used for the study of *Drosophila* host-pathogen interaction.

1.3 *Serratia marcescens*

The bacterium

S. marcescens is a Gram(-) rod that belongs to the family of *Enterobacteriacae*. The bacteria are peritrichous flagellated and therefore motile, facultative anaerobe and chemoorganotrophic with both a respiratory and a fermentative type of metabolism. They are usually found in water, soil, and on plant and animal surfaces (Van Houdt *et al.* 2007). Many strains of *Serratia* are capable of producing prodigiosin, a red pigment, which led to some “miracles” in the Middle Ages, since consecrated wafers or other food happened to start “bleeding” due to *Serratia* growth. *Serratia* strains are commonly associated with raw food materials and cause spoilage of various foods. In 2004, *Serratia* contamination caused a shortage in flu vaccine in the US (Erickson *et al.* 2004). In addition, *Serratia* is capable of colonizing a wide variety of surfaces as the eye and the digestive tract of rodents, insects, fish and humans, and is a health hazard as it is an opportunistic pathogen (Grimont *et al.* 1978). *S. marcescens* is an important nosocomial pathogen that can cause pneumonia, intravenous catheter-associated infections, urinary tract infections, ocular and skin infections, osteomyelitis and endocarditis. Its pathogenicity is often exacerbated by multiple-antibiotic resistance (Arakawa *et al.* 2000; Knowles *et al.* 2000; Traub 2000). Its drug resistance is mediated by the production of β-lactamases and active multi drug efflux pumps. Swimming and swarming motility as well as extracellular enzymes, such as nuclease, proteases, lipase and hemolysin, contribute to its pathogenicity (Hejazi *et al.* 1997).

Serratia strains are also capable of quorum sensing, a way of communication and sensing bacterial density. The bacteria secret small molecules, in the case of *Serratia* N-acetyl-homoserine lactones and autoinducer 2, which they are also able to sense. This cell-cell-communication relies on the principle that when the bacterial density is low the extracellular concentration of these small molecules stays below a certain threshold. But, when the cell density reaches a critical level, this threshold concentration is reached and allows the bacteria to sense and respond to these signaling molecules. This results in a change of the transcription profile of the bacteria. Swarming motility, hemolytic activity, biofilm formation, and production of extracellular enzymes have been reported to be regulated in a quorum sensing-

dependent manner. Therefore quorum sensing might be crucial for virulence (reviewed in (Van Houdt *et al.* 2007)).

An important virulence factor of *S. marcescens* is the hemolysin it produces. The hemolysin activity has for a long time been overseen on blood agar plates because this hemolysin is a high molecular weight protein with a short half-life, which therefore is not diffusing far and leads to small zones of lysis around the colonies (Braun *et al.* 1987). This effect is even strengthened by the rapid aggregation and subsequent inactivation of the enzyme after release form the bacteria (Schiebel *et al.* 1989), and by degradation through exoproteases. In addition, hemolysin production is only switched on strongly under iron-limiting growth conditions, which is not the case on blood agar plates (Poole *et al.* 1988).

Hemolysins are amphiphilic proteins since they need to be hydrophilic to be soluble when secreted, and lipophilic to insert themselves into the plasma membrane of eukaryotic cells. The *S. marcescens* hemolysin ShlA is distinct from *E. coli* type α-hemolysins or the “thiol-activated” cholesterol binding hemolysins. It requires a protein for secretion through the outer membrane, the secretory protein ShlB. This secretion is distinct from type I–IV secretion systems and has been named two partner secretion system (TPSS or type V, (Jacob-Dubuisson *et al.* 2001)). ShlA and ShlB are secreted through the cytoplasmic membrane by the Sec-system. ShlB then integrates into the outer membrane, and activates and secretes ShlA (Figure 12). In addition to ShlB, ShlA binding to several molecules of phosphatidylethanolamine, the major component of the *S. marcescens* outer membrane, is essential for its activation (Hertle *et al.* 1997). When secreted and activated, ShlA integrates, possibly with the help of bound phosphatidylethanolamine molecules, into eukaryotic plasma membranes and forms pores (Hertle *et al.* 1997). These pores are formed by monomers or dimers of ShlA, which when inserted are able to form large oligomers. ShlA is not forming pores in prokaryotic membranes. Membrane binding and pore formation are highly dependent on phosphatidylserine, which targets the toxic activity to eukaryotic membranes since this component is almost absent from prokaryotic membranes (Hertle 2002).

ShlA plays an important role in pathogenicity. This hemolysin is also a cytolysin, which damages tissues and may contribute to invasion of host cells. Pores formed by ShlA lead to ATP depletion of host cells and induce vacuolation. However, the pathogenicity of *S. marcescens* is not limited to hemolysin and depends also on

multiple virulence factors. Hemolysin, fimbriae, proteases, lipase, nuclease and factors that facilitate invasion act in concert. Resulting effects are bacterial adherence, actin-independent invasion, cytotoxicity, and finally cytolysis (reviewed in (Hertle 2005)).

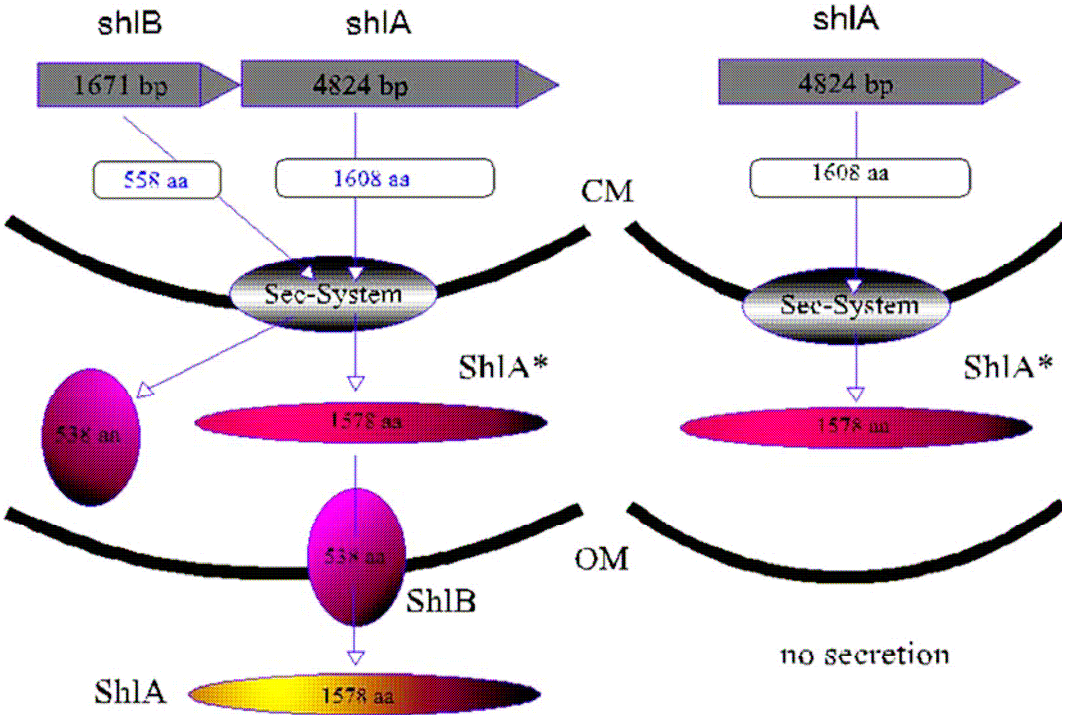

Figure 12: **Secretion of *Serratia* hemolysin.** ShlA and ShlB are secreted into the periplasmic space by the Sec-system. ShlB thereafter integrates into the outer membrane (OM) and secretes and activates ShlA. In the absence of ShlB nonhemolytic ShlA* remains in the periplasm. From (Hertle 2005).

In our laboratory the nonpigmented *S. marcescens* strain Db11 is used. Db11 is a spontaneous streptomycin resistant mutant of the strain Db10 that was isolated from moribund flies (Flyg *et al.* 1980). The genome of *S. marcescens* Db11 has been sequenced by the Sanger Institute (Hinxton, UK) in collaboration with the laboratory of Jonathan Ewbank (Marseille, F). The genome data is by now unpublished, but yet available under:
http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html.

S. marcescens infection in *Drosophila*

S. marcescens is a natural pathogen of *Drosophila*. The inoculation of as little as one bacterium into the body cavity of the fly by a septic injury kills it within 24h. Once introduced in the body cavity of the fly, the bacteria proliferate strongly and the fly succumbs to septicaemia. *S. marcescens* triggers an IMD-dependent systemic

immune response, but seems to be resistant to the action of subsequently produced AMPs, since it is killing wild-type and IMD mutant flies at the same rate (Nehme *et al.* 2007). It has already been shown decades ago that *S. marcescens* is very resistant to the action of Cecropin (Samakovlis *et al.* 1990) and is actually resistant to high concentrations of all AMPs that have been tested in the laboratory (P. Bulet, C. Hetru, personal communication). Nadine Nehme has established an oral infection model using *S. marcescens* in our laboratory. In this model flies are constantly fed on a bacteria-containing sucrose solution. The flies succumb to the infection within 6 days. Flies mutant for the IMD pathway and flies in which phagocytosis is blocked die more rapidly than wild-type flies, thus indicating a role of the IMD pathway and phagocytosis in host defense against ingested Db11. Phagocytosis of *S. marcescens* is, at least partially, mediated by the phagocytic receptor Eater, since *eater* mutant flies are less efficient in phagocytosing *S. marcescens* than wild-type flies (Kocks *et al.* 2005). When fed to the fly the bacteria are capable of overcoming the gut epithelial barrier and reach the hemolymph within 2 hours. Although found in the hemolymph very early during infection, the bacteria do not kill as rapidly as in the septic injury model. This suggests that the bacterial virulence program is downregulated after passage through the gut epithelium (Nehme *et al.* 2007). Even though *S. marcescens* is triggering an IMD-dependent local immune response in the gut, it does not trigger a systemic immune response after passage through the midgut epithelium. Nehme *et al.* suggested that wild-type flies are dying from gut damage and not from septicemia.

1.4 *Pseudomonas aeruginosa*

The bacterium

P. aeruginosa is a Gram(-) opportunistic human pathogen of the family *Pseudomonadaceae* that can be found in soil and water. It is able to infect plants, insects, nematodes, and mammals. *P. aeruginosa* strains are a major threat to weakened and especially to immunocompromised people. Patients who suffer from bronchiectasis or cystic fibrosis (CF) are predisposed for *P. aeruginosa* respiratory tract infections. Most serious infections are seen in health-care settings and comprise bacteraemia, pneumonia, urosepsis, and wound infections, including secondary infections of burn wounds. Infection often follows surgical incisions and catheter

insertion (reviewed in (Kerr *et al.* 2009)). These infections, as well as infection of patients suffering from cystic fibrosis, are facilitated by the ability of the bacteria to form biofilms. In the US, *P. aeruginosa* was the sixth most frequent occurring pathogen, the second commonest cause of ventilator-associated pneumonia and the seventh most common reason for catheter-related blood stream infection in 2006 and 2007 (Hidron *et al.* 2008). A major challenge in curing *P. aeruginosa* infections is the intrinsic resistance to multiple classes of antibiotics of the bacterium. The natural resistance, together with acquired or mutational resistances, is strongly limiting therapeutic options. Amongst others, multi-drug efflux pumps, aminoglycoside-modifying enzymes, and β-lactamases are responsible for these resistances. 18% of *P. aeruginosa* isolates have been reported to be multi-drug resistant, and pandrug-resistant strains occur more and more often (reviewed in (Kerr *et al.* 2009)). Overall, *P. aeruginosa* infections can be considered as a growing problem in health-care facilities.

P. aeruginosa possesses a large arsenal of virulence factors. Amongst them, proteases, elastases and phenazine pigments, such as pyocyanin, and rhamnolipids. The bacteria have several different export systems to secrete virulence factors, including two-step secretion systems like the type II secretion system (reviewed in (Filloux 2004)) and one step secretion systems like the type III secretion system. The type III secretion system, which allows injecting effector molecules directly into the cytoplasm of the host cells, is of particular importance (Engel *et al.* 2009). Its effector proteins are: ExoS, ExoT, ExoU and ExoY. ExoS and ExoT are capable of inhibiting phagocytosis by disrupting actin cytoskeletal rearrangements, focal adhesion and important signal transduction cascades (Barbieri *et al.* 2004). ExoU and ExoY are cytotoxins with phospholipase and adenylate cyclase activities respectively (Yahr *et al.* 1998; Sato *et al.* 2004). *P. aeruginosa* strains express different combinations of these proteins, that have profound and often devastating effects on epithelial barrier function and wound healing (Kerr *et al.* 2009). Growth of the bacteria in environmental niches, such as pipes and taps, is facilitated by its ability to form biofilms (Tart *et al.* 2008). When growing in a biofilm attached to a surface, *P. aeruginosa* is significantly more resistant to biocides than when in the planktonic (free-floating) state (Smith *et al.* 2008). This has important consequences for hospital cleaning procedures. The formation of biofilms can also take place in the host, and is

associated with pathogenesis in patients for example those suffering from cystic fibrosis.

Biofilm formation as well as production of virulence factors is regulated by quorum sensing (QS). QS regulation is very complex and influences, positively and negatively, the transcription of 5-10% of the *P. aeruginosa* genes (Hentzer *et al.* 2003; Schuster *et al.* 2003; Wagner *et al.* 2003). There are three QS systems known in *P. aeruginosa*: the *las* and *rhl* systems, using N-acylhomoserine lactones as signaling molecules, and the *pqs* system, that is dependent on quinolones. The *las* and *rhl* systems are conventional systems: LasI and RhlI produce the N-acylhomoserine lactone (N-acyl-HSL) signaling molecules 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and butanoyl-homoserine lactone (C_4-HSL) respectively. These signaling molecules induce their associated transcriptional regulators LasR and RhlR, which activate numerous QS-controlled genes. Amongst the target genes are the genes coding for LasI and RhlI. Therefore, N-acyl-HSLs are called autoinducers since they induce their own production and thereby boost the QS signal (Pesci *et al.* 1997; Juhas *et al.* 2005) (Figure 13). The *pqs* system depends on the signaling molecule 2-heptyl-3-hydroxy-4-quinolone that was termed Pseudomonas Quinolone Signal (PQS). PQS binds to the transcriptional regulator MvfR (also known as PqsR) that in turn induces target gene expression. The biosynthesis of PQS requires several enzymes encoded in the *pqs* operon. Therefore PQS bound to MvfR triggers its own production, as do the signal molecules of the other QS systems.

The *las* and the *rhl* systems are organized in a hierarchical manner such that the *las* system exerts transcriptional control over both *rhlR* and *rhlI* (Latifi *et al.* 1996). The quinolone system is modulated by both the *las* and the *rhl* systems (Dubern *et al.* 2008) (Figure 13), while the *pqs* system itself positively influences the *rhl* system (Cao *et al.* 2001). Since the *las* system activates the *rhl* system, it has been commonly thought, until recently, that mutations in *lasR* will block the *las* and the *rhl* systems, even though several groups had found previously that pyocyanin and other *rhl* dependent effectors, are expressed in *lasR* mutant strains in the stationary phase of bacterial growth. Lately, Dekimpe *et al.* were able to show that, most likely, basal *rhlR* and *RhlI* transcription as well as environmental conditions lead to an autoinduction of the *rhl* system in a *lasR* mutant background. In addition, they showed that many genes that were thought to be strictly *las* dependent are also

regulated by the *rhl* system, including *lasI* and *pqsH*. Therefore, the *rhl* system is not absolutely dependent on LasR; its activation is just delayed in *lasR* mutants (Dekimpe *et al.* 2009) (Figure 13). On top of the QS systems, there is a complex regulatory network that controls the QS systems at the transcriptional and the posttranscriptional level. At least 18 QS regulators have been identified, even though the targets in the QS network are only known for less than half of them (Diggle *et al.* 2006; Venturi 2006; Williams *et al.* 2007; Liang *et al.* 2009; Siehnel *et al.* 2010).

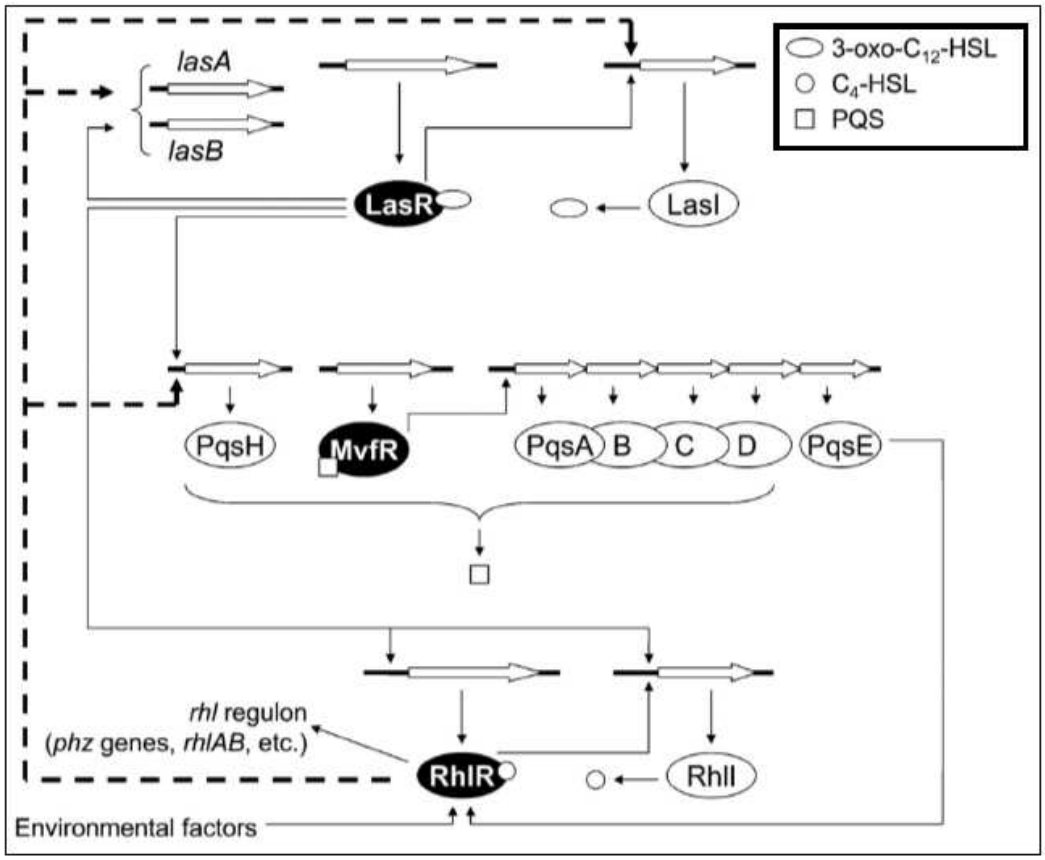

Figure 13: **Interactions between the different quorum sensing systems.** The Las system regulates the Rhl and the quinolone system. Effectors of the quinolone system are able to induce the Rhl system. The Rhl system in turn regulates at least partially the quinolone system and induces effectors of the Las system. Dashed arrows: interaction recently proposed by Dekimpe *et al.*; Figure modified from (Dekimpe *et al.* 2009).

P. aeruginosa is used in many laboratories to study host-pathogen interactions. The genome of *P. aeruginosa* has been sequenced (Stover *et al.* 2000) and genetic manipulation is easier than in *S. marcescens*. The wild-type strain we are using is PA14, which has been isolated from a burn wound patient. PA14 is a Rifampicin resistant strain that expresses *exoU*, *exoT*, and *exoY*, but does not seem to express *exoS*. A nonredundant transposon insertion library has been created in

the PA14 background in the laboratory of Frederick Ausubel (Liberati *et al.* 2006), to which we have access. This library combined with all the mutations available in *Drosophila* gives us the possibility to study host-pathogen interactions from both, the host and the pathogen side at the same time.

P. aeruginosa infections in *Drosophila*

Since it is a major human pathogen, *P. aeruginosa* is a well-studied microorganism. Whereas the basic biology of the bacterium has been studied intensively, *P. aeruginosa* infections in many model organisms, including *Drosophila*, are also a focus of active research. To infect flies with *P. aeruginosa*, as for *S. marcescens* infections, two basically different infection models have been used, the septic injury model and the oral infection model. Variations (see Annex) of both models have been used widely. Systemic infection with *P. aeruginosa* induces both the IMD and the Toll pathway (Lau *et al.* 2003).

Highly virulent strains of *P. aeruginosa* have been suggested to suppress NF-κB signaling, thereby downregulating the humoral immune response (Apidianakis *et al.* 2005; Kravchenko *et al.* 2008). In cell culture, this repression seems to be dependent on 3-oxo-C_{12}-HSL (Kravchenko *et al.* 2008). Quorum sensing in general seems to be important for *P. aeruginosa* virulence. The expression of several virulence factors, *e.g.* pyocyanin, rhamnolipids, and proteases, has been shown to be QS dependent (reviewed in (Dekimpe *et al.* 2009; Williams *et al.* 2009)). Another group of important virulence factors is injected directly into host cells via the T3SS: ExoS, ExoT, ExoU and ExoY. As mentioned above these virulence factors can have devastating effects on epithelial barrier function and wound healing (Kerr *et al.* 2009). Several other mechanisms and factors have been found to be important for *P. aeruginosa* virulence. For an overview of the recent advances in understanding *P. aeruginosa-Drosophila* host-pathogen interactions, please, refer to the Annex.

1.5 Aim of this work

The major aim of my PhD was to gain a better understanding of host-pathogen interactions in *Drosophila* oral infection with bacteria. Nadine Nehme previously established an oral infection model using *Serratia marcescens* as pathogen. In the first part of this study I describe the use of this model system to perform a genome-wide RNAi screen for genes implicated in resistance or susceptibility of the fly to oral

infection by *S. marcescens*. By using inducible drivers (ubiquitous and tissue-specific (intestine, hemocytes)) to express the different RNAi-hairpin constructs it was possible to circumvent developmental lethality and thereby to investigate the role of essential as well as non-essential genes for infection. Several hundred genes that seem to be implicated in the infectious process have been found. I joined the laboratory while the screen was running (performed by Shane Cronin and Nadine Nehme) and was implicated in the characterization of the intestine-specific genes found in the screen.

Several members of the JAK/STAT pathway were found to be important for response to infection. The tissue-specific screen revealed that those genes are needed in the intestinal epithelium during infection. Samuel Liégeois and I established that the JAK/STAT pathway is implicated in triggering compensatory proliferation in the midgut epithelium to counteract bacteria-induced tissue damage.

While further investigating the gut phenotype of infected flies, Samuel Liégeois, Richard Bou Aoun and I found that the gut epithelium is severely damaged by ingested *S. marcescens* within six hours of infection. Surprisingly, the intestinal epithelium of the fly is able to repair this damage within the following 6-18 hours, which means that the gut epithelium is repaired at an amazing speed. This unexpected finding is described in the second half of the *S. marcescens* part.

To further decipher host-pathogen interaction in the oral infection model it would be helpful to not just be able to genetically manipulate the host, but also the pathogen. Unfortunately *S. marcescens* genetics are not very advanced. Therefore we decided to work with another opportunistic pathogen, *Pseudomonas aeruginosa*. As described in the second part of this manuscript, I established and characterized a *P. aeruginosa* oral infection model. Resistance to *P. aeruginosa* infection is depending on all major defense mechanisms of the fly, the humoral immune response mediated by both the IMD and the Toll pathway and the cellular response. Flies deficient for one of these arms of the immune system are susceptible to the infection. Thanks to the possibility of manipulating both the host and the pathogen, my Master student Samantha Haller and I were further able to demonstrate that the *P. aeruginosa* quorum sensing regulator RhlR is needed to overcome the cellular immune response of the fly during infection.

2 *Serratia marcescens* infections

2.1 Genome-Wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection

Introduction

In the last decades forward genetic screens have been used to identify genes implicated in diverse biological processes. Many genes implicated in *Drosophila* immunity have been found in screens using chemical mutagenesis (Reichhart *et al.* 1992; Jung *et al.* 2001) or transposon mutant libraries (Gottar *et al.* 2002; Gobert *et al.* 2003; Gottar *et al.* 2006). The generation of random mutations using chemicals like ethylmethanesulfonate (EMS) is easy. The problem of this method lies in the identification of the mutated genes, once mutants have been isolated in a phenotypic screen. In this perspective, transposon insertion mutations are much easier to work with, since the molecular tag provided by the transposon allows rapid cloning of the mutated gene. Chemical and transposon insertion mutagenesis are approaches that are very useful to find genes implicated in development. However, genetic screening at the adult stage raises another problem that is less limiting in the case of developmental genes: all genes essential for development cannot be tested for their function in processes taking place in the adult as the corresponding mutants never reach the adult stage. In *Drosophila*, around one third of the genes are essential genes.

To find new genes implicated in oral infection of *Drosophila* with *S. marcescens*, we therefore used another technique. In collaboration with the Penninger laboratory (Vienna), we were able to have access to the transgenic *Drosophila* collection developed by Barry Dickson (Dietzl *et al.* 2007). These fly lines carry a transgenic RNA interference hairpin construct targeting a specific gene of interest. RNA interference (RNAi) (Kennerdell *et al.* 2000) depends on the presence of dsRNA. This dsRNA is encoded by the transgene, which contains a fragment of the gene of interest cloned as an inverted repeat, leading to the transcription of a RNA that forms a hairpin as secondary structure (Lam *et al.* 2000). The dsRNA is processed into small interfering RNAs (siRNAs) by a ribonuclease (RNAse) III enzyme called Dicer. These siRNAs are subsequently loaded into the RNA-induced silencing complex (RISC), which then targets and cleaves mRNAs with sequences

homologous to the siRNA (Hannon 2002). The RNAi pathway is cell autonomous in *Drosophila* (Van Roessel *et al.* 2002). In the transgenic flies, the RNA hairpin sequence is under the control of the Gal4-responsive UAS promoter. This allows transgene expression in specific tissues using relevant Gal4 drivers (Brand *et al.* 1993). The UAS-Gal4 system can be combined with the expression of the thermosensitive repressor of Gal4, $Gal80^{ts}$. The $GAL80^{ts}$ molecule regulates GAL4 in a temperature dependent fashion with optimal repression observed at 19℃ and derepression at temperatures around 30℃. The RNAi technique combined with the UAS-Gal4 and $Gal80^{ts}$ system allows to control the timing of the expression of the RNAi hairpin transgene and thus bypasses developmental effects of gene silencing. A nonexclusive possibility is to use Gal4 drivers that are inducible (like heat-shock drivers) or just expressed in the adult.

This inducible RNAi system used to silence genes in the adult fly allowed us to perform a genome wide screen for resistance and susceptibility to oral *S. marcescens* infection. We used an inducible heat-shock (hsp)-driver combined with $Gal80^{ts}$ for the primary screen, that is an ubiquitous inactivation of each targeted gene that was triggered only when flies had reached adulthood by placing flies at the permissive temperature. Tissue specific drivers (intestine, hemocytes) were used for secondary screens, in combination with the $Gal80^{ts}$ system for some experiments.

Genome-Wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection

Genome-Wide RNAi Screen Identifies Genes Involved in Intestinal Pathogenic Bacterial Infection

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Innate immunity represents the first line of defense in animals. We report a genome-wide in vivo *Drosophila* RNA interference screen to uncover genes involved in susceptibility or resistance to intestinal infection with the bacterium *Serratia marcescens*. We first employed whole-organism gene suppression, followed by tissue-specific silencing in gut epithelium or hemocytes to identify several hundred genes involved in intestinal antibacterial immunity. Among the pathways identified, we showed that the JAK-STAT signaling pathway controls host defense in the gut by regulating stem cell proliferation and thus epithelial cell homeostasis. Therefore, we revealed multiple genes involved in antibacterial defense and the regulation of innate immunity.

Drosophila melanogaster provides a powerful model that allows the dissection of the innate immune response at the organism level. In *Drosophila*, innate immunity has a humoral and a cellular immune response. The majority of our knowledge of *Drosophila* immunity is based on injection of nonpathogenic bacteria (*1*–*3*); however, this bypasses the initial steps of naturally occurring infections—namely, the physical barriers and the local, mucosal immune response. Intestinal immunity is currently the focus of intense research (*4*). In contrast to the human digestive tract, *Drosophila* lacks mammalian-like adaptive immunity and so relies entirely upon an innate immune system for protection against invading pathogens.

The intestinal infection model using pathogenic *Serratia marcescens* allows for the detailed analysis of local intestinal immunity and phagocytosis (*5*). *S. marcescens* is a gram-negative, opportunistic pathogen that can infect a range of hosts including *Drosophila*, *Caenorhabditis elegans*, and mammals (*6*, *7*). Using ubiquitous RNA interference (RNAi)–mediated suppression, we performed an inducible genome-wide in vivo screen in *Drosophila* for novel innate immune regulators after *S. marcescens* infection (*8*) [Fig. 1A, fig. S1, A and B, and supporting online material (SOM) text]. To confirm our experimental approach we assayed various members of the Immune deficiency (IMD) and Toll pathways, the two major fly immune signaling cascades (Fig. 1B) (*1*–*3*). RNAi lines targeting several IMD members resulted in significantly reduced survival on infection with *S. marcescens*, whereas suppression of Toll pathway components had a less dramatic effect, which supports previous reports that the immune response to *S. marcescens* is IMD-dependent and Toll-independent (Fig. 1B) (*5*). Notably, not all members of the IMD pathway, such as *imd*, *rel*, and *ird5*, were picked up by our screening criteria, most likely because of inefficient RNAi silencing (Fig. 1B) (*9*).

We assayed 13,053 RNAi lines (*9*) representing 10,689 different genes (78% of the genome) against intestinal infection with *S. marcescens* (fig. S2A and tables S1 and S2). Of these, 8.3% (885 genes) were defined as hits, the majority of which (89.3%; 790 genes) were susceptible candidates (fig. S2A and table S3). On the basis of gene ontology (GO) annotations, susceptible candidates were classified according to their predicted biological processes. Genes involved in signaling, intracellular protein transport, and transcriptional regulation were overly represented among the entire data set (Fig. 1C). We also found marked enrichment for genes that regulate phagocytosis, defense responses, vesicle trafficking, and proteolysis. Several candidate RNAi lines represented genes that have been previously implicated in mounting an effective immune response (*10*–*19*) (table S3).

Our approach also allowed us to identify negative regulators of *Drosophila* host defense (Fig. 1A). We identified 95 genes (10.7% of the total hits) that confer resistance to *S. marcescens* infections when silenced (fig. S2, A and B, table S4), none of which had previously been characterized as negative regulators of innate immunity. Thus, our genome-wide screen revealed previously

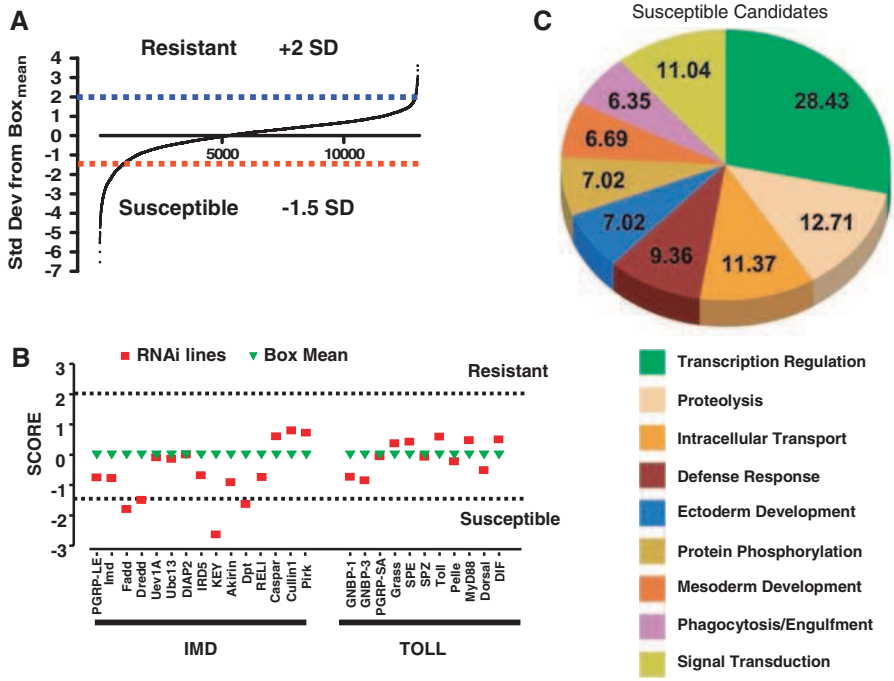

Fig. 1. Analysis of genome-wide in vivo RNAi screen. (**A**) Total data of all RNAi lines screened for survival after *S. marcescens* infections. Data were analyzed as the time, in days, when 50% of the total number of flies had died. All data were normalized to the daily median time-to-death (LT_{50}) mean of an experimental cohort. In all experiments, the cohort ranged from 80 to 200 lines. Hits were defined by susceptible (red dashed line) and resistant (blue dashed line) cut-offs, i.e., 1.5 SD below the mean and 2 SD above the mean, respectively, based on the pilot screen and controls. (**B**) Effect of RNAi knockdown of IMD and Toll pathway components on their survival against *S. marcescens* infection. SCOREs are shown for each line as described in (*8*). The dashed lines indicate the cut-offs used for resistance (+2 SD) and susceptibility (−1.5 SD) candidates. (**C**) Percentage distribution of GO annotated genes to biological processes for susceptible candidates.

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known genes associated with *Drosophila* immunity and more than 800 additional candidate genes implicated in innate immunity, 40% of which had unknown function.

We retested some of our susceptible and resistant RNAi hits in the gut epithelium and the macrophage-like hemocytes, the two major cell types associated with our infection model, using cell type–specific driver lines, NP1-GAL4 and HML-GAL4, respectively (*5*, *20*). We prioritized genes of interest by selecting the primary hits that have mammalian (mouse and/or human) orthologs. Of the 358 susceptible hits tested with the HML-GAL4 driver, RNAi against 98 genes (27%) resulted in significantly reduced survival as compared with RNAi controls, which indicated that these genes function in hemocytes to combat intestinal *S. marcescens* infections (Fig. 2A, fig. S3A, and table S5). When we used the NP1-GAL4 driver (fig. S4) to test 337 genes, RNAi against 129 genes (38%) resulted in significantly reduced survival, which suggested that these genes play an important role in host intestinal defense (Fig. 2B, fig. S3B, and table S6). Of the resistance hits, 37 HML-GAL4 RNAi candidates (79%) and 28 NP1-GAL4 RNAi candidates (61%) exhibited markedly enhanced survival (Fig. 2, C and D, fig. S3, and tables S7 to S9). Of the candidate genes, 79 functioned in both hemocytes and gut (fig. S3). Multiple susceptibility and resistance genes were tested 3 to 15 independent times, using ≥2 RNAi transformants to exclude position effects and second independent RNAi hairpins to confirm the target gene when available (Fig. 2, A to D, fig. S3, and tables S5 to S8). To exclude a potential developmental phenotype, we have tested most candidate lines by feeding flies on a sugar diet in the absence of bacteria (table S9). Thus, we have identified multiple regulators in hemocytes and/or gut epithelium that confer susceptibility or resistance to *S. marcescens* infections.

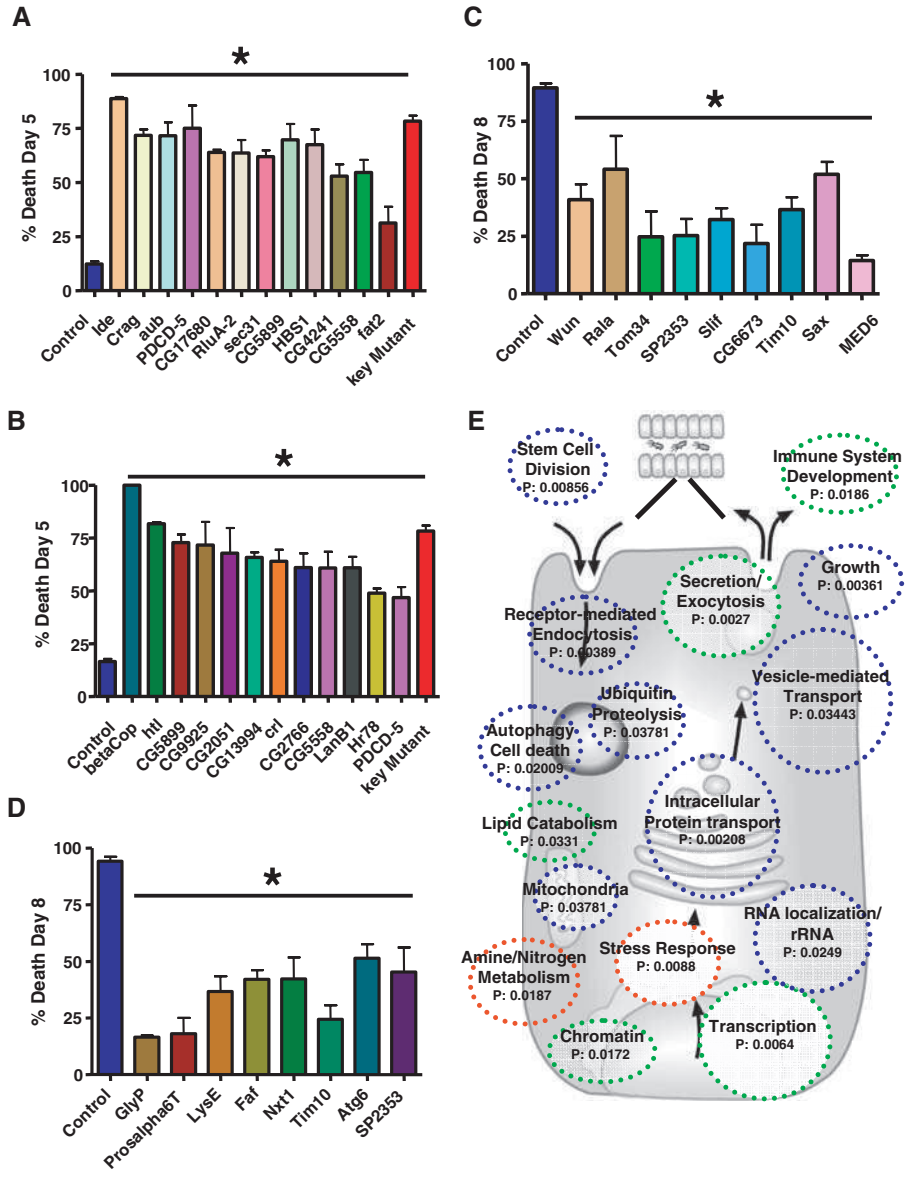


Fig. 2. Mapping and validation of conserved hits in the gut and hemocytes. (**A** and **B**) Survival graphs showing susceptible hits, (A) HML-susceptible genes and (B) NP1-susceptible genes, tested 3 to 15 times with several transformants and hairpins in hemocytes and gut epithelium, respectively. The *kenny* mutant line (key Mutant) is shown as a positive control. Means ± SEM, $n \geq 3$ experiments with 20 flies in each. *$P < 0.05$ (Welch *t* test). (**C** and **D**) Survival graphs showing resistant hits, (C) HML-resistant genes and (D) NP1-resistant genes, tested 3 to 15 times with several transformants and hairpins in hemocytes and gut epithelium, respectively. Means ± SEM, $n \geq 3$ experiments with 20 flies in each. *$P < 0.05$ (Welch *t* test). (**E**) Statistically enriched biological processes superimposed on a sketch depicting a gut epithelial cell, with the corresponding *P* value in the gut associated with *S. marcescens* infection. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates can be attributed. See also table S10 for annotation of genes involved in each process. All processes shown display $P < 0.05$ (Fisher's exact test).

Using GO enrichment analysis, we classified our tissue-specific candidates into statistically significant biological processes. In the intestinal tract, intracellular processes such as endocytosis and exocytosis, proteolysis, vesicle-mediated transport, and stress response all appeared significantly enriched (Fig. 2E, figs. S5 to S7, and table S10). We also observed a marked enhancement of genes associated with immune system development, growth, stem cell division, and cell death, which suggested an important role for these processes in the gut during *S. marcescens* infection. In hemocytes, ontology enrichment analysis revealed a strong enrichment in several processes linked to phagocytosis including endocytosis, response to external stimuli, and vesicle trafficking (figs. S8 to S10 and table S11). In both cell types, deregulation of the stress response, as well as amine and/or nitrogen metabolism, resulted in enhanced resistance to *S. marcescens* challenge (Fig. 2E and fig. S8).

We next performed Kegg pathway analysis to identify enriched gene sets that might be involved in *S. marcescens* infections. Kegg profiling on the susceptible genome-wide candidates (table S12) showed the importance of the IMD pathway in our infection model and also pointed to a possible role of Notch and transforming growth factor-β signaling pathways, which have previously been difficult to study in an infection setting because of a lack of adult viable mutants (*21*, *22*). Moreover, our analysis revealed prominent involvement of the Janus kinase–signal transducers and activators of transcription (JAK-STAT) pathway during *S. marcescens* infection. In *Drosophila*, the JAK-STAT pathway plays an important role in hematopoiesis, stress responses, stem cell proliferation, and antiviral immunity, but its role in the defense against natural bacterial pathogens is unknown (*23*–*26*). We therefore sought to validate our analysis and focused on how JAK-STAT signaling regulates the host response during *S. marcescens* infection.

To investigate whether the JAK-STAT pathway is activated during *S. marcescens* infection, we used transgenic reporter lines (*24*, *27*, *28*) in which green fluorescent protein (GFP) is expressed under the control of *unpaired* (*upd*) and *upd-3*, which encode two ligands for Domeless (the receptor of the JAK-STAT pathway). We observed *upd*-GFP and *upd3*-GFP expression in the gut of *S. marcescens*–infected flies (Fig. 3A and figs. S11 and S12). Moreover, we demonstrated intestinal activation of the JAK-STAT pathway by using a *stat92E*–binding site–GFP reporter line (Fig. 3B) (*27*, *28*). On ligation of UPD or UPD3 to Domeless, Stat92E translocates to the nucleus and activates reporter GFP gene expression (*27*). To confirm the relevance of JAK-STAT activation for *S. marcescens* infections, we performed global (Fig. 3, C and D) and gut-specific (Fig. 3E) RNAi-mediated silencing of PIAS [also called Su(var)-10] and PP1α96A, two negative regulators of JAK-STAT signaling (*29*, *30*). In both RNAi lines, we observed significantly earlier death compared with that of control flies (Fig. 3, C to E). The role of PP1α96A in intestinal immunity was also validated using a

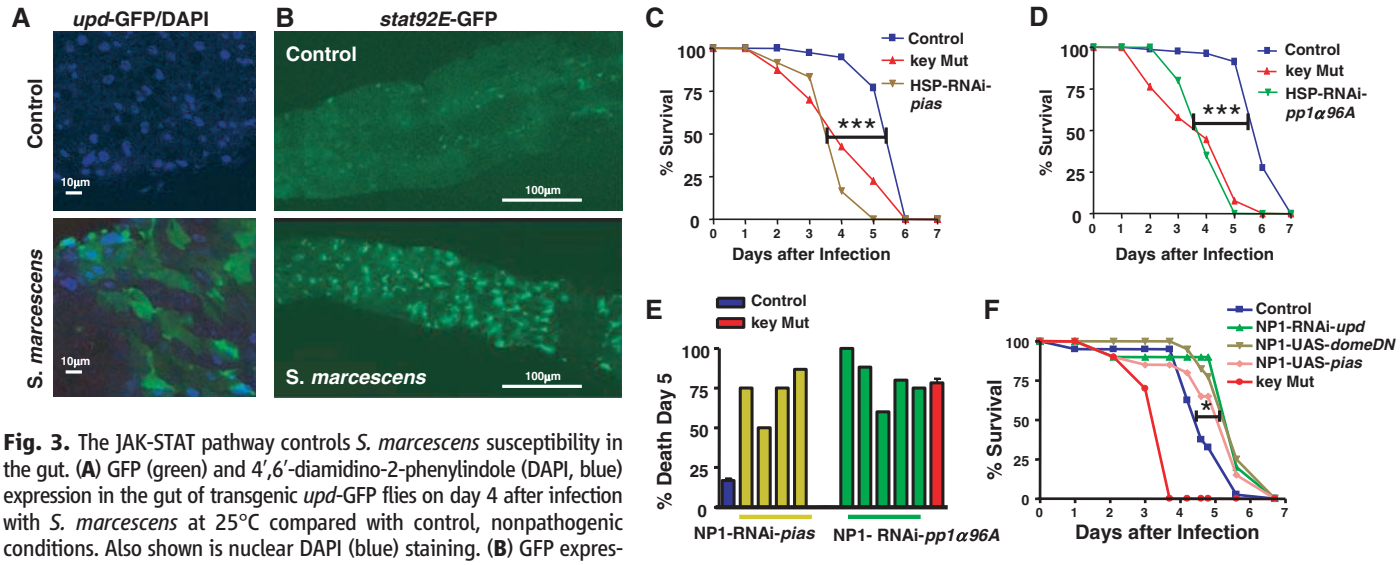
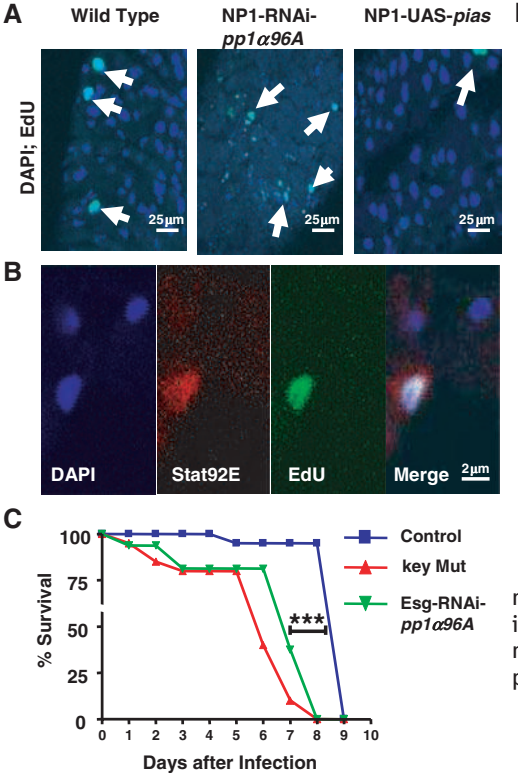


Fig. 3. The JAK-STAT pathway controls *S. marcescens* susceptibility in the gut. (**A**) GFP (green) and 4′,6′-diamidino-2-phenylindole (DAPI, blue) expression in the gut of transgenic *upd*-GFP flies on day 4 after infection with *S. marcescens* at 25°C compared with control, nonpathogenic conditions. Also shown is nuclear DAPI (blue) staining. (**B**) GFP expression in the gut of transgenic *stat92E*-GFP flies under *S. marcescens*–infected and control conditions on day 4 at 25°C. (**C**) Survival curves of *S. marcescens*–infected RNAi lines against the negative JAK-STAT pathway regulator PIAS driven by the ubiquitously expressed HSP-GAL4 driver compared with control and *key* mutant flies. (**D**) Survival curves of *S. marcescens*–infected RNAi lines targeting the negative JAK-STAT regulator PP1α96A driven by the ubiquitously expressed HSP-GAL4 driver compared with control and *key* mutant flies. (**E**) Survival graph representing individual tests of RNAi-mediated silencing of PIAS and PP1α96A specifically in the gut (NP1 driver) after *S. marcescens* challenge at 29°C, compared with control and *key* mutant flies. (**F**) Survival curves of lines shown at 29°C compared with control and *key* mutant flies following *S. marcescens* feeding. *$P \leq 0.05$; ***$P \leq 0.0001$ (logrank test). *upd*, *unpaired*; DN, dominant-negative.



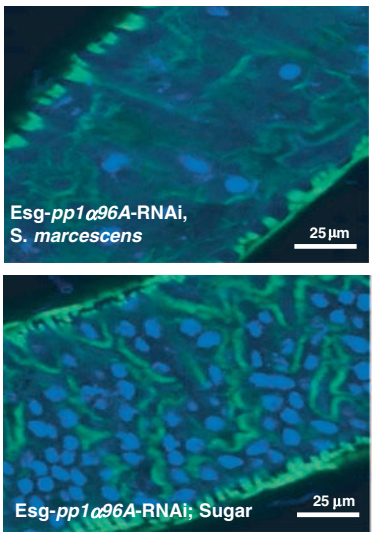


Fig. 4. Impaired epithelial integrity and control of intestinal stem cell homeostasis upon *S. marcescens* challenge. (**A**) Analysis of gut epithelium integrity using DAPI (blue) and intestinal proliferation using EdU (green) staining; EdU was injected into flies just 3 hours before dissections. Samples were assayed on day 5 after *S. marcescens* infection at 25°C. (**B**) Representative confocal image showing JAK-STAT pathway activation in EdU-positive nuclei in an intestinal stem cell using the *stat92E*-GFP reporter line. Data are from day 5 following *S. marcescens* challenge. In a total of three experiments and 39 gut dissections, we detected 13 cells with small nuclei (DAPI) that were positive both for 10xSTAT-GFP (red) and positive for EdU staining in the region anterior to the copper cells, although no such cells were observed in 42 noninfected control guts ($P < 0.003$, Student's *t* test). EdU was injected 3 hours before dissection. (**C**) Survival curves of *S. marcescens*–infected *Drosophila* in which PP1α96A is specifically silenced in intestinal stem cells of adult flies using Esg-GAL4;tubulinGal80^{ts} at 25°C. Control and *key* mutant lines are shown for comparison. ***$P < 0.0001$ (logrank test). (**D**) Analysis of gut epithelium integrity in Esg-*pp1a96A*-RNAi lines kept for 5 days after *S. marcescens* infections at 25°C (top) or under nonpathogenic conditions (bottom). Nuclei were visualized with DAPI, and actin was visualized with phalloidin (green).

sensitized background (fig. S12). In contrast, partial pathway inhibition via gut-specific overexpression of PIAS (NP1-UAS-*pias*), dominant-negative *domeless* (NP1-UAS-*domeDN*), or RNAi-mediated silencing of the *domeless* ligand, UPD (NP1-RNAi-*upd*) significantly increased the survival of *Serratia*-challenged flies (Fig. 3F). Thus, the JAK-STAT pathway activation in the gut negatively regulates survival in response to an intestinal *S. marcescens* infection.

To elucidate a possible mechanism in which JAK-STAT is involved in host defense against *S. marcescens*, we analyzed the effects of infection on gut epithelium. Infected flies exhibited massive death of intestinal epithelial cells (fig. S14A) and compensatory proliferation (fig. S14, B and C). Enhanced JAK-STAT signaling, through the use of NP1-RNAi-*pp1α96A* flies, resulted in a marked reduction in the number of large, polyploid nuclei, which signify differentiated enterocytes (*31*), after 5 days of infection (Fig. 4A). Epithelial morphology (fig. S15A) and survival on sucrose solution under nonpathogenic conditions (fig. S15B) were comparable for control, NP1-RNAi-*pp1α96A*, NP1-UAS-*pias*, and NP1-UAS-*domeDN* fly lines. We next assessed whether JAK-STAT signaling affected cellular proliferation of the epithelium. We found that DNA synthesis in epithelial cells was reduced when JAK-STAT signaling was impaired and significantly increased by silencing *pp1α96A* in the gut, both in the presence and absence of infection (Fig. 4A and fig. S16). Thus, JAK-STAT signaling enhances epithelial cell death and positively regulates compensatory proliferation of intestinal cells, also after *S. marcescens* infection.

We next examined whether the JAK-STAT pathway was affecting intestinal cell homeostasis specifically through the resident stem cell compartment (*32*). Basal intestinal stem cells (ISCs) can be distinguished from apical enterocytes on the basis of a characteristic smaller nuclear morphology (*31*, *33*). By using the *stat92E*-GFP reporter line to image JAK-STAT activation, the JAK-STAT pathway was selectively induced in the ISCs but not in mature enterocytes (fig. S17). Moreover, on infection of *stat92E*-GFP flies with *S. marcescens*, we observed GFP expression also in small, 5-ethynyl-2′-deoxyuridine (EdU)–positive cells, which suggests that JAK-STAT signaling regulates ISC proliferation during *S. marcescens* infection (Fig. 4B). To definitively demonstrate that this pathway acts in gut stem cells and that this compartment controls susceptibility to *S. marcescens* infections, we silenced *pp1α96A* in adult ISCs using an escargot-GAL4 driver line. Escargot is a specific marker of ISCs (*31*). ISC-specific suppression of PP1α96A resulted in early lethality in response to *S. marcescens* infection, whereas flies remained viable under nonpathogenic conditions (Fig. 4C and fig. S18). Furthermore, the guts of infected escargot-GAL4-*pp1α96A*-RNAi flies showed a phenotype similar to that obtained using the gut-specific NP1 driver, namely, severely depleted mature enterocytes (Fig. 4, A and D). Thus, our data demonstrate that JAK-STAT signaling is required for ISC homeostasis and implicates ISCs as a critical component of host defense to mucosal *S. marcescens* infections.

Our global experimental approach allows a comprehensive dissection of the biological processes that may regulate host defense against a bacterial infection at the organism level. Besides revealing previously known immune pathways, we uncovered more than 800 additional genes, many of which were of unknown function. Furthermore, our data demonstrate that host defense may involve many processes that are not limited to classical innate immune response pathways, as exemplified here by the role of the JAK-STAT pathway in the regulation of epithelial homeostasis in response to infection. In addition, we validate and map conserved candidates to intestinal cells and hemocytes, which allows us to propose a blueprint of the processes involved in host defense against *S. marcescens* infection. As all genes analyzed here are conserved during evolution, it is likely that some of the processes that are important in flies are also relevant to mammalian host defense (*34*, *35*).

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S19
Tables S1 to S12
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Supporting Online Material for

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Supporting Online Material

Materials and Methods

Bacterial strains and culture.

The non-pigmented *S. marcescens* Db10 strain was isolated almost three decades ago from a moribund fly (Db; *Drosophila* bacterium). Db11 is a spontaneous streptomycin-resistant mutant of Db10 (*3*). Strains were grown in LB (Luria Bertani) medium at 37°C and supplemented with 100 μg/ml ampicillin antibiotic.

Infection by feeding.

Batches of 20 adult flies were used for each RNAi line assayed. The food solution containing *S. marcescens* was prepared from the culture grown exponentially at 37°C to an optical density of OD (600nm) = 1. This culture was diluted with a sterile 0.05M sucrose solution to a final OD (600nm) = 0.1. Two absorbent filters (37mm; Millipore) were placed into fly culture tubes and soaked with 2ml of the *S. marcescens* sucrose solution. The flies were then transferred to these infectious vials. The flies were counted each day for 8 days. Unless otherwise stated, all experiments have been performed at 29°C.

Fly strains.

RNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC; https://stockcenter.vdrc.at) and all lines that we have tested are Wolbachia positive. Stocks were raised on standard cornmeal-agar medium at 25°C. The *kenny* (*key*) mutant (*2*), *UAS-Diptericin* (*2*), HSP70-GAL4 (*9*), NP1-GAL4 (*5, 6*), HML-GAL4 (*7, 8*), UAS-PIAS (*4*), UAS-DomeDN (*10*), *upd*-GFP (*11, 12*), *upd3*-GFP (*13*), *stat92E*-GFP (*11*) and escargot (Esg)-GAL4 (*14*) lines have all been previously described. We incorporated a tubulinGAL80^{ts} transgene onto the HSP70, NP1, HML, and Esg-GAL4 lines. The mutant PP1α96A line used for sensitization of RNAi-*pp1α96A* is: y[1]w[67c23];P{w[+mC]y[+mDint2]=EPgy2}Pp1alpha-96A[EY12810] (http://flybase.org/reports/FBal0159997.html). *cn bw* flies were used as controls in fig. S1A for which the *key* mutant is on the same genetic background; for RNAi experiments a random set of RNAi lines plus RNAi directed against CG12333, which had been shown to behave as *cn bw* flies as well as the mean of the 13053 RNAi lines analysed in *S. marcescens* survival assays (fig. S19), were used as control lines.

Screening procedure.

Male flies containing the UAS-RNAi transgene against gene X where crossed with 5 virgin HSP70-GAL4; TubGAL80^{ts} female flies at 18^{o}C. One week later, parents were removed. Two weeks after crossing, vials were moved to 25^{o}C for an additional week after which time the progeny were transferred to new vials and put at 29^{o}C for two days. 20 adult flies were sorted and placed in separate vials which were subsequently heat shocked. Based on pilot experiments the most effective heat shock protocol is as follows: 30mins at 37^{o}C; 30mins at 18^{o}C; 30mins at 37^{o}C. The flies were then allowed to rest for 3 hours at 29^{o}C before being transferred to fresh vials containing bacterial/sugar solution. For the HML-GAL4 and NP1-GAL4 drivers, crosses were set up at 25^{o}C for two weeks after which time the progeny were transferred to 29^{o}C for two days as before and then infected. To study long-lived immune-resistant mutants, flies were transferred to fresh bacterial/sugar solution on day 4. The flies were counted each day for eight days.

Data analysis and orthologue retrieval.

LT_{50} (lethal time in days at which 50% of the flies died) analysis was calculated using the GraphPad Prism 4.00 (GraphPad Software, San Diego, CA, USA). The Box mean and standard deviation (SD) was calculated by determining the average LT_{50} of all the lines in a given box and the standard deviation among them using Excel statistical analysis tool. SCORE is defined as [(LT_{50}Mean – LT_{50}Line)/SD of the Mean LT_{50}]. Information regarding individual genes was retrieved from FlyBase. Biological process classification (Fig. 1C) was compiled using FlyBase QueryBuilder (www.flybase.org). Tissue-specific candidates (Fig. 2) were analyzed using Welch t-test comparing all samples to RNAi control lines. $P < 0.05$ was considered to indicate statistical significance. Survival data were analysed using a Logrank (Mantel-Cox) test. Survival experiments shown are representative of at least three independent experiments. Mouse and human orthologues were retrieved from the orthology resources, inparanoid, orthomcl, or ensembl.

Gene ontology enrichment analysis.

The Gene Ontology annotation of CG genes was retrieved from the ensemble biomart database (*15*) for *Drosophila melanogaster* (BDGP5.4) using the biomaRt (*16*) package from Bioconductor (*12*). The gene ontology enrichment analysis was performed with the "topGO" package (*17*). For all computations we used the R version R-2.7.1 (http://www.r-project.org/).

For each Gene Ontology term of the class "biological process" a Fisher exact test was performed where the number of genes assigned to this term is compared between the candidate set and all other genes from the screen. The enrichment analysis was performed for the candidate gene sets in gut and macrophages. GO terms with a nominal p-value < 0.05 were selected as significantly enriched. The candidate genes were then classified manually by inspecting the subgraphs that are induced by significantly enriched terms connected in the GO graph. The subgraphs were visualized using the Rgraphviz package (*18*) from Bioconductor (*19*). In the graphical representation non-significant parental terms were removed where its child terms are reconnected to the parental terms of the deleted term.

Determination of cell death and proliferation.

The positively marked mosaic lineage (PMML) strategy was used to induce mitotic clones, as previously described (*20*). Briefly, the mitotic clones were induced by two consecutive heat shocks (37°C 20 min - 18°C 30 min - 37°C 20 min - recovery at 29°C during one hour) followed by *S. marcescens* infection. Flies were dissected 5 days after and pictures were taken by conventional epifluorescence microscope. DNA synthesis in proliferating cells was detected using 5-ethynyl-2′-deoxyuridine (EdU - Invitrogen). Briefly, The EdU Click-iT™ EdU Alexa Fluor® 488 HCS Assay (Invitrogen, ref A10027) was used to stain the replicating DNA. A 69nL volume of a 0,5mM EdU solution in PBS was injected in the fly hemolymph with Nanoject II (Drumond Scientific). 3h after EdU injection, guts were dissected, fixed, and stained as described by the manufacturer. For the TUNEL assay, 4 days-old Oregon (wild-type)flies were fed in 50mM sucrose with and without *S. marcescens*, during 24h. Guts were fixed in 4% PFA in PBS for 30 min, rinsed once in PBS 1X and incubated overnight in a 0,44M sucrose solution in PBS. Guts were then cryo-embedded in PBS + 0,22M sucrose + 7,5% gelatine, and cut with a Leica CM3050S cryostat. Apoptosis in the midguts of 2-day-old flies was detected by TUNEL, using the in situ cell death detection kit (Roche).

Fluorescent microscopy and imaging.

Intestines were dissected in PBS and immediately observed using a Zeiss SteREO Lumar.V12 dissection microscope equipped with an AxioCam camera and AxioVision 4.1 software. For Apotome microscopy, intestines were dissected in PBS, mounted in Vectashield, and observed immediately using a Zeiss Axiovert 200 inverted microscope equipped with an AxioCam camera and AxioVision 4.1 software. Optical sections through the fluorescent sample were taken using

the Apotome fringe projection system. To visualize GFP, a FITC filter set was used. For confocal microscopy, dissected guts were fixed 30 min in 4% paraformaldehyde (PFA). Guts were observed under an inverted Zeiss Axiovert 100 M microscope equipped with the LSM510 laser scanning confocal module. Images were processed with LSM510 (version 2.5) and ImageJ (version 1.37h) software.

Quantitative reverse-transcription PCR.

This analysis was performed as previously described (*9*). The primers used were as follows:

Diptericin: forward 5'GCTGCGCAATCGCTTCTACT3',

reverse: 5'TGGTGGAGTGGGCTTCATG3'.

RP49: forward 5'GACGCTTCAAGGGACAGTATCTG3',

reverse: 5'AAACGCGGTTCTGCATGAG3'.

Supporting Text

A *Drosophila* high throughput assay for natural bacterial infection

Recently, RNA interference (RNAi) has enabled the generation of gene expression knockdowns at the level of mRNA in transgenic flies, and thus provides an efficient tool to silence every gene in a temporal and/or spatial manner (*1*). We studied the effect of three different ubiquitous GAL4 drivers – daughterless (da) and actin which are constitutively active, as well as the inducible heat-shock promoter-70 (HSP70) driver lines – on the survival of RNAi against the essential local immune regulatory gene *key*. The HSP70-GAL4-RNAi-*key* line displayed the strongest phenotype when fed on *S. marcescens* compared to the other candidate GAL4 lines (fig. S1A). To further enhance adult inducibility of the GAL4 driver, we incorporated a temperature-sensitive, tubulin promoter-GAL80^{ts} transgene. At the permissive temperature, GAL80^{ts} represses the GAL4 transcription factor while it fails to do so at the restrictive temperature, thus allowing the expression of the GAL4 driver. An additional benefit with this experimental approach is that we bypass developmental lethality which occurs in ~30% of *Drosophila* coding genes (*2*). The functional efficacy of our approach was further demonstrated by showing that RNAi-mediated suppression of *key* resulted in reduced induction of *Diptericin* mRNA (encoding for an antimicrobial peptide) by septic injury, a reduction that was maintained for up to 6 days after the initial heat shock (fig. S1B).

For pilot screening, twenty adult flies per RNAi line were sorted, heat shocked to allow full Gal4 activation, put on bacteria and then survivals were recorded for 8 days. We analyzed the data using the lethal time 50 (LT_{50}) for each line as defined as the time, in days, when 50% of the total number of flies have died. After testing some 600 different and randomly chosen RNAi lines over several days in which RNAi-*key* lines were included as controls, we set ≤ -1.5 standard deviations (-1.5 SD) from the mean LT_{50} of batches of 80-200 lines (see Methods) to be considered an infection-susceptible hit. Due to the lack of any known RNAi lines or mutants that confer resistance to *S. marcescens* infections, we decided upon ≥ +2 SD from the mean LT_{50} as an infection-resistant hit, to identify those genes which, when suppressed, display the strongest resistance to *S. marcescens* challenge. Thus, we have successfully set-up a high throughput RNAi screen of intestinal *S. marcescens* infection that mimics all the characteristics of a local bacterial innate immune response.

S. marcescens – a model for natural bacterial infection

S. marcescens is a Gram-negative, opportunistic and versatile pathogen that has developed the ability to adapt to a large number of environmental conditions and infects a range of hosts including *D. melanogaster, Caenorhabditis elegans,* and mammals (*3, 4*). Upon oral entry, *S. marcescens* (strain Db11) proceeds through the gut where it triggers local expression of antimicrobial peptides (AMP) such as Diptericin (*5*). In contrast to all other bacterial species studied to date, *S. marcescens* is the only bacterium which crosses the intestinal barrier to reach the hemolymph without activating the systemic immune response (*5*). Intestinal infection by *S. marcescens* kills adult flies within a week of continuous bacterial ingestion. The host defense against infection occurs on two levels; firstly, a local, Kenny (*key*)-regulated anti-microbial immune response in the intestine and secondly, a cellular immune response that relies on macrophage-like hemocytes that phagocytose bacteria that have escaped from the gut (*5, 6*). *Key* is the *Drosophila* orthologue of *IKKγ/Nemo* and an essential component of the imd pathway and AMP induction (*7*). Of note, expression of AMP in response to *S. marcescens* is solely dependent on the IMD pathway with the Toll pathway playing no obvious role (*5*). Another novel and important aspect to intestinal *S. marcescens* infection is that *S. marcescens* is pathogenic to a wild type, immune-competent fly thus allowing identification of genes which negatively regulate host immunity (*5*). Therefore, *S. marcescens* infection permits an in-depth *in vivo* analysis on the role of hemocytes and innate intestinal immunity in bacterial pathogenesis.

Genome-wide *in vivo* RNAi screen

In order to address the issue of sequence-specific numbers of off-targets (OTEs), we only included those lines representing 10268 genes that fit the previously defined criteria of specificity for further analyses, i.e. only lines with an S19 score ≥ 0.8 were considered specific (Table S2) (*1*). As a measure of targeting specificity previously defined (*1*), a specificity score, s_{19}, is the number of all on-target 19-mer matches divided by the total number of matches of a given RNAi hairpin (that is s_{19} = Σon-target matches / (Σ on-target matches + Σoff-target matches)). Thus, s_{19} = 1 for a RNAi construct with no off-target hits, and s_{19} = 0 for a (hypothetical) construct with only off-target hits. Each line was systematically monitored and viability registered each day, representing 1.83 million individual data points for the global screen. All survival data were normalized to the daily mean of all RNAi lines in the same

experimental cohort (cohorts ranged from 80-200 lines) to exclude day-to-day variations such as bacterial load or possible changes in the environmental milieu.

Mapping of the function of mammalian orthologues to hemocytes or/and intestinal epithelium

Note that a lower number of orthologous candidates were screened for the NP1-GAL4 driver line compared to the HML-GAL4 driver due to developmental lethality indicating an essential role of these genes in gut development (Table S7). Since multiple candidate gene may play a role in non-immune functions and their phenotypes might manifest during stress conditions, we assayed a large proportion of the strongest hits using a special diet depleted of nitrogen to ensure that the reduced survival phenotype of our hits is not due to developmental defects or the consequence of this particular stress (Tables S5 and S6).

The JAK/STAT pathway controls *S. marcescens* susceptibility

We performed all our *S. marcescens* infections at 29^{o}C because yeast GAL4 is more efficient at this temperature. Since there is no effect of the NP1-driven PP1α96A RNAi line at 25^{o}C, we could therefore further confirm the role of PP1α96A in intestinal immunity using a sensitized background. When one copy of the PP1α96A gene was removed by P-element insertion i.e. Null/NP1-RNAi-*pp1α96A*, the flies were reproducibly short-lived in the context of infection (fig. S13). It is likely that *pp1α96A* mutant flies succumb because of the overproliferation of ISCs that do not appear to differentiate properly into mature enterocytes, a phenotype reminiscent to that observed in aged flies (*8*).

Figure S1. GAL4 driver selection and efficiency.

(A) Survivals of RNAi-*key* flies driven by various ubiquitously-expressed GAL4 lines compared to control and the reference *key* mutant line against oral *S. marcescens* infections. Da, daughterless; HSP, heat shock promoter; *key*, *kenny* (IKKγ). ***, $p \leq 0.0001$ (Logrank test). (B) Efficiency of HSP70-GAL4/80-driven *key* suppression was functionally determined by Diptericin induction at the indicated days after heat shock. On each day flies received septic injury with *E. coli* 6 hrs before analyzing Diptericin expression using quantitative RT-PCR. The *kenny* mutant (*key Mut*) is shown as a control. Values are mean ± SEM, n = 3 experiments with 20 flies in each. *, $p < 0.05$ (one-way ANOVA with a Dunnett post test). See supporting online material (SOM) Text for detailed descriptions.

Figure S2. Genome-wide immunity screen analysis.

(A) Table showing the number of genes analyzed in the *S. marcescens* infection screen as well as the hit rate. Also shown is the breakdown of total hits. See SOM text for details. OTEs; off target effects. (B) Percentage distribution of gene ontology (GO) annotated genes to biological processes for resistant candidates.

Figure S3. Tissue-specific immunity screen analysis.

(A and B). Tables summarizing the tissue-specific analysis of RNAi lines that confer susceptibility and resistance to oral *S. marcescens* challenge in hemocytes (A) and gut (B). Only genes with conserved mammalian homologues were assayed. A hit is defined as an RNAi line which shows statistically significant differences in survival compared to control RNAi lines (see Methods). The number of statistically significant genes assayed with more than one transformant and distinct RNAi hairpin is also shown. Lethality rate refers to developmental lethality of HML-GAL4 and NP1-GAL4 RNAi lines. Also shown is the overlap of candidates between the two cell types.

Figure S4. NP1 driver expression is midgut-specific.

(A) The NP1 driver line used for gut-specific RNAi silencing and overexpression studies was crossed to UAS-GFP to visualise its expression pattern in the gut. (B) A schematic of the data is also shown. Green indicates expression of NP1-driven GFP. Circled sections represent the areas that were examined in Figures 3 and 4, and Figures S14-S17.

Figure S5. Enriched biological intestinal processes during *S. marcescens* challenge (1/3)

First of three gene ontology enrichment graphs depicting candidate intestinal genes classified to annotated biological processes of increasing specificity. Boxed processes are hierarchal arranged in which upper terms are parental to those below i.e. lower terms are subgroups to those connected higher. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Each biological term is represented by its gene ontology (GO) identifier, the expected number of genes one would expect from a random sample of similar size (Exp.), the number of candidates present as a fraction of the total number of genes classified to a particular term, as well as the p-value of the enrichment. The candidate genes were then classified as described in Methods (see also Suppl. Table S10).

Figure S6. Enriched biological intestinal processes during *S. marcescens* challenge (2/3)

Second of three gene ontology enrichment graphs depicting candidate intestinal genes classified to annotated biological processes of increasing specificity. Boxed processes are hierarchal arranged in which upper terms are parental to those below i.e. lower terms are subgroups to those connected higher. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Each biological term is represented by its gene ontology (GO) identifier, the expected number of genes one would expect from a random sample of similar size (Exp.), the number of candidates present as a fraction of the total number of genes classified to a particular term, as well as the p-value of the enrichment. The candidate genes were then classified as described in Methods (Suppl. Table S10).

Figure S7. Enriched biological intestinal processes during *S. marcescens* challenge (3/3)

Third of three ontology enrichment graphs depicting candidate intestinal genes classified to annotated biological processes of increasing specificity. Boxed processes are hierarchal arranged

in which upper terms are parental to those below i.e. lower terms are subgroups to those connected higher. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Each biological term is represented by its gene ontology (GO) identifier, the expected number of genes one would expect from a random sample of similar size (Exp.), the number of candidates present as a fraction of the total number of genes classified to a particular term, as well as the p-value of the enrichment. The candidate genes were then classified as described in Methods (Suppl. Table S10).

Figure S8. Affected biological processes in hemocytes during *S. marcescens* infection.

Shown are statistically enriched biological processes with their corresponding p-value in hemocytes associated with *S. marcescens* infection. Candidate genes are classified to their respective process according to gene ontology enrichment analysis. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Processes are superimposed upon a sketch depicting a hemocyte cell engulfing a bacterium and localized to appropriate cellular organelles/compartments. R, resistant; S, susceptible, P, p-value. All processes shown display $p < 0.05$ (Fischer Test).

Figure S9. Enriched biological processes in hemocytes during *S. marcescens* challenge (1/2)

First of two gene ontology enrichment graphs depicting candidate hemocyte genes classified to annotated biological processes of increasing specificity. Boxed processes are hierarchal arranged in which upper terms are parental to those below, i.e. lower terms are subgroups to those connected higher. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Each biological term is represented by its gene ontology (GO) identifier, the expected number of genes one would expect from a random sample of similar size (Exp.), the number of candidates present as a fraction of the total number of genes classified to a particular term, as well as the p-value of the

enrichment. The candidate genes were then classified as described in Methods (Suppl. Table S11).

Figure S10. Enriched biological processes in hemocytes during *S. marcescens* challenge (2/2)

Second of two gene ontology enrichment graphs depicting candidate hemocyte genes classified to annotated biological processes of increasing specificity. Boxed processes are hierarchal arranged in which upper terms are parental to those below i.e. lower terms are subgroups to those connected higher. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong. Each biological term is represented by its gene ontology (GO) identifier, the expected number of genes one would expect from a random sample of similar size (Exp.), the number of candidates present as a fraction of the total number of genes classified to a particular term, as well as the p-value of the enrichment. The candidate genes were then classified as described in Methods (Suppl. Table S11).

Figure S11. Induction of the JAK/STAT pathway ligand UPD in the gut after *S. marcescens* infection.

GFP expression in the whole gut of transgenic *upd*-GFP flies following natural infection with *S. marcescens* compared to control, non-pathogenic conditions. Blue images show DAPI stained guts for visualisation. White arrows indicate areas of intense transgene expression. Data are shown on day 4 after *S.* marcescens infections.

Figure S12. upd3 is upregulated in the gut after feeding with *S. marcescens*.

GFP expression in the whole gut of transgenic *upd3*-GFP flies following natural infection with *S. marcescens* compared to control, non-pathogenic conditions. Blue image shows DAPI stained control gut for visualisation. White arrows indicate areas of *upd3*-driven GFP expression. Data are shown on day 4 after *S. marcescens* infections.

Figure S13. PP1α96A controls intestinal immunity.

Survival curves of NP1-RNAi-*pp1α96A*, Null/+;NP1-RNAi-*pp1α96A*, and Null/+;NP1-driver (no RNAi) lines at 25°C compared to control and *key* mutant flies following *S. marcescens* feeding. ***, $p \leq 0.0001$ (Logrank).

Figure S14. Oral *S. marcescens* infections result in increased death and proliferation of intestinal cells.

(A) TUNEL (red) and DAPI (blue) staining of day 2 *S. marcescens*-infected as well as non-infected, control gut epithelium. (B) Mitotic GFP clonal expression in the midgut of control wild type flies on day 5 after *S. marcescens* challenge and under non-pathogenic (control) conditions, using positively marked mosaic lineage clones. (C) EdU (green) staining to visualize DNA synthesis as a marker for proliferation in epithelial cells of wild type flies on day 5 of *S. marcescens*-infection and non-infection (control) conditions; EdU was injected 3h prior to dissection. DAPI (blue) nuclear staining is also shown.

Figure S15. Normal intestinal epithelium and survivals under non-pathogenic conditions.

(A) EdU (green) and DAPI (blue) staining of epithelial cells under normal feeding conditions in NP1-UAS-*pias*, NP1-UAS-*domeDN*, NP1-RNAi-*pp1α96A* and control flies at 25°C. Note normal appearance of the gut epithelium in all the indicated lines. Arrow points to a single EdU positive cell. (B) Survival curves of control, NP1-UAS-*pias*, NP1-UAS-*domeDN*, and Null/+,NP1-RNAi-*pp1α96A* flies on non-pathogenic, sugar conditions at 25°C.

Figure S16. Quantification of EdU positive cells in infected and non-infected anterior midguts.

NP1-UAS-*pias*, NP1-UAS-*domeDN*, NP1-RNAi-*pp1α96A* and control flies were fed for 4 days on *S. marcescens* or sugar solution. EdU was injected 3h prior to dissection. EdU positive cells were counted in the region of the anterior midgut that is proximal to the copper cell region. Values correspond to the mean of 20 guts for each line. * $p<0.03$, ** $p<0.01$, *** $p<0.0015$ (Students t-test).

Figure S17. JAK/STAT activation in intestinal stem cells after oral *S. marcescens* infection.

JAK/STAT pathway activation in intestinal stem cells (small nuclei, white arrows) using the *stat92E*-GFP reporter line. The stellate shape of the cells is also evocative of intestinal stem cells. Note that the GFP signal is absent from enterocytes (larger nuclei, yellow arrows). Confocal gut sections are shown at day 5 following *S. marcescens* challenge. Green indicates GFP expression. DAPI (blue) stains the nuclei. Note that intestinal stem cells (small nuclei) reside predominantly in the basal compartment, while enterocytes (large nuclei) are found primarily in more apical

compartments of the intestine (*14*). Note that only small nuclei, defining stem cells as previously characterized (*14*), co-localize with GFP expression. Bottom panels show enlarged images of the same region.

Figure S18. Intestinal stem-cell-specific silencing of PP1α96A show normal survivals in the absence of *S. marcescens* infection.

Survival curves of non-infected (sugar) flies in which PP1α96A is specifically silenced in intestinal stem cells of adult flies using escargot (Esg)-GAL4;tubulinGal80^{ts} at 25^{o}C. Key is also shown.

Figure S19. Survival comparison of control flies.

Graph showing the LT_{50} (lethal time at which 50% of flies die in days) of total RNAi lines screened (n=13092 experiments), the control RNAi line silencing the control gene CG12333 (n=23 experiments), the wild type strain *cn bw* (n=45 experiments), as well as *cn key bw* (*key*) mutant *Drosophila* (n=41 experiments). Data are shown as the mean LT_{50} +/- SEM.

Table S1. Total lines screened.

All experimental data for the entire list of lines screened against natural *S. marcescens* infections are shown. Lines are sorted from the strongest susceptible to the strongest resistant candidates. The LT_{50}Mean was calculated for the total number of lines within a given cohort on a given day. The CG number, the gene symbol, SCORE results (see Methods), the transformant identification and construct identification numbers for each line analysed as well as its respective S19 value and number of predicted off-targets are indicated. *S. marcescens* infections were performed at 29°C.

Table S2. Total lines screened excluding off-target effects.

The Table shows all lines that were included into the final analyses of the *S. marcescens* screen. Based on previous data (*1*) S19 scores < 0.8 are deemed to have significant off-target effects and were therefore discarded from further analysis. The CG number, the gene symbol, SCORE results (see Methods), the transformant identification and construct identification numbers for each line analysed as well as its respective S19 value and number of predicted off-targets are indicated. *S. marcescens* infections were performed at 29°C.

Table S3. Susceptible hits from genome-wide screen.

Those genes from Table S2 which show a SCORE ≤ -1.5 were selected as susceptible candidates upon *S. marcescens* challenge. The CG number, the gene symbol, SCORE results (see Methods), the transformant identification and construct identification numbers for each line analysed as well as its respective S19 value and number of predicted off-targets are indicated. *S. marcescens* infections were performed at 29°C.

Table S4. Resistant hits from genome-wide screen.

Those genes from Table S2 which show a SCORE ≥ +2 were selected as resistant candidates upon *S. marcescens* challenge. The CG number, the gene symbol, SCORE results (see Methods), the transformant identification and construct identification numbers for each line analysed as well as its respective S19 value and number of predicted off-targets are indicated. *S. marcescens* infections were performed at 29°C.

Table S5. HML specific susceptible candidates.

The Table shows the conserved genes of Table S3 retested specifically in hemocytes using the HML driver line. For each gene assayed the Table shows: the CG number; the gene symbol and closest human and/or mouse orthologue id (see methods); mean % death on day 5 after *S. marcescens* infection (% Death Day 5) and its standard deviation (SD); the number of times the lines were assayed (N); the number of transformants (TFs) tested; number of different RNAi hairpins (HPs) tested for each gene; a subset of RNAi lines were also tested for lethality under non-pathogenic conditions (L); also included in the Table are the p-values and whether a given line exhibits statistically significant earlier death as compared to control RNAi lines using t-test analysis. Yes (Y) indicates a statistically significant susceptible hit ($p<0.05$). No (N) indicates genes that are not short lived in response to *S. marcescens*. Green indicates those genes which die significantly earlier than control RNAi lines. Yellow are those lines which were only tested one time and therefore were not included in the statistical analysis. Blue indicates lines that did not show a significant difference from the controls. NA, not applicable (due to being tested only once), V, viable; L, lethal; SL, semi-lethal. *S. marcescens* infections were performed at 29^{o}C.

Table S6. Susceptible candidates in the gut.

The Table shows the conserved genes of Table S3 retested specifically in the intestine using the NP1 driver line. For each gene assayed the Table shows: the CG number; the gene symbol and closest human and/or mouse orthologue id; mean % death on day 5 after *S. marcescens* infection (% Death Day 5) and its standard deviation (SD); the number of times the lines were assayed (N); the number of transformants (TFs) tested; number of different RNAi hairpins (HPs) tested for each gene; a subset of RNAi lines were also tested for lethality under non-pathogenic conditions (L); also included in the Table are the p-values and whether a given line shows statistically significant differences in survival as compared to control RNAi lines using t-test analysis. Yes (Y) indicates a statistically significant susceptible hit ($p<0.05$). No (N) indicates genes that are not short lived in response to *S. marcescens*. Green indicates those genes which die significantly earlier than control RNAi lines. Yellow are those lines which were only tested one time and therefore were not included in the statistical analysis. Blue indicates lines that did not show a significant difference from the controls. NA, not applicable (due to being tested only once), V, viable; L, lethal; SL, semi-lethal. *S. marcescens* infections were performed at 29^{o}C.

Table S7. HML specific resistant candidates.

The Table shows the conserved genes of Table S4 retested specifically in hemocytes using the HML driver line. For each gene assayed the Table shows: the CG number; the gene symbol and closest human and/or mouse orthologue id; mean % death on day 8 after *S. marcescens* infection (% Death Day 8) and its standard deviation (SD); the number of times the lines were assayed (N); the number of transformants (TFs) tested; number of different RNAi hairpins (HPs) tested for each gene; also included in the Table are the p-values and whether a given line shows statistically significant differences in longevity as compared to control RNAi lines using t-test analysis. Yes (Y) indicates a statistically significant resistant hit ($p<0.05$). No (N) indicates genes that are not long-lived in response to *S. marcescens*. Green indicates those genes which survive significantly longer than control RNAi lines. Blue indicates lines that did not show a significant difference from the controls. *S. marcescens* infections were performed at 29^{o}C.

Table S8. Resistant candidates in the gut.

The Table shows the conserved genes of Table S4 retested specifically in the midgut using the NP1 driver line. For each gene assayed the Table shows: the CG number; the gene symbol and closest human and/or mouse orthologue id; mean % death on day 8 after *S. marcescens* infection (% Death Day 8) and its standard deviation (SD); the number of times the lines were assayed (N); the number of transformants (TFs) tested; number of different RNAi hairpins (HPs) tested for each gene; also included in the Table are the p-values and whether a given line shows statistically significant differences in longevity as compared to control RNAi lines using t-test analysis. Yes (Y) indicates a statistically significant resistant hit ($p<0.05$). No (N) indicates genes that are not long-lived in response to *S. marcescens*. Green indicates those genes which survive significantly longer than control RNAi lines. Blue indicates lines that did not show a significant difference from the controls. *S. marcescens* infections were performed at 29^{o}C.

Table S9. Developmentally lethal genes in the gut.

Presented is a list of genes and their respective transformant and construct id which, when silenced specifically in the gut, cause larval lethality. The CG number, the gene symbol, the transformant identification and construct identification numbers are indicated. The crosses were done at 25^{o}C and lethality was scored as larval lethality or failure to eclose.

Table S10. Enriched intestinal processes upon *S. marcescens* infection.

Shown are statistically enriched biological processes with their corresponding p-value in the gut associated with *S. marcescens* infection. Candidate genes are classified to their respective process according to gene ontology enrichment analysis. For each gene, the Table shows its CG number as well as its corresponding gene symbol where available. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong.

Table S11. Enriched processes within hemocytes upon *S. marcescens* infection.

Shown are statistically enriched biological processes with their corresponding p-value in the hemocyte cells associated with *S. marcescens* infection. Candidate genes are classified to their respective process according to gene ontology enrichment analysis. For each gene, the Table shows its CG number as well as its corresponding gene symbol where available. Green indicates processes to which susceptible candidates are exclusively attributed. Red indicates processes to which resistant candidates are exclusively attributed. Blue indicates processes to which both susceptible and resistant candidates belong.

Table S12. KEGG analysis on global susceptible candidates.

The Table lists KEGG pathways showing their name, total number of genes assayed from a given pathway, numbers and percentages of those genes picked up in our screen, as well as the CG numbers for the genes themselves. Pathways are ranked according to their percentage hit rate with a given pathway. As the IMD pathway is not annotated in KEGG we added it manually ranked according to the numbers of hits in order to demonstrate that we hit the IMD pathway in our screen.

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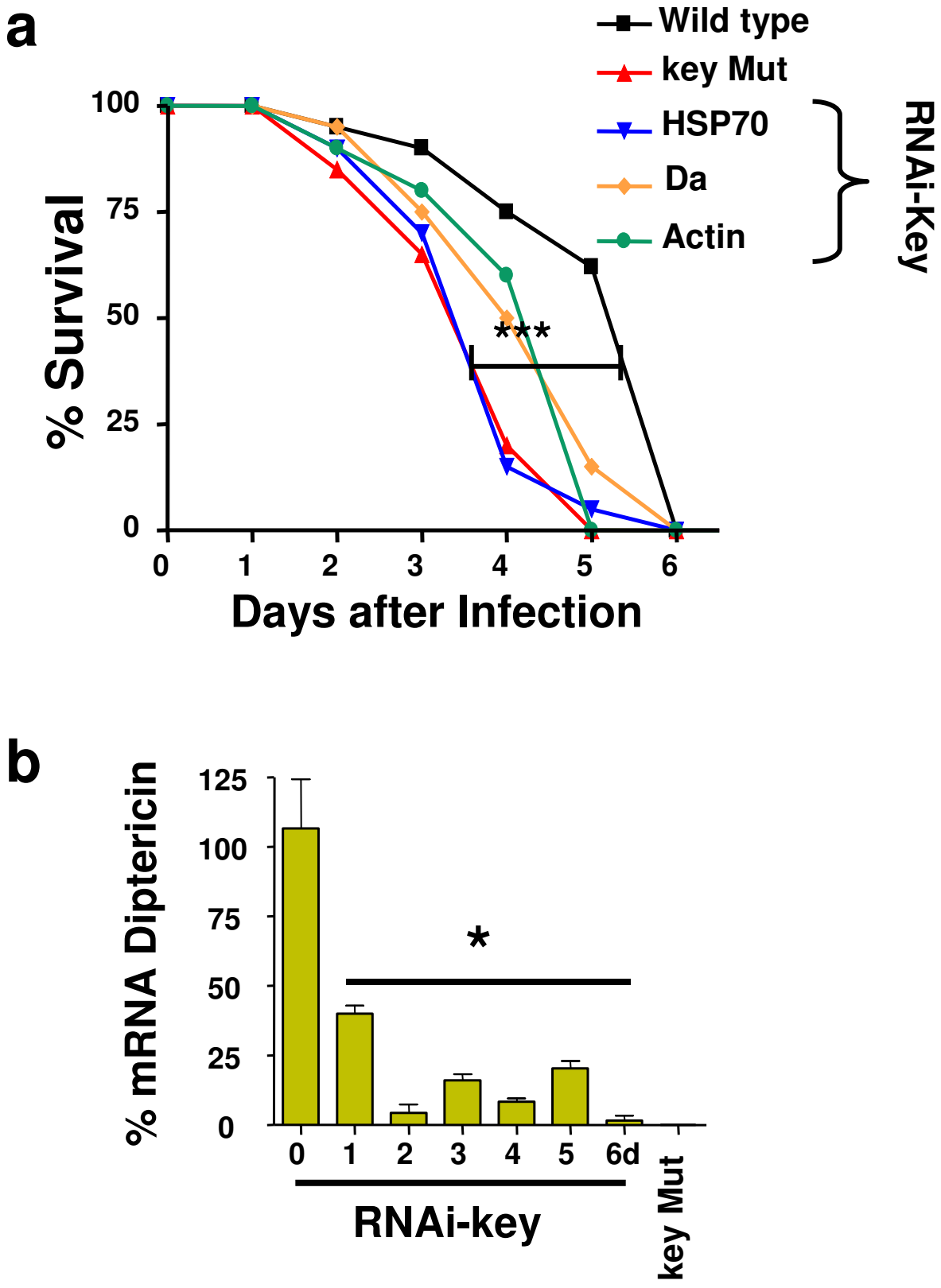
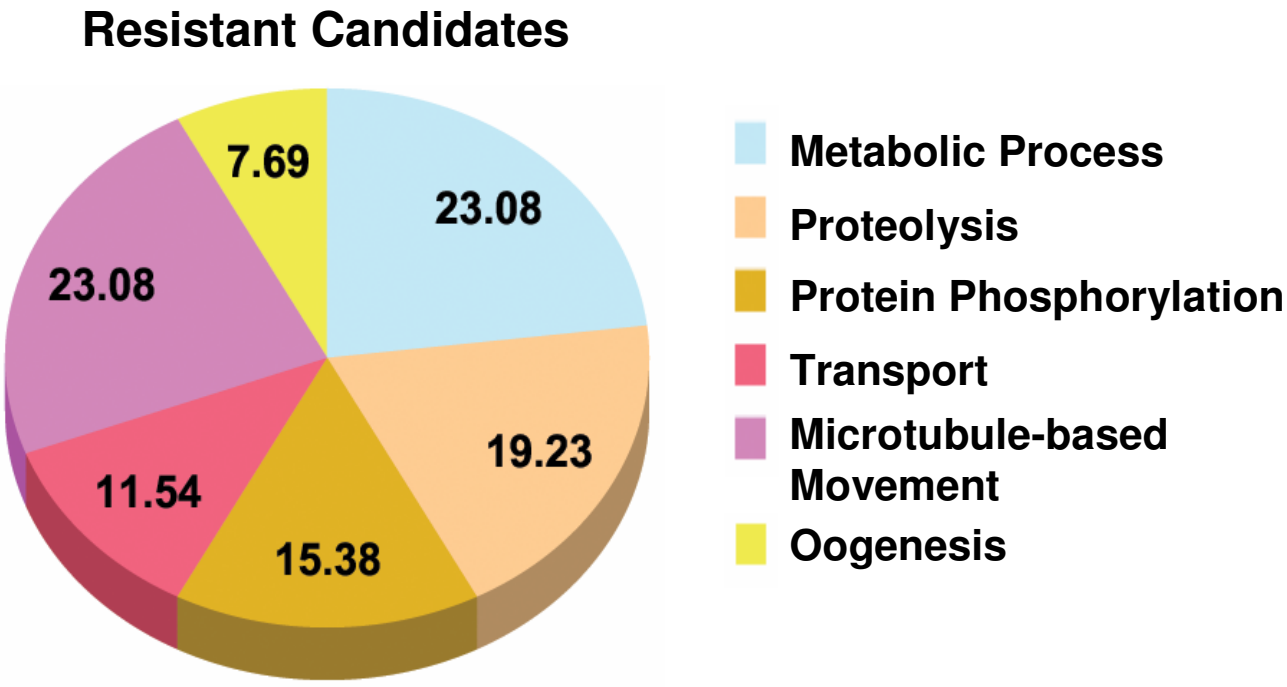


Suppl. Figure S1

a

	Number	Percentage
Total genes in *Drosophila* genome	~14000	100
Total genes analyzed	10689	78
Total genes minus OTEs	10268	73
Total Hits	885/10268	8.6
≥ +2 SD: Resistant Hits	95/885	10.7
≤ -1.5 SD: Susceptible Hits	790/885	89.3

b



Suppl. Figure S2

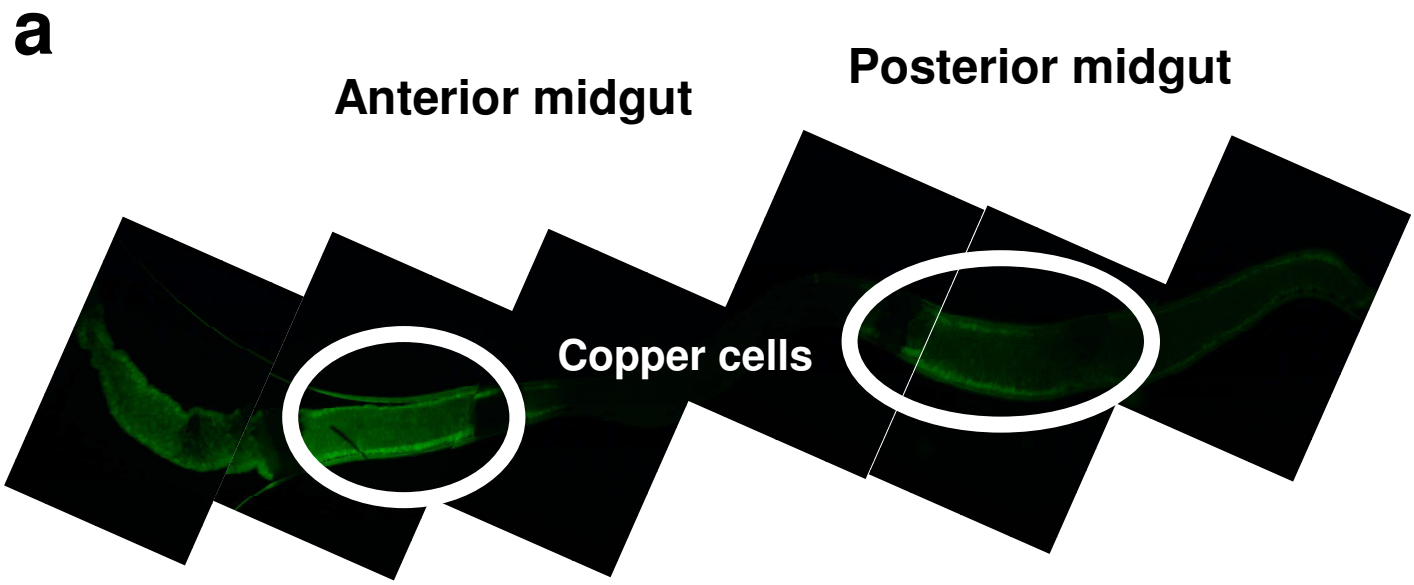
a

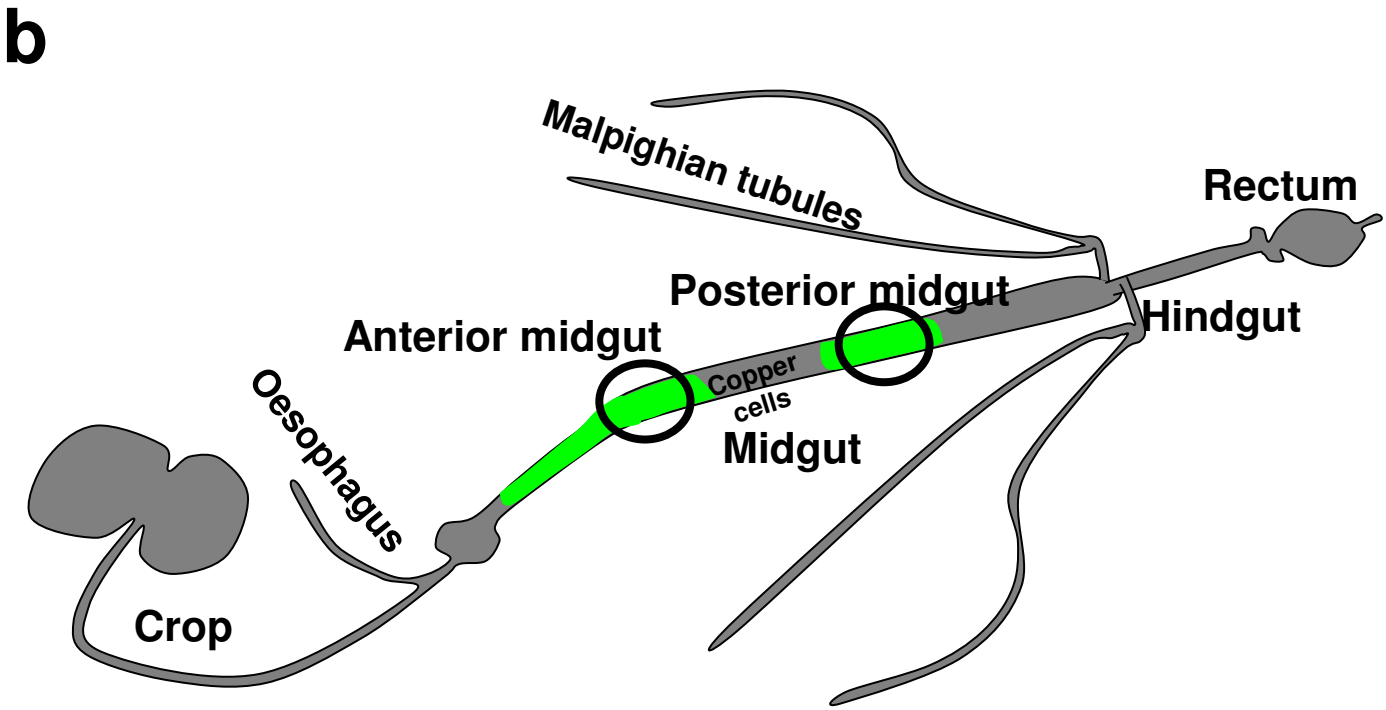
HML (Hemocytes)	Susceptible		Resistant	
	Gene #	%	Gene #	%
Assayed	358		47	
HIT (p<0.05)	98/358	27	37/47	79
Transformants ≥ 2	60/98	61	23/37	62
Hairpins ≥ 2	12/98	12	5/37	14
Lethality Rate	0/39	0	0	0
NP1 Overlap	54/98	55	25/37	68

b

NP1 (Gut)	Susceptible		Resistant	
	Gene #	%	Gene #	%
Assayed	337		46	
HIT (p<0.05)	129/337	38	28/46	61
Transformants ≥ 2	79/129	61	24/28	86
Hairpins ≥ 2	17/129	13	5/28	18
Lethality Rate	4/74	5	0	0
HML Overlap	54/129	42	25/28	89

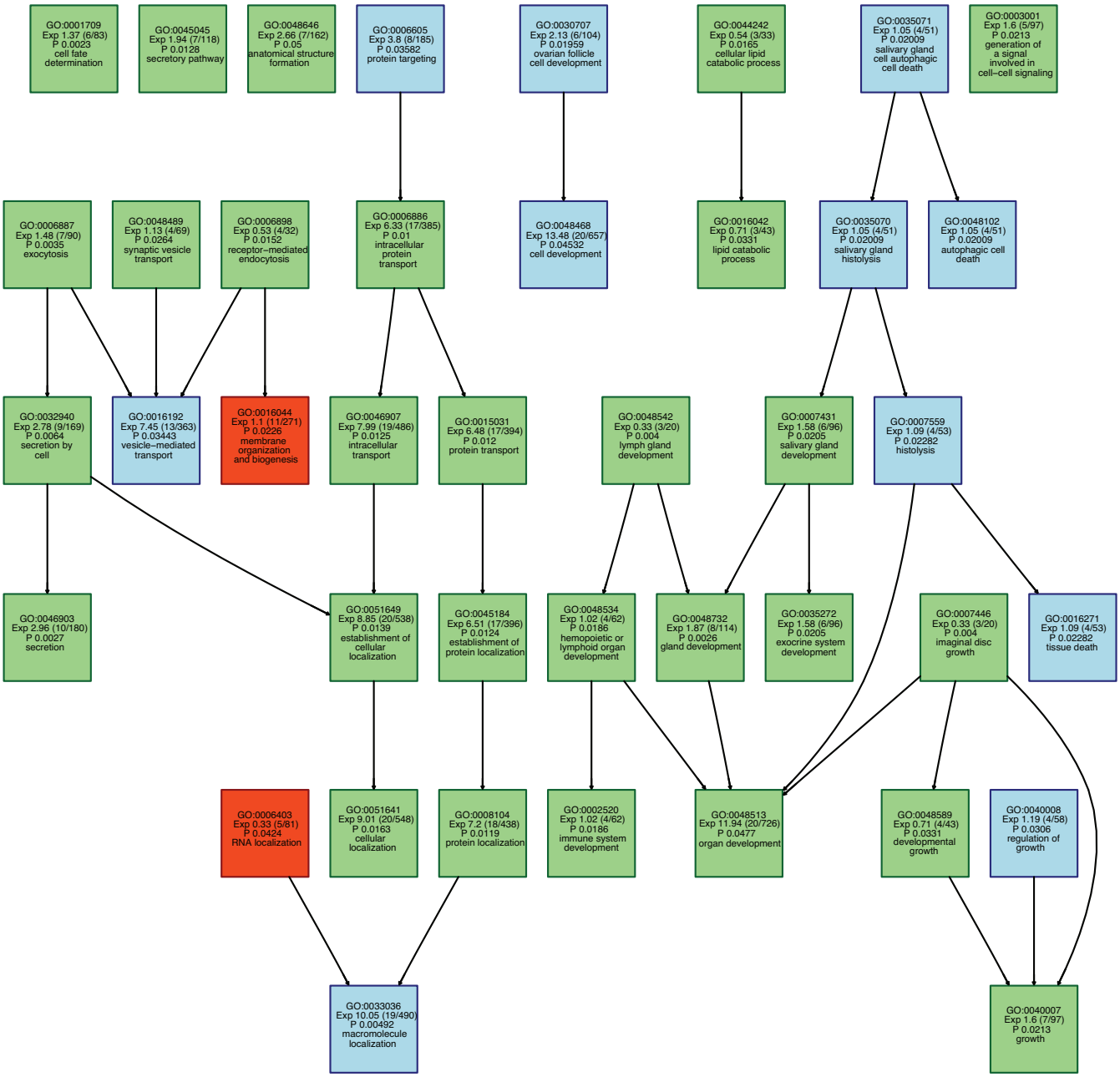
Suppl. Figure S3





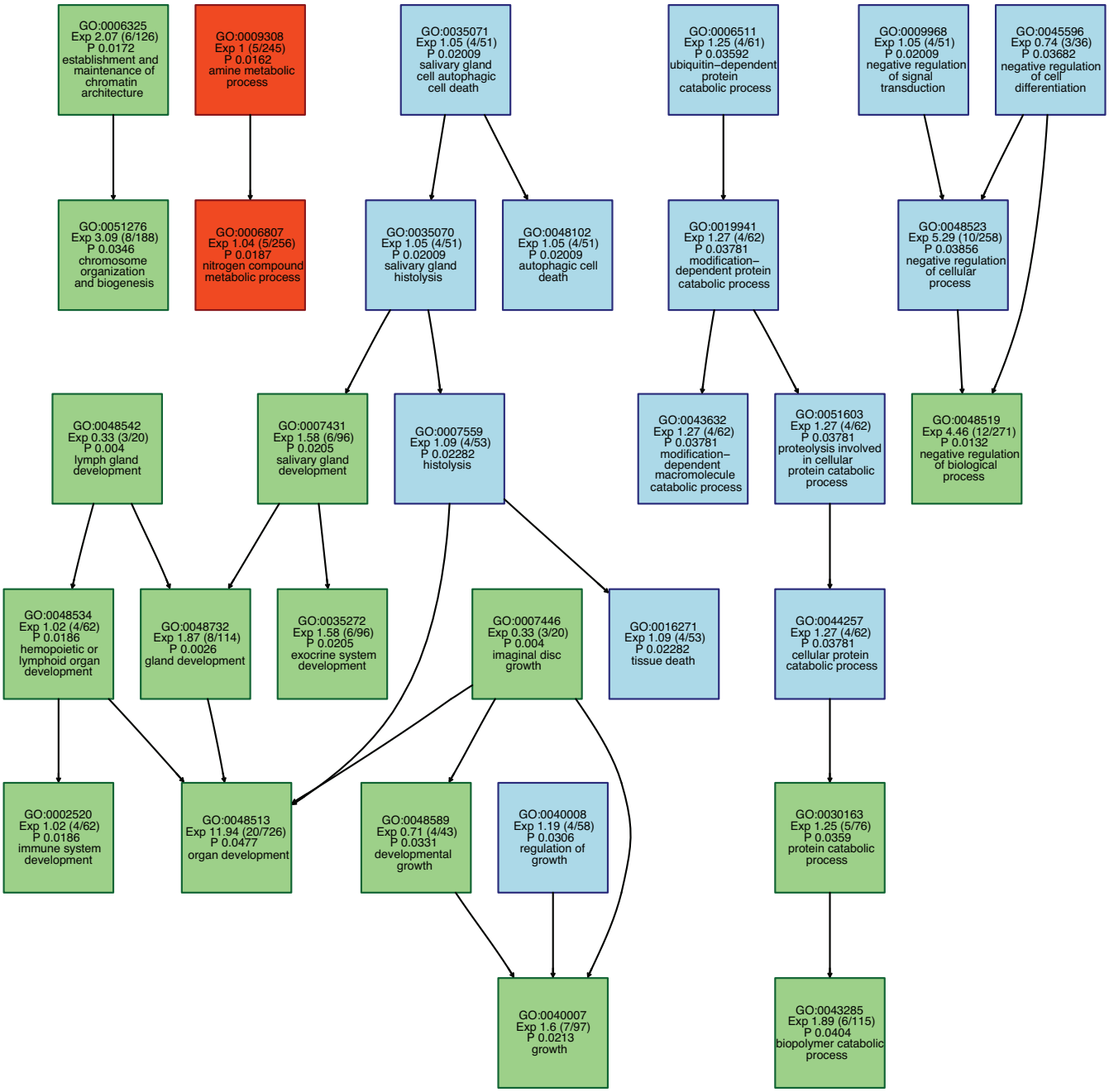
Suppl. Figure S4

Gut - Gene Ontology Enrichment 1/3



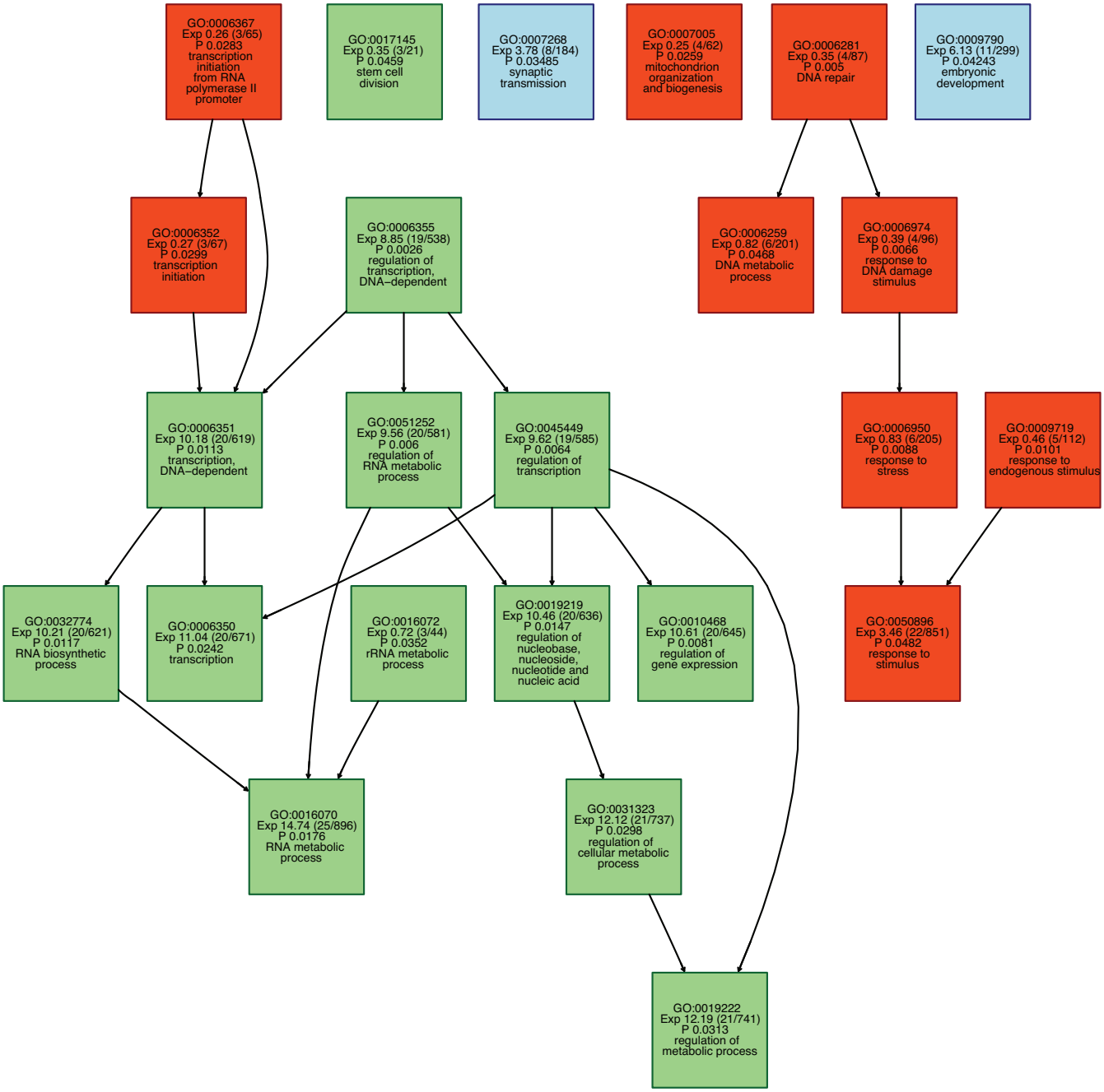
Suppl. Figure S5

Gut - Gene Ontology Enrichment 2/3

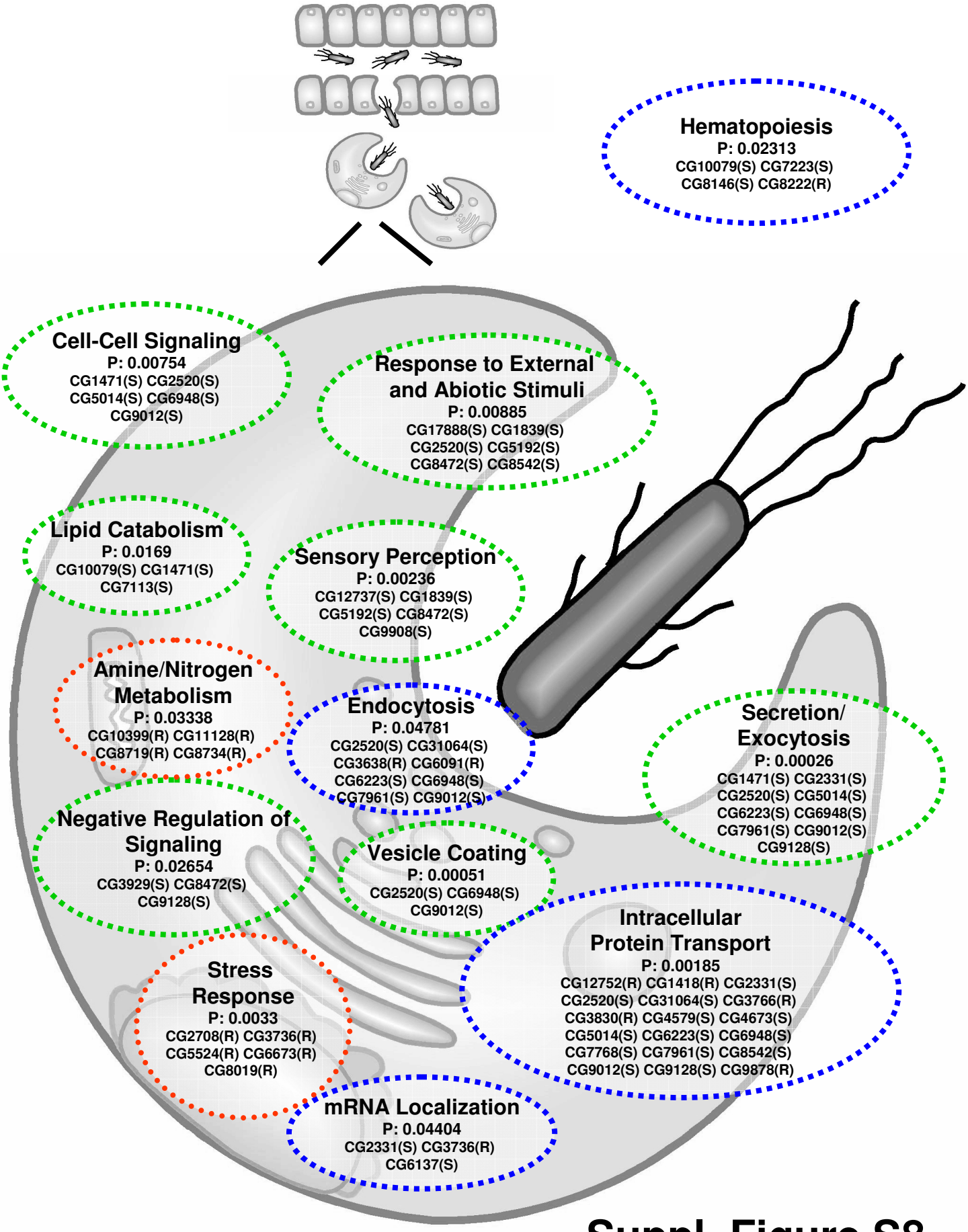


Suppl. Figure S6

Gut - Gene Ontology Enrichment 3/3

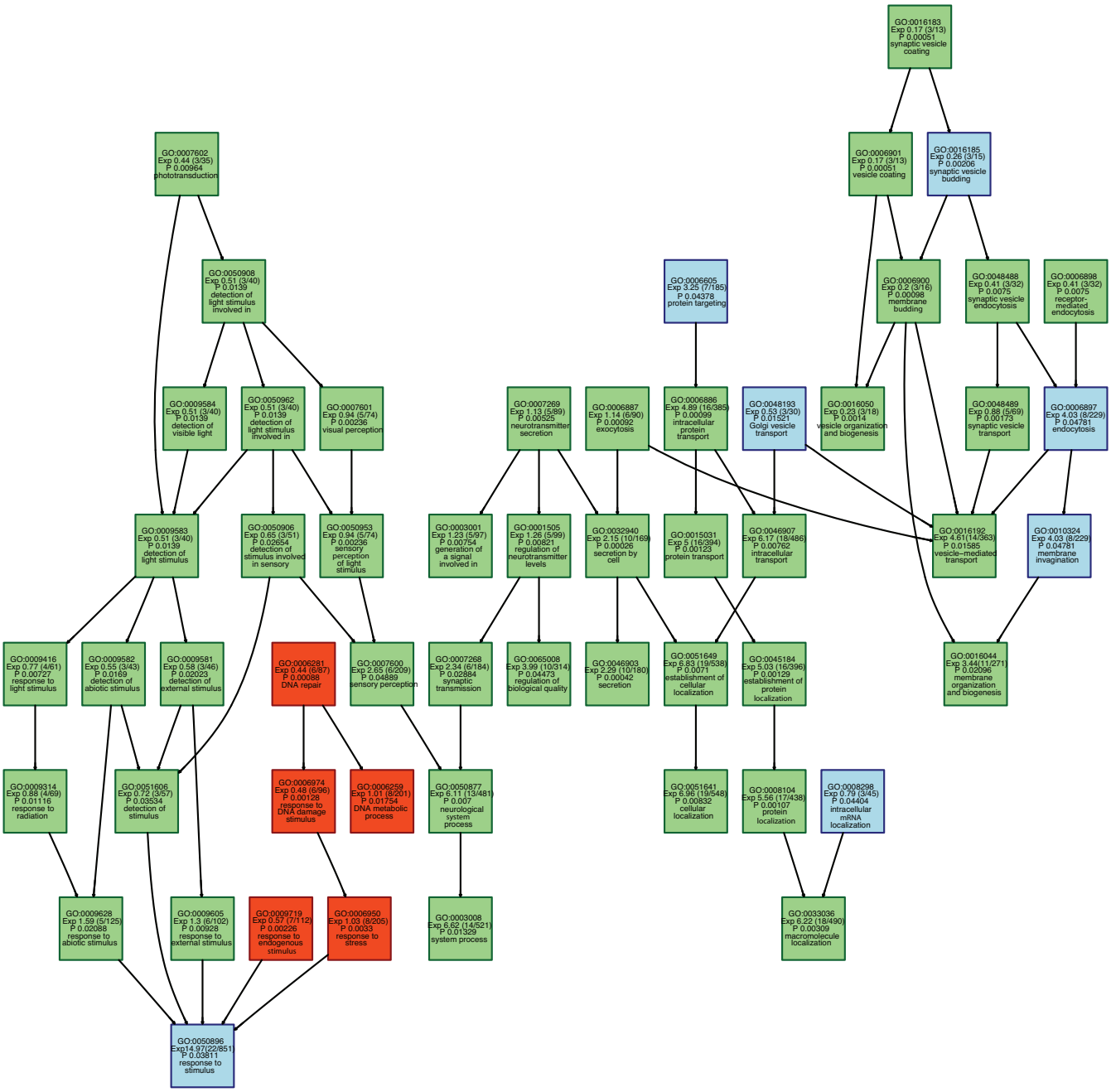


Suppl. Figure S7



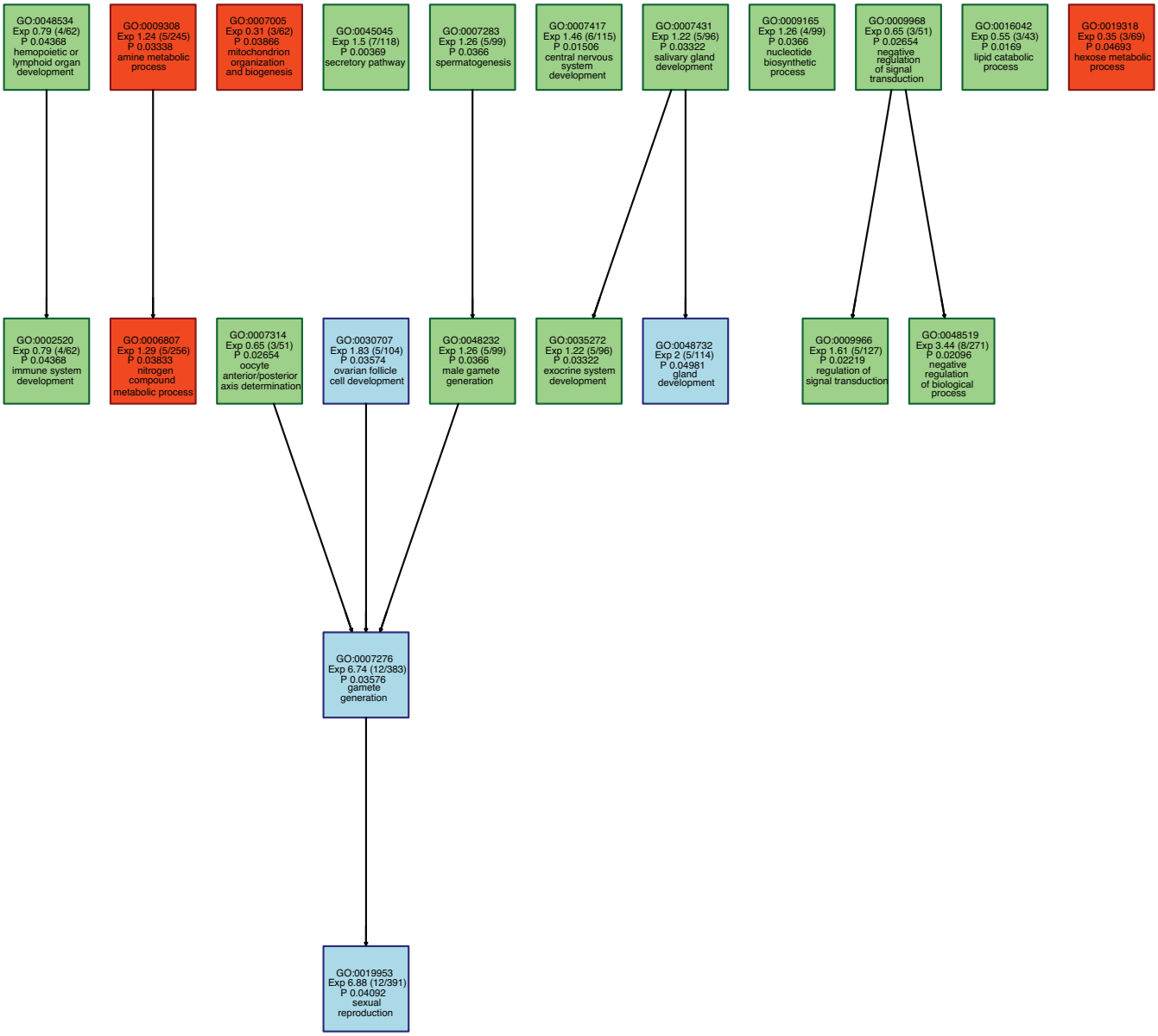
Suppl. Figure S8

Hemocyte - Gene Ontology Enrichment 1/2

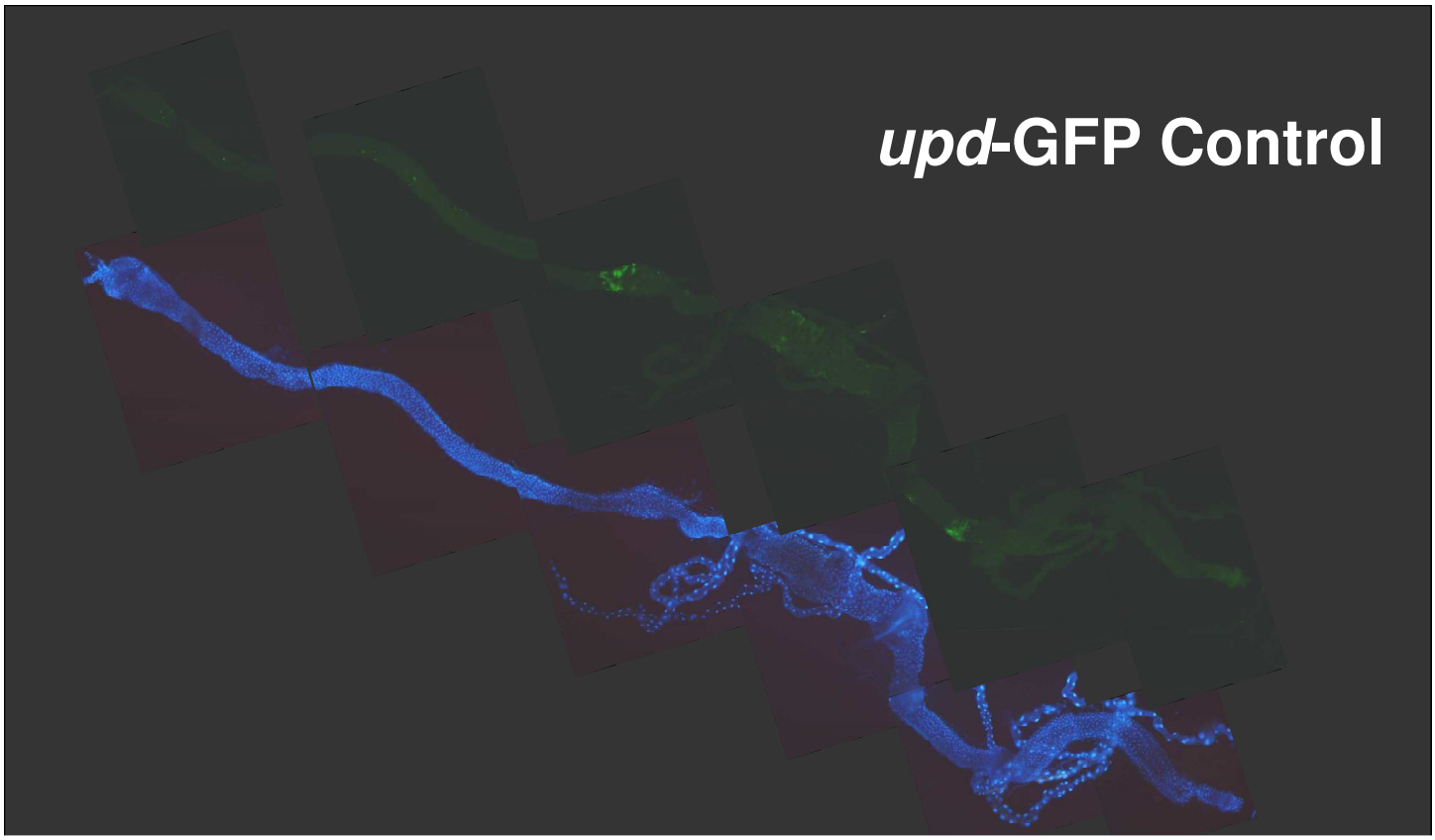


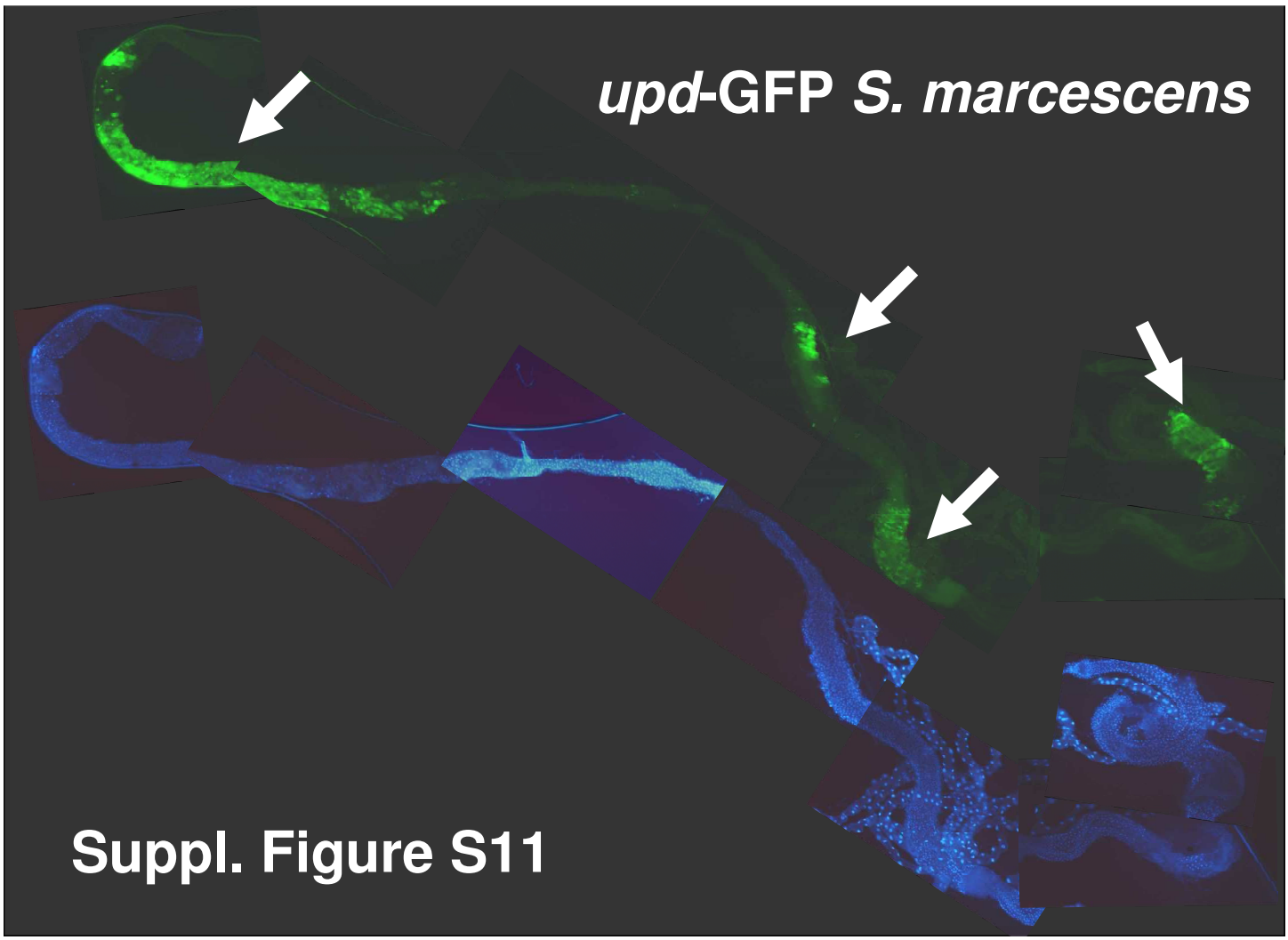
Suppl. Figure S9

Hemocyte - Gene Ontology Enrichment 2/2

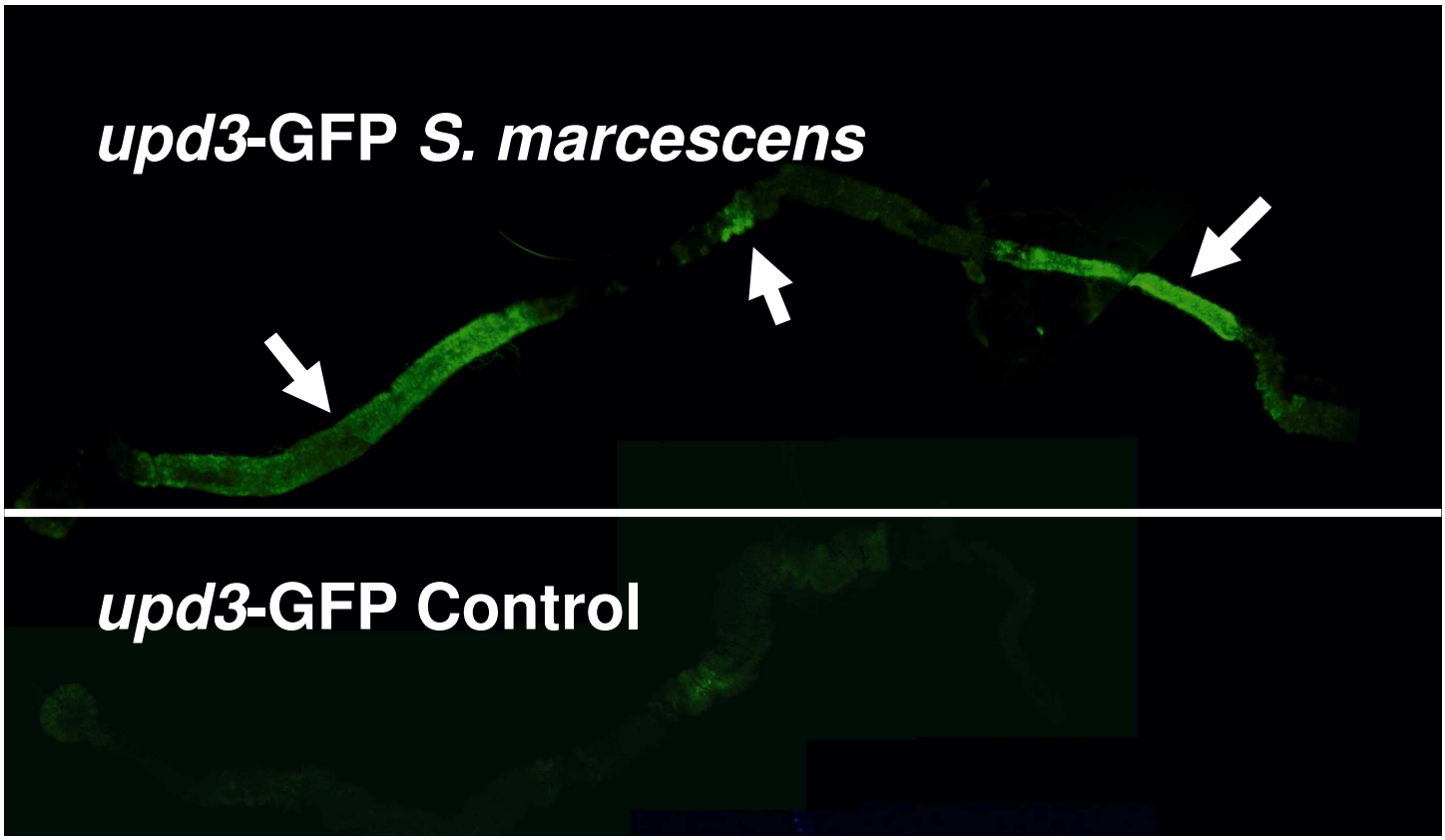


Suppl. Figure S10





Suppl. Figure S11



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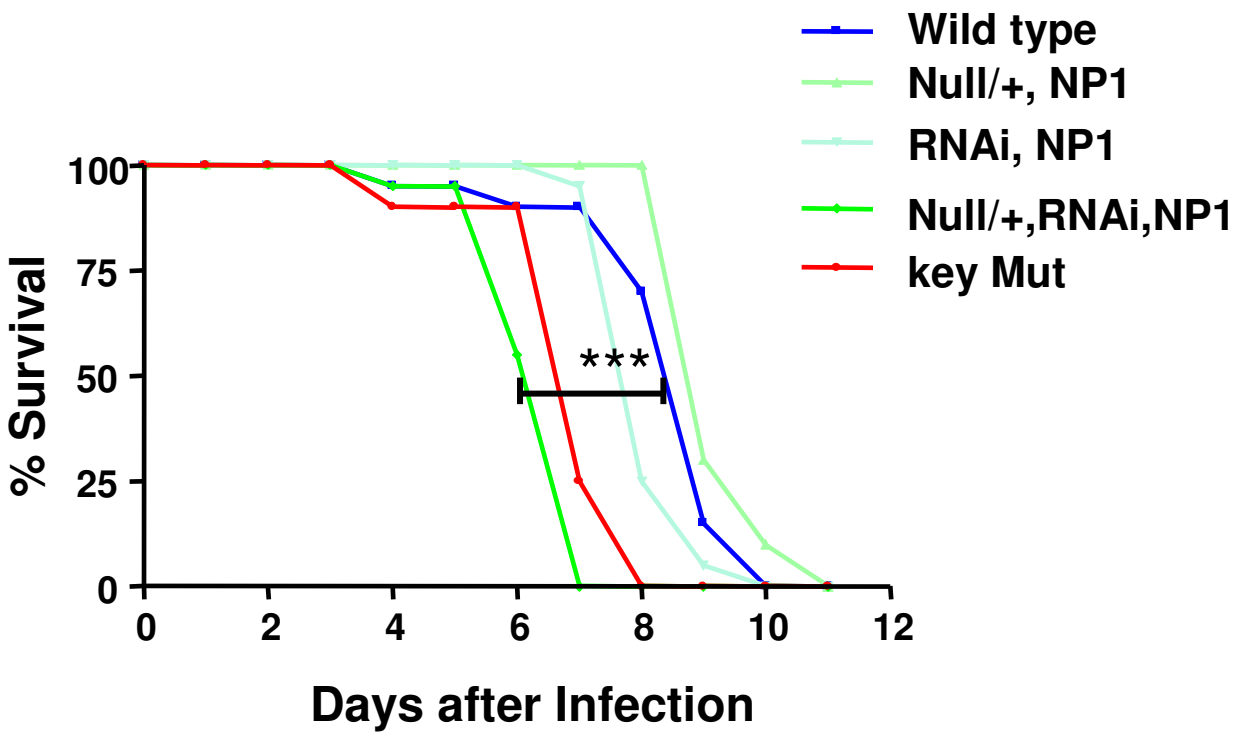
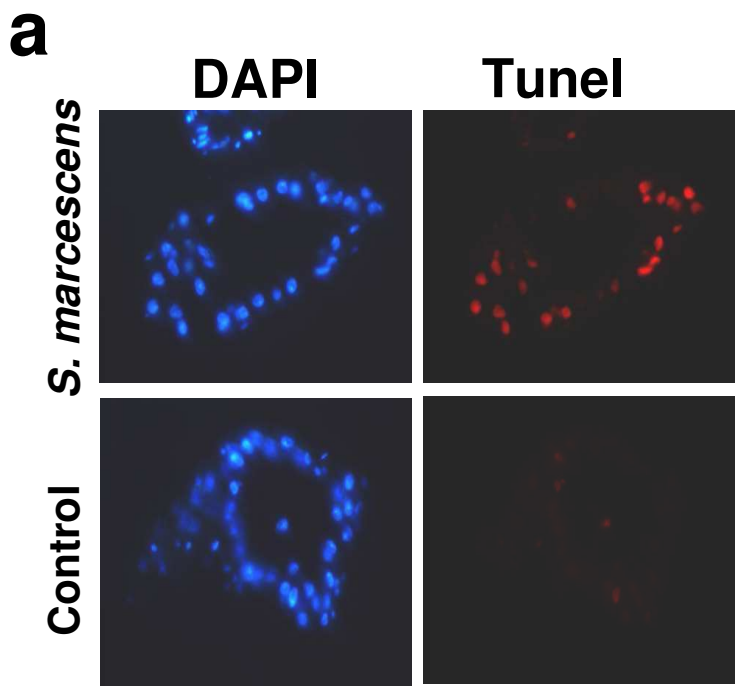
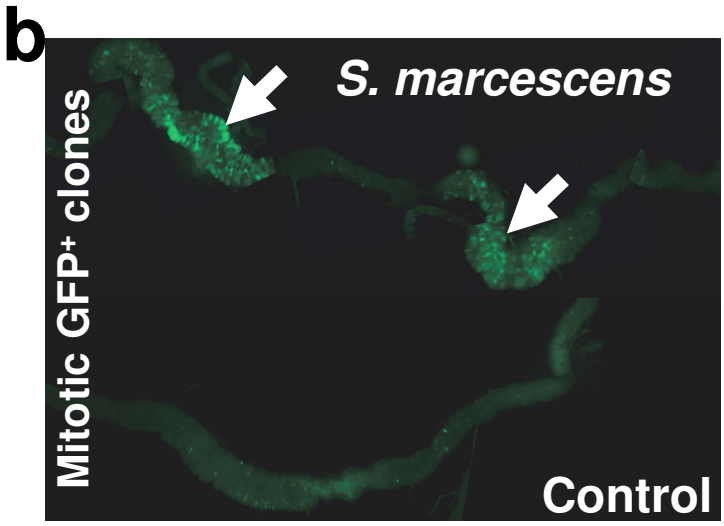
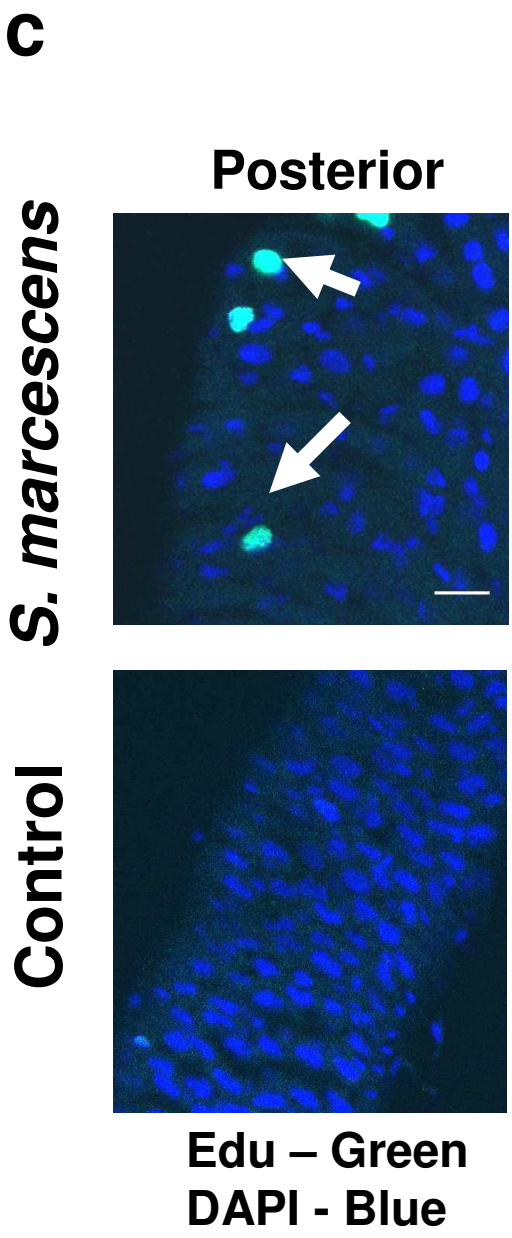


Suppl. Figure S13

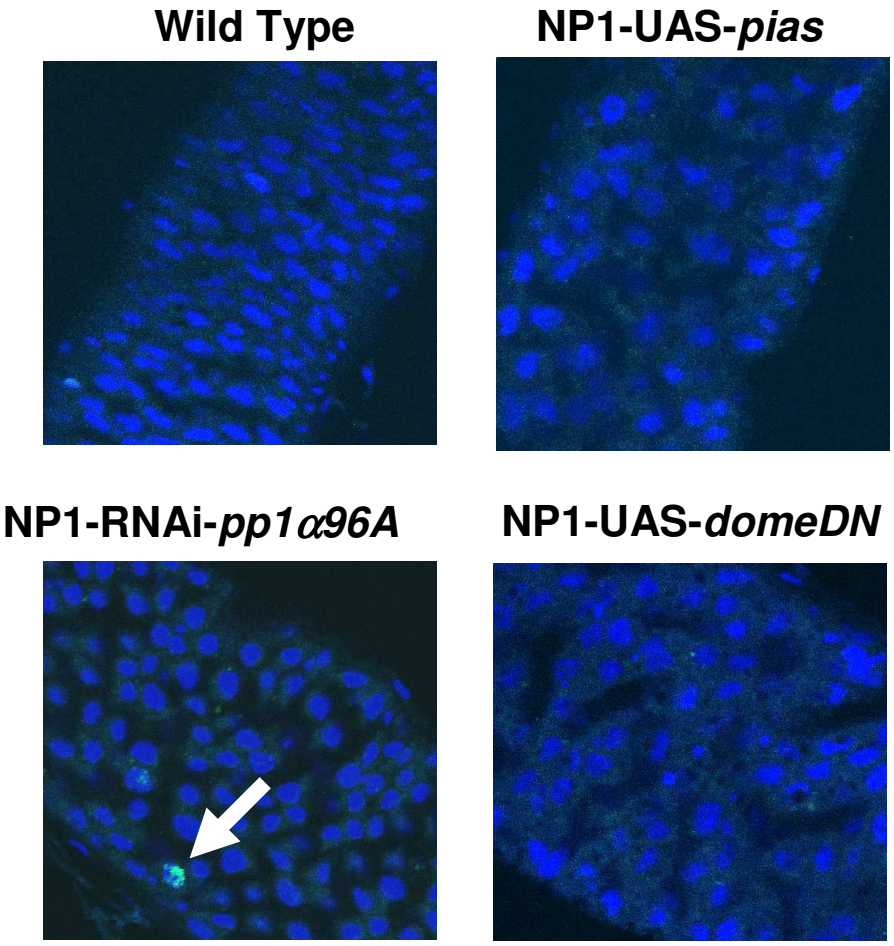






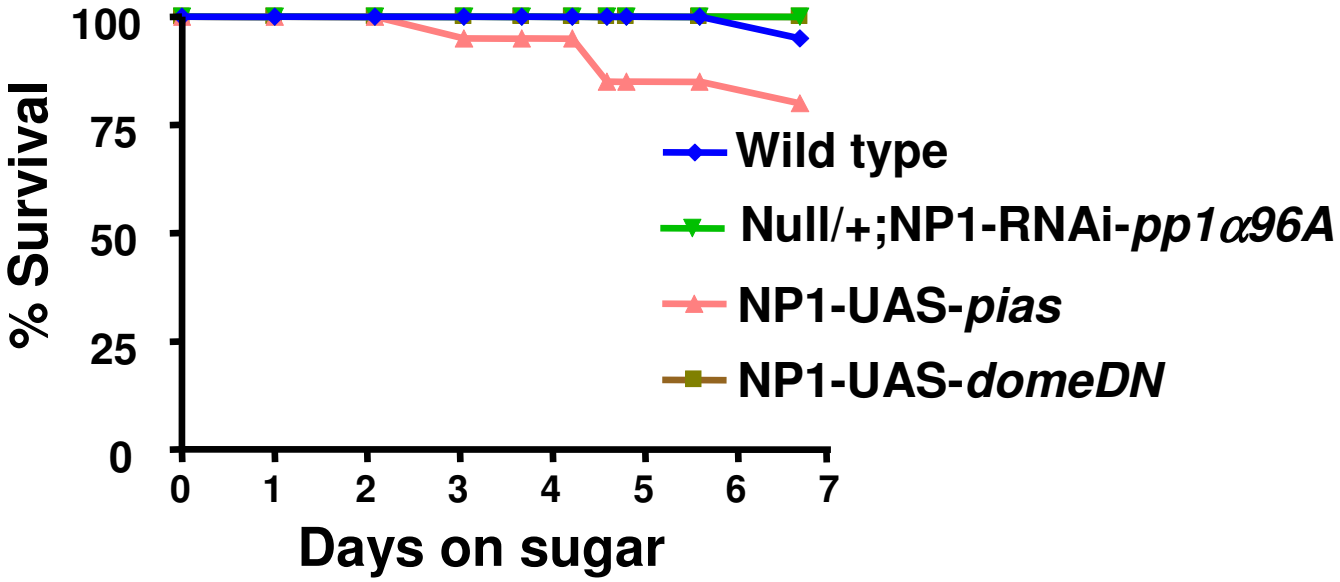
Suppl. Figure S14

a

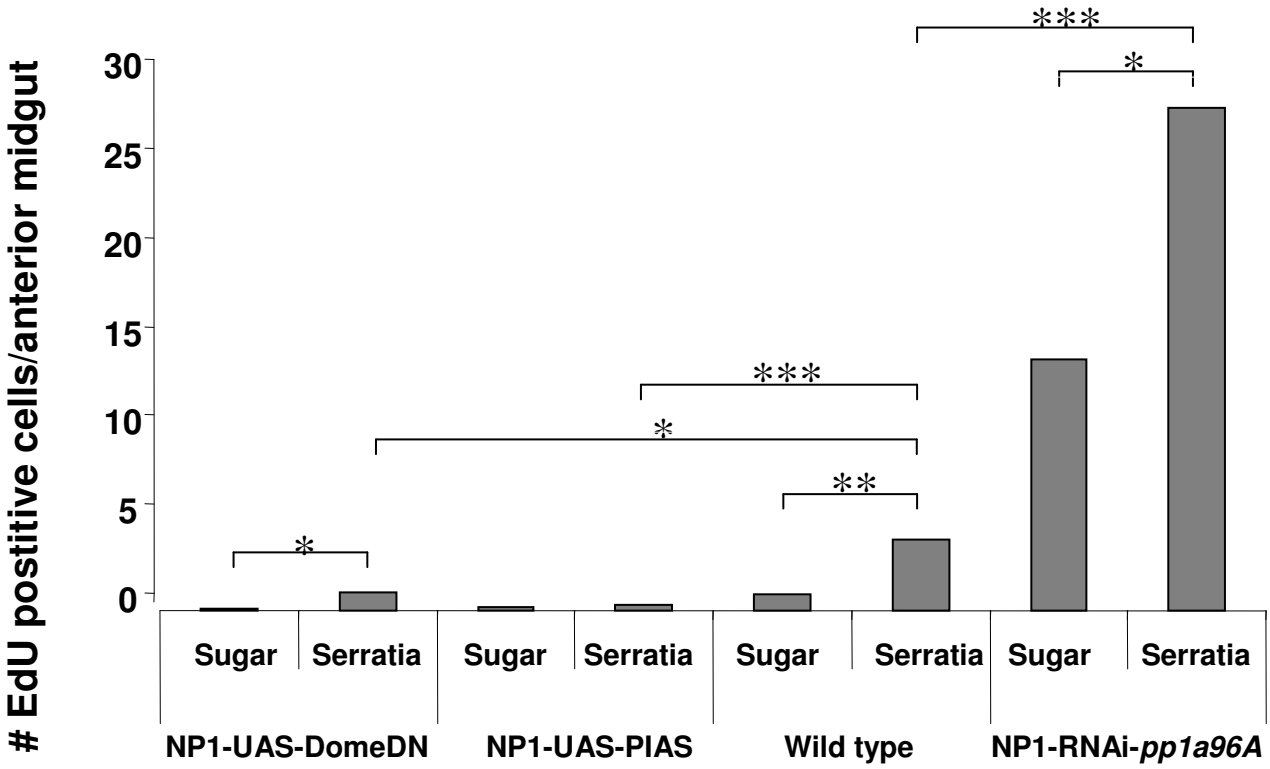


EdU – Green
DAPI - Blue

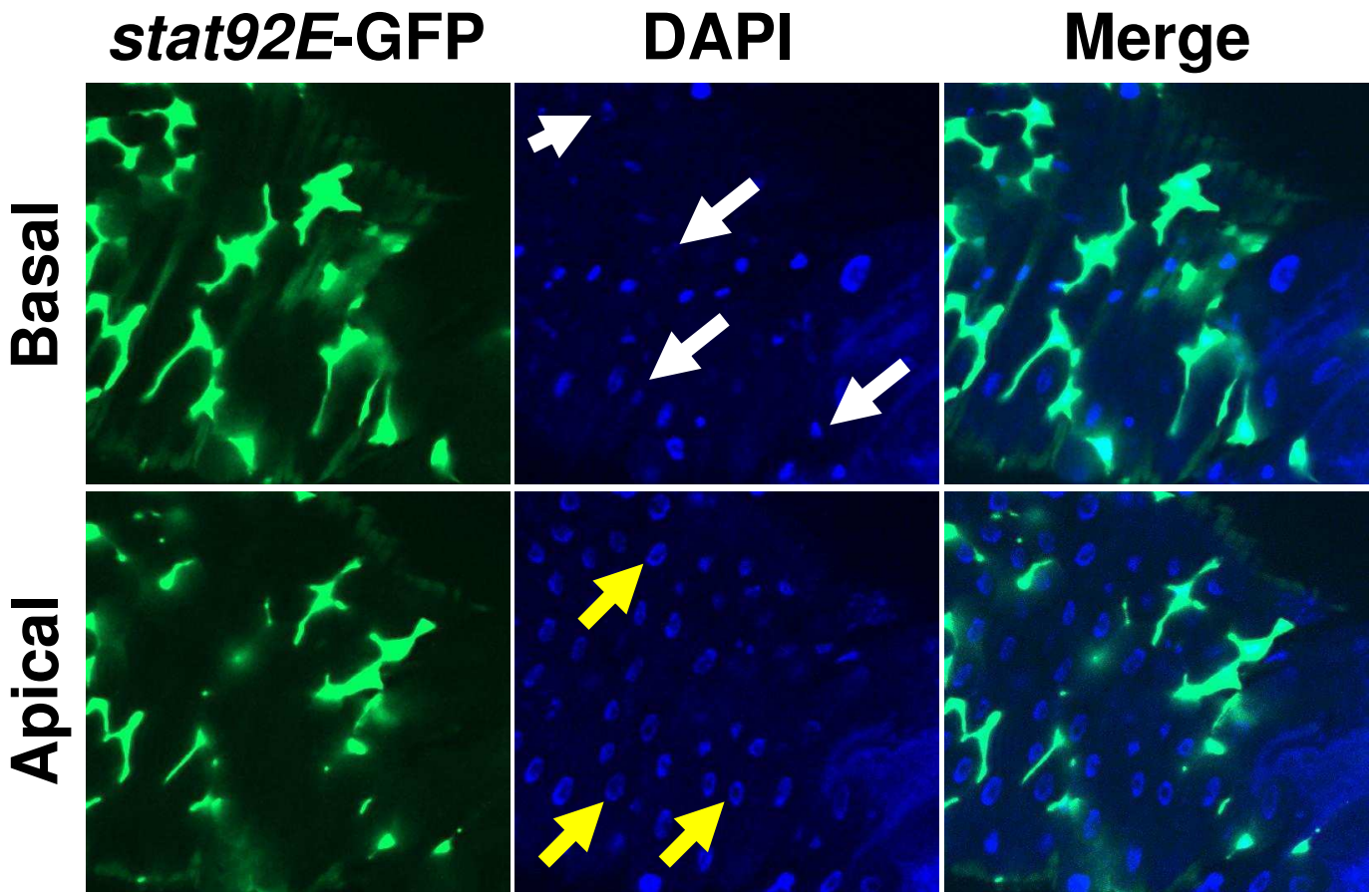
b

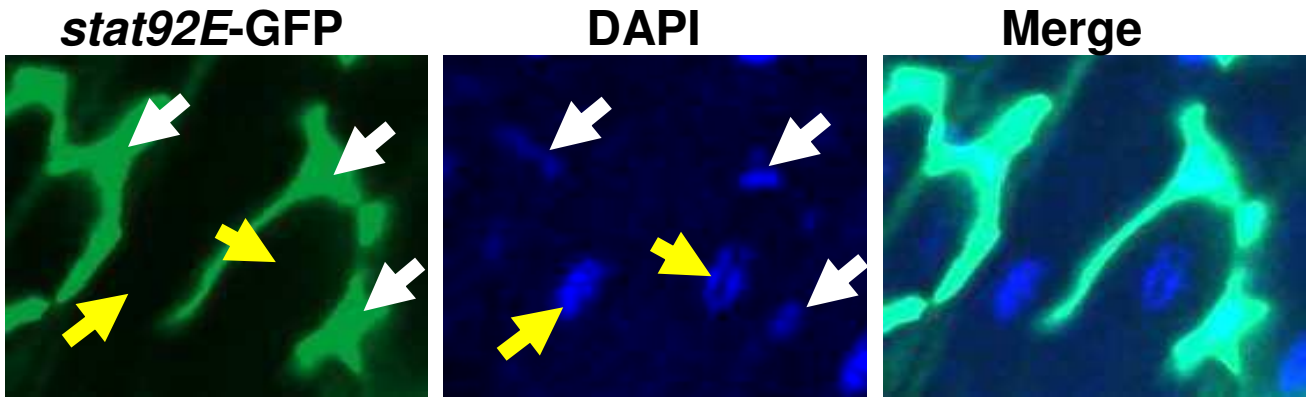


Suppl. Figure S15

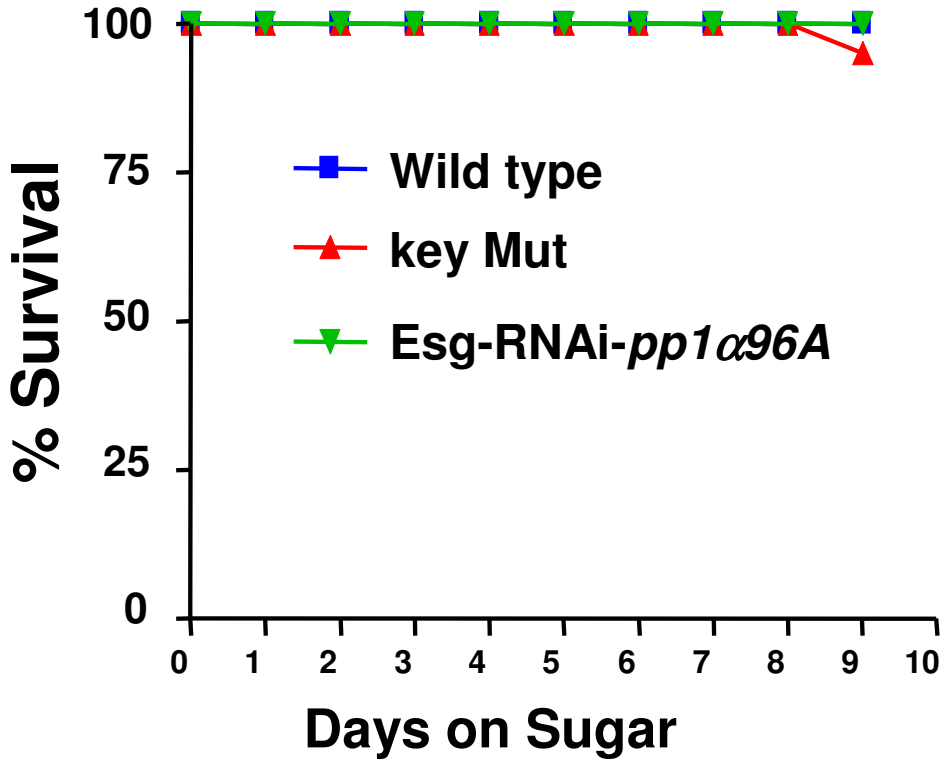


Suppl. Figure S16

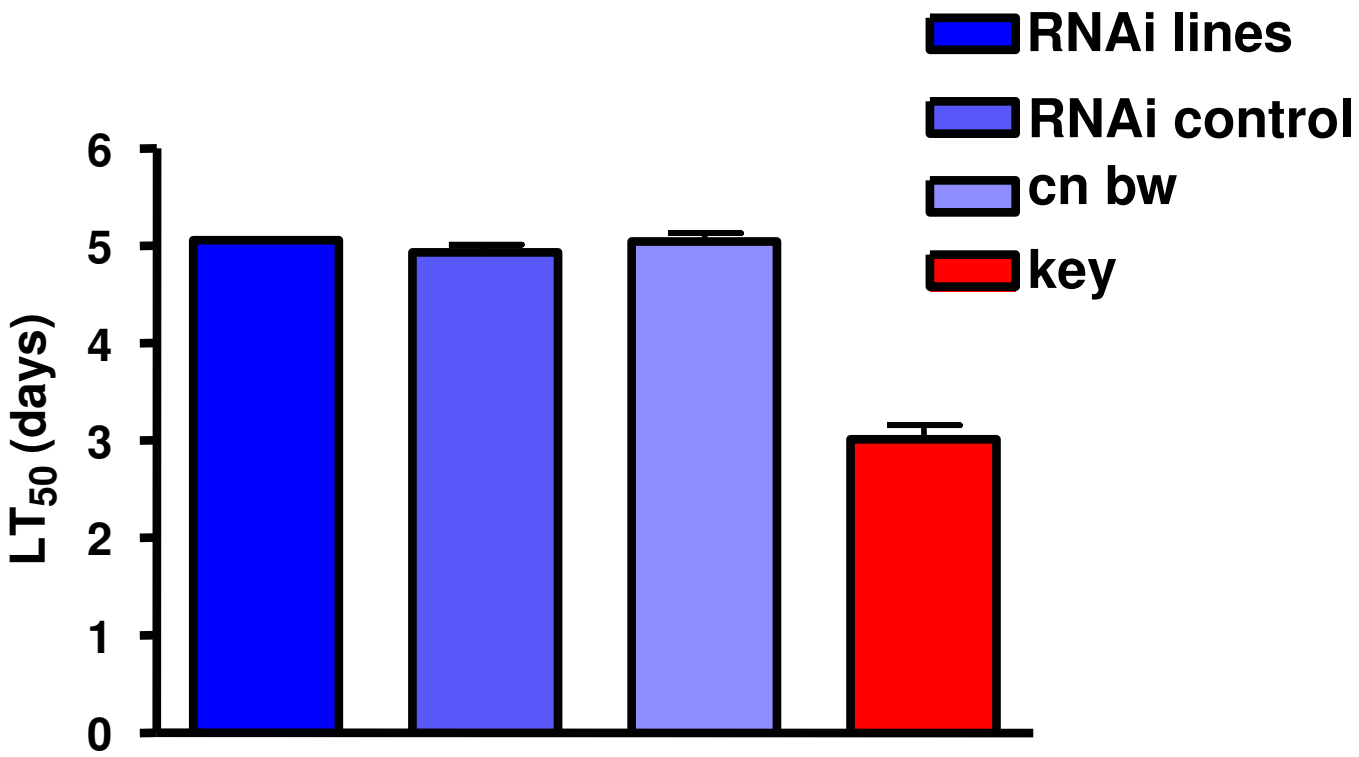




Suppl. Figure S17



Suppl. Figure S18



Suppl. Figure S19

Additional results and discussion

Validation of candidate genes

We found in the genome-wide RNAi screen hundreds of genes that are potentially implicated in *Drosophila*'s defense against oral infection by *S. marcescens*. The secondary screens allowed us to identify the tissues in which some of the genes are required for host defense. I further validated some of the hits using a genetic approach. Indeed, there are two major limitations of the RNAi approach. One is that the construct may affect the expression of other genes, besides the one that is targeted. This off-target effect can be modeled to some extent by a mathematical approach. Each transgenic construct is characterized by a specificity index that reflects the predicted specificity of the construct. The identity of each gene potentially affected by the RNAi construct is known. Of note, constructs with low specificity that target simultaneously hundreds of genes were excluded from the screen. The second limitation is that it is highly likely that the RNAi effect does not fully abrogate gene function and thus yields hypomorphic phenotypes. Therefore, a strategy to validate hits, especially those that were not confirmed by secondary screens possibly because they are required in other tissues, is to study the phenotype of RNAi transgenes in a sensitized background. This is achieved by expressing the RNAi transgene in flies that are heterozygous for a null mutation or a deficiency of the gene of interest. The use of homozygous mutants was not possible for most candidate genes since there were either no mutants available or the mutant or deficiency was homozygous lethal. When the RNAi transgene is not 100% efficient, the inactivation of the targeted gene should be more efficient when just one copy of the gene is present in the fly since one copy should give a lower number of transcripts that need to be targeted by the produced siRNAs. If the RNAi itself was already very efficient one would expect no difference between the phenotype of the RNAi alone and the RNAi in sensitized background (Figure 14). Phenotypes caused by off-target effects should also not be stronger in the targeted gene sensitized background, unless the off-target gene is also removed by the deficiency, which is not the case for the deficiencies we used.

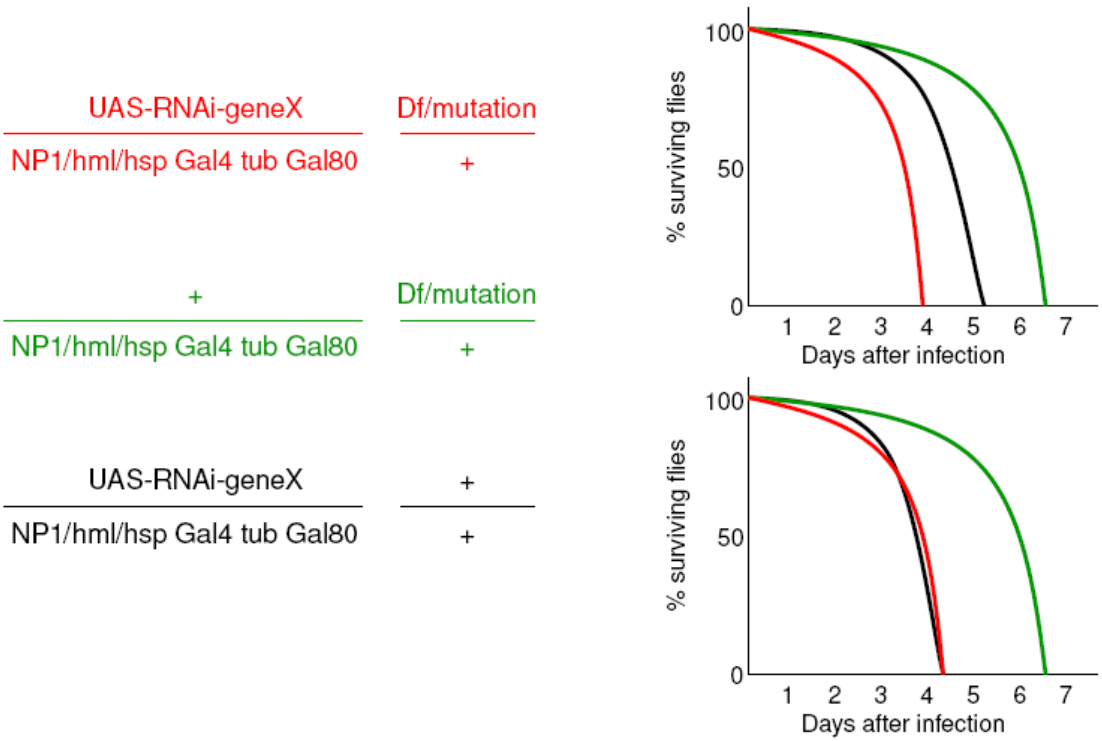


Figure 14: **RNAi in sensitized background**. The scheme shows the different fly genotypes used. The RNAi line in sensitized background is shown in red. As control, a strain carrying one copy of the deficiency (Df) or the null mutation and the driver was used (green). Those fly strains should show wild-type survival. As another control we used flies carrying the RNAi transgene and the driver, which are equivalent to those flies used in the screen (black). RNAi lines that are not very efficient (black) should display an intermediate phenotype between the wild-type control (green) and the RNAi in sensitized background (red, upper right panel). In the case of a very efficient RNAi line or an off-target effect, no difference in survival occurs between the RNAi in sensitized background (red) and the RNAi in wild-type background (black, lower right panel).

I generated the different genotypes shown in figure 14 for a limited number of genes (22) found in the screen to test the validity of this strategy. The approach is very time consuming since a mutation or deficiency needs to be crossed into the background of several transgenes, the RNAi construct, the Gal4 driver and the ubiquitously expressed $Gal80^{ts}$. This requires complicated crossing schemes that differ depending on the transgene insertion sites and the chromosomal position of the mutation or the deficiency (Figure 15). The phenotype that has been found in the primary (heat-shock driver, ubiquitous) and in the secondary (tissue specific drivers) screens was confirmed for 55% of the RNAi lines tested with this approach, that is, flies succumbed to infection earlier than wild-type animals (Table 1). The relatively low rate (55%) of confirmation requires further analysis. One has first to take into

account whether the candidate had been selected for retest using the hemocyte or gut specific drivers, that is that the corresponding genes have mammalian homologs. We found that only four out of nine genes that had not been retested were confirmed. This may be due to a limitation of the hsp driver that, even though it was the strongest available driver we tested turned out to be somewhat variable in its effect. Quite often, flies would be more susceptible in only two out of three experiments. This is one of the reasons that led us to design a strategy of retests using tissue-specific drivers, which yield more reproducible phenotypes. Indeed, we find that for the four genes that were not confirmed in our sensitized background approach, the hsp driver was used for retests (CG16771, CG17065, coro, hsp70Ba).

The RNAi in sensitized background showed an enhanced phenotype in 83% of the confirmed cases. For those 10 genes where the sensitized background leads to a stronger phenotype, it is very likely that the observed effect is due to the targeted gene and not to off-target effects. This genetic approach of validating the hits of the primary and secondary screens appears to work, but unfortunately it is very time consuming. Strikingly, the exact function in host defense of most of the genes retested in sensitized background as well as of most of the other genes found in the primary and secondary screens remains to be established.

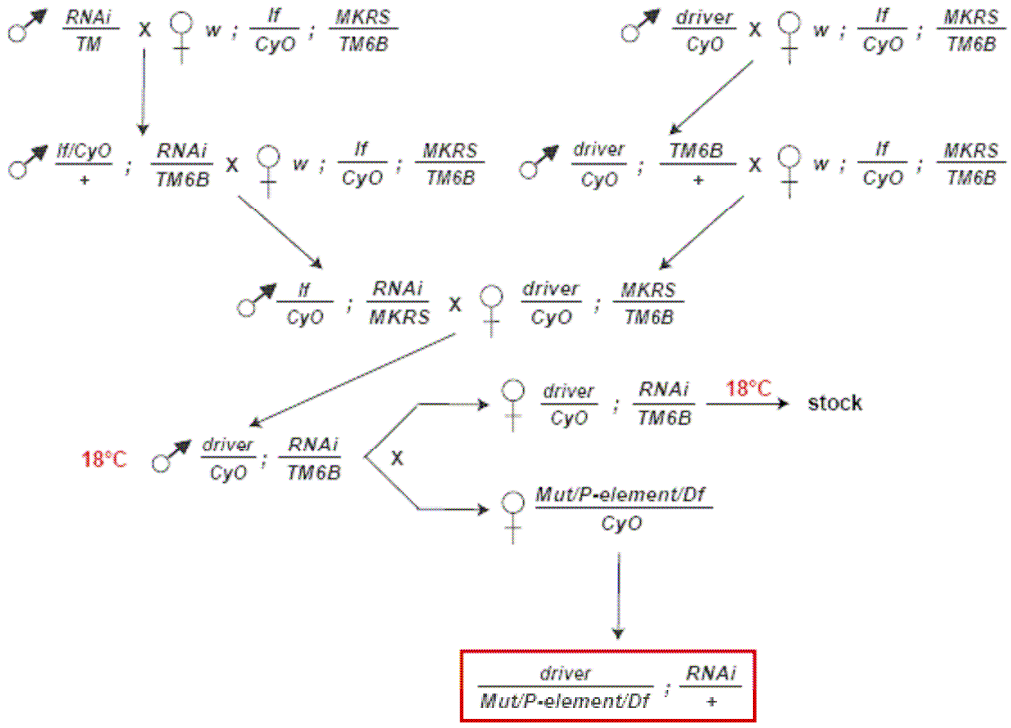


Figure 15: **Example of a crossing scheme**. This is the crossing scheme that was followed to generate flies having a driver (gut/hemocyte/heat-shock) on the 2nd chromosome and a mutation, P-element, or deficiency on the other 2nd chromosome and an RNAi-transgene on the 3rd chromosome. To get these flies four generations are needed. Similar crossing schemes were used for the different combinations of insertion sites and gene locations. The driver-transgenes used for tissue specific RNAi expression (gut, hemocytes) are integrated on the second chromosome. For the heat-shock driver, we have two lines, one having the transgene on the 2nd and one having the transgene on the 3rd chromosome. Therefore, in the case of the gene of interest and the RNAi transgene both being located on the 2nd chromosome, we always used the heat-shock driver on the 3rd chromosome to drive the RNAi-transgene expression. Another very time-consuming possibility would have been to recombine the RNAi-transgene and the mutation/deficiency/P-element or the driver-transgene and the mutation/deficiency/P-element on one chromosome. Depending on the exact positions on the chromosome, it may be very difficult to get recombinants.

Table 1: **Hits retested in sensitized background**. The RNAi lines have all been found as hits in the primary screen using a heat-shock driver. The phenotype found in the primary (heat-shock driver) and the secondary (tissue specific drivers) screens was confirmed for 55% of the RNAi lines tested. Of those 55% 83% showed an enhanced phenotype in sensitized background.

symbol	name	function	off-target predicted genes	hit in tissue specific retests?	driver used for test in sensitized back-ground	phenotype of primary and/or secondary screens confirmed?	phenotype enhanced in sensitized background?
Aprt	Adenine phosphor-ribosyl-transferase	adenine phosphoribosyl-transferase		-	heat-shock	no	no
Best1	Bestrophin 1	chloride channel		hemocytes	heat-shock	yes	yes
CG10858		sodium channel		hemocytes	hemocytes	yes	yes
CG16771		alkaline phosphatase	CG34120	hemocytes	heat-shock	no	no
CG17065		N-acetylglucos-amine-6-phosphate deacetylase	CG14830	hemocytes	heat-shock	no	no
CG17919		Phosphatidyl-ethanolamine binding	CG10298	-	heat-shock	no	no
CG32160		molecular function unknown		hemocytes	hemocytes	yes	yes
CG6074		carbonate dehydratase	CG5543	hemocytes	hemocytes	yes	yes
CG7598		cytochrome complex assembly		hemocytes	heat-shock	yes	no
CG8331		small GTPase regulator	CG4960	-	heat-shock	no	no
CG4757		carboxylesterase activity		hemocytes	hemocytes	yes	no
coro	coronin	actin binding		hemocytes	heat-shock	no	no
hsp70Ba	heat shock protein 70Ba	heat shock-mediated polytene chromosome puffing	CG18743 CG31366 CG7756 CG4264 CG5436	gut	heat-shock	no	no
Med1	Mediator complex subunit 1	RNA polymerase II transcription mediator activity		-	heat-shock	yes	yes
Obp19c	Odorant-binding protein 19c	Odorant binding		-	heat-shock	no	no
PP1α96	Protein phosphatase 1alpha at 96A	protein phosphatase type 1	CG2096 CG5650	gut	gut	yes	yes
Pros 26	Proteasome 26kD subunit	endopeptidase		-	hemocytes	no	no
Pros35	Proteasome 35kD subunit	endopeptidase		-	heat-shock	yes	yes
rhea	Tendrils/talin/ rhea	actin binding		gut	heat-shock	yes	yes
Sec61beta	Sec61beta	protein transporter		-	heat-shock	no	no
Ser12	Serine protease 12	serine carboxypeptidase		-	heat-shock	yes	yes
Spn43Ab	Serine protease inhibitor 43Ab	serine-type endopeptidase inhibitor		-	heat-shock	yes	yes

JAK/STAT pathway and compensatory proliferation

As described in the article above, the JAK/STAT pathway plays an important role in maintaining gut homeostasis during infection. Therefore the JAK/STAT pathway is indirectly implicated in the infectious process. As mentioned in the introduction, other laboratories have also been working on gut homeostasis in the last years. It has been shown that ISCs respond to stress-induced epithelial damage by increasing their division rate (Amcheslavsky *et al.* 2009). In addition, Buchon *et al.* showed that the ingestion of *Erwinia carotovora carotovora* leads to a downregulation of digestive and absorptive functions in the gut, and the expression of immune genes (Buchon *et al.* 2009b). The JAK/STAT pathway is induced in the intestinal epithelium. Infection with *Erwinia carotovora carotovora* leads to the activation of stress-response, tissue repair pathways, and ISC proliferation (Buchon *et al.* 2009b). Afterwards, Jiang *et al.* reported that the gut epithelium is able to recover from damage even when a large portion of the cells is ablated (Jiang *et al.* 2009). They suggest that gut repair is a two-step process, which involves first a size-increase of enterocytes involving rounds of endoreplication resulting in a gut of normal size, but with fewer and therefore larger enterocytes. ISC proliferation also starts at this stage. Second, when the gut integrity is assured, ISCs keep on proliferating to increase the number of intestinal epithelial cells back to normal. JAK/STAT pathway signaling is upregulated in the damaged guts and implements the repair process. Besides triggering compensatory proliferation, it has also been shown that the JAK/STAT pathway is important for enteroblast differentiation into mature enterocytes (Jiang *et al.* 2009). Overall, the JAK/STAT pathway is upregulated in response to gut damage caused by apoptosis, ROS (Buchon *et al.* 2009a), bacterial infection or JNK pathway activation, which is itself triggered by cellular stress and bacterial infection, but not by apoptosis (see also Annex).

Jiang *et al.* overexpressed UPD with the gut specific driver that we also used for our study (NP1, also known as MyoIA), which is expressed in enterocytes. No expression in ISCs could be detected using a UAS-GFP-transgene. The overexpression of UPD in the enterocytes led to the induction of JAK/STAT signaling in ISCs and subsequent proliferation. The overexpression of Hopscotch, the *Drosophila* JAK kinase, in the ISCs (using an esg driver, which is specific of diploid cells and therefore expressed in ISCs and enteroblasts) also triggered proliferation, while blockade of JAK/STAT signaling in ISCs prevented compensatory proliferation.

We found that downregulation of the JAK/STAT pathway in enterocytes using the NP1 driver by expression of either a dominant negative form of the Domeless receptor (DomeDN) or the negative regulator PIAS blocked ISC-dependent compensatory proliferation. In keeping with this, expression of RNAi against the negative regulator PP1α96A led to a higher proliferation of the ISCs. These results are difficult to interpret since the 10xSTAT92E-GFP reporter was just found to be activated in ISCs and not enterocytes, in keeping with the model that damaged enterocytes produce UPD-cytokines to activate JAK/STAT signaling in ISCs and thereby trigger compensatory proliferation. To investigate the role of JAK/STAT signaling in enterocytes it would be important to cross the STAT92E-GFP reporter into a background in which the JAK/STAT pathway is blocked or activated in enterocytes. Thereby it might be possible to visualize JAK/STAT signaling in enterocytes and its possible effects on JAK/STAT signaling in ISCs. A positive feedback loop of the JAK/STAT pathway that triggers the expression of the cytokines that activate JAK/STAT signaling in ISCs would be one explanation for our findings. To my knowledge such a feedback loop has not been reported yet. The alternative explanation is that NP1 is expressed at a low level in ISCs and that the JAK/STAT pathway is exquisitely sensitive to its regulators. The exact level of activation of this pathway would be very important in the control of ISC proliferation.

Surprisingly, flies in which the JAK/STAT pathway is putatively downregulated in enterocytes using the NP1 driver survived longer to the infection than wild-type flies (see article Figure 3F). If compensatory proliferation were completely blocked in these flies, one would expect them to die faster, since they would not be able to deal with the damage caused by the bacteria on the intestinal epithelium as reported by other studies (Buchon *et al.* 2009b; Jiang *et al.* 2009). The 5-ethynyl-2′-deoxyuridine (EdU) incorporation experiments suggest that proliferation is indeed downregulated in the flies, even though it is not blocked completely (see article above and Figure 18).

We do not know what exactly is killing the flies. The compensatory proliferation might at one point be so fast that the newly formed enteroblasts are not differentiating fast enough anymore, which would cause a loss of gut integrity and ultimately the flies' death. If this was the case, slowing compensatory proliferation down, but not blocking it completely, might result in enhanced survival to infection, while enhancing it (by expressing RNAi against the negative regulator PP1α96A)

leads to premature death. Indeed, NP1>PP1α96A RNAi flies displayed a gut with less differentiated enteroblasts (see article Figure 4A).

As stated before, overactivation of the JAK/STAT pathway in ISCs, as in enterocytes, leads to the earlier demise of the flies upon *S. marcescens* infection. Interestingly, the downregulation of the JAK/STAT pathway in ISCs (esg-driven DomeDN or PIAS), in contrast to downregulation in enterocytes, also results in susceptibility to *S. marcescens* infection (Figure 16). The midgut epithelia display in both cases a severe loss of mature enterocytes (Figure 17), as can be seen by the lack of large, polyploid nuclei. When proliferation is blocked the cells that die from *S. marcescens*-induced damage can probably not be replaced, while in the case of overproliferation, differentiation of enteroblasts into enterocytes might not be fast enough.

To test this theory, we performed EdU staining of infected intestines to see how much the ISCs are proliferating in both cases (Figure 18). As expected, the ISCs in which the JAK/STAT pathway is blocked hardly proliferate, probably even less than when the JAK/STAT signaling is blocked in enterocytes using the NP1 driver. To our surprise, the ISCs in which JAK/STAT signaling was presumably upregulated (expression of PP1α96A-RNAi), did not proliferate at all. This result is completely unexpected. Therefore it has to be reproduced to exclude that the wrong transgene has been crossed to the esg driver. If the data were right, it would suggest that PP1α96A, in ISCs, acts as a positive regulator of the JAK/STAT pathway. Compared to the results we got when downregulating PP1α96A in enterocytes, this would mean a change of behavior. This might be possible, *e.g.*, if PP1α96A is acting in a complex with a by now unknown protein. PP1α96A could build a complex with different proteins depending on the cellular context, leading to differential effects on JAK/STAT signaling. To uncover the mechanism by which PP1α96A is acting in enterocytes and ISCs further experiments need to be performed. A first experiment would be to monitor STAT92E-GFP expression in the background of PP1α96A-RNAi expression in enterocytes and ISCs.

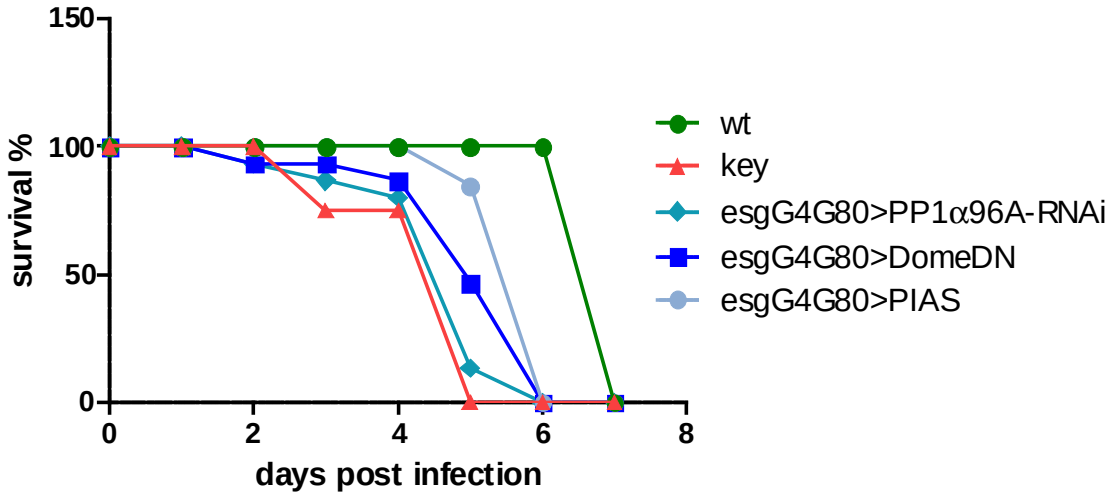


Figure 16: Overexpression as well as inhibition of the JAK/STAT pathway in ISCs using the esg-driver lead to susceptibility to *S. marcescens* infection. As shown before, the overactivation of the JAK/STAT pathway in the ISCs by inhibiting the negative regulator PP1α96A leads to susceptibility to *S. marcescens* Db11 infection (p=0.001; n=3). In contrast to inhibition of the JAK/STAT pathway in enterocytes, inhibition in ISCs by overexpression of a dominant-negative form of the Domeless receptor (DomeDN; p=0.009; n=3) or the negative regulator PIAS (p=0.006; p=3) leads to a susceptibility phenotype. n: number of independent experiments (20 flies used for each experiment). Survival experiments were performed at 29℃.

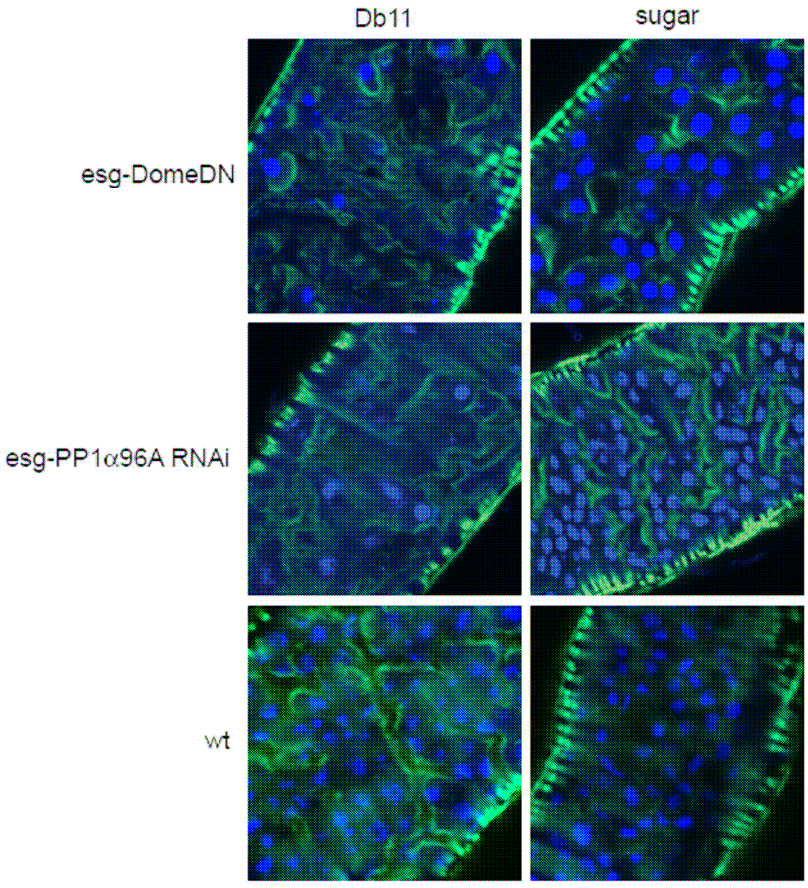


Figure 17: **JAK/STAT mis-regulation in ISCs using the esg-driver leads to the loss of enterocytes in *S. marcescens* infected flies**. Confocal images of dissected anterior midguts. Nuclei are visualized by DAPI-staining (blue) and the actin network of intestinal cells is stained using fluorescein isothiocyanate (FITC)-labeled phalloidin (green). After four days of infection the intestines in which JAK-STAT signaling is misregulated in ISCs show much less differentiated enterocytes (big nuclei) than wild-type guts.

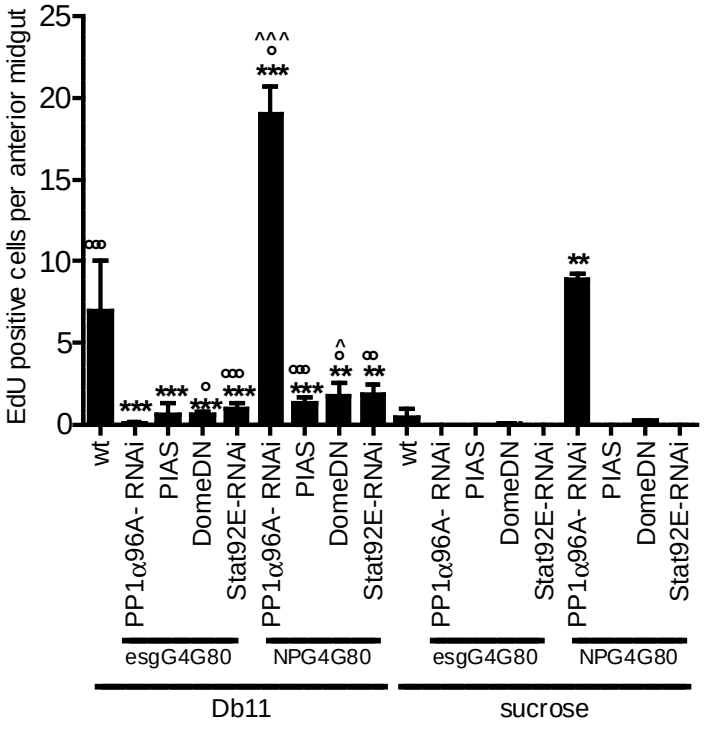


Figure 18: **Proliferation of the anterior midgut epithelium in *S. marcescens* infected flies**. EdU was injected into infected (day four of infection) or non-infected flies three hours prior to dissection. 10 guts per sample were dissected, stained and EdU positive nuclei in the anterior midgut were counted. *p-values compared to wild-type flies having undergone the same treatment (infected or non-infected). ℗p-values comparing infected to non-infected flies of the same genotype. ^p-values comparing NP-driven transgenes to the same transgene driven by esg. */℗/^: p<0.05, **/℗/^^: p<0.01, ***/℗/^^^: p<0.001, n=2. n: number of independent experiments.

Overall, we do not yet really understand the role of JAK/STAT signaling in enterocytes and the mechanisms leading to enhanced survival when JAK/STAT signaling in enterocytes is downregulated. Nevertheless, our data suggest that the fine-balance of JAK/STAT signaling activation in the gut epithelium is crucial for maintaining gut homeostasis in the case of infection.

The JAK/STAT signaling and subsequent proliferation of ISCs is upregulated in response to damage caused by infection, ROS, cellular stress and apoptosis. In the case of cellular stress, and maybe also during some infections, the JNK pathway is triggered in enterocytes, leading to an activation of JAK/STAT signaling in ISCs (Buchon *et al.* 2009a). Stressed enterocytes, in addition, secrete UPD-cytokines to directly activate JAK/STAT signaling in ISCs (see article above and (Buchon *et al.* 2009b; Jiang *et al.* 2009)). Lin *et al.* reported that stem cell self-renewal and proliferation are dependent on Wingless (wg) and JAK/STAT signaling from the intestinal muscles (Lin *et al.* 2010). In the case of *P. aeruginosa* infection, we have detected JAK/STAT pathway signaling in the muscles (see next chapter). The compensatory proliferation of ISCs can be triggered by different means, but it seems to always implicate JAK/STAT signaling. By now however, it remains unknown what triggers JAK/STAT activation in different tissues in the different infection models.

Further research with *Erwinia carotovora carotovora, S. marcescens, P. entomophila,* and *P. aeruginosa* will lead to a better understanding of the mechanisms leading to JAK/STAT pathway-dependent compensatory proliferation in the intestinal epithelium of orally infected *Drosophila*.

The effect of JAK/STAT signaling on survival to infection shows that the ability to tolerate damage caused by an infectious microorganism, is essential for host survival. In contrast to resistance, which is the ability to attack the pathogen or limit its growth, tolerance is the ability to withstand and repair damage caused by the pathogen or possibly by the host's own immune response, as for example in the case of *Erwinia carotovora carotovora* infection that leads to the production of ROS, which harm both the pathogen and the epithelial cells (Buchon *et al.* 2009a). The compensatory proliferation controlled by the JAK/STAT pathway is a mechanism that maintains intestinal integrity despite the damage caused by *S. marcescens*. The fly is thereby able to tolerate the presence of the bacterium to some extent. Resistance combined with tolerance capacities of the host define the outcome of infection. Therefore research should not just focus on resistance mechanisms, but turn also more towards tolerance when aiming to fully understand an infectious process.

2.2 Six hour-long regeneration of the *Drosophila melanogaster* midgut following its partial degradation by ingested *Serratia marcescens*

Introduction

S. marcescens oral infection causes intestinal damage and triggers thereby JAK/STAT dependent compensatory proliferation. From day one of infection onwards, *S. marcescens* constantly damages the gut while ISCs proliferate to replace damaged cells and maintain gut integrity. In the early days of infection, no apparent gut damage could be observed, even though we were able to detect a high number of apoptotic cells (see article above). While having a closer look at the intestinal damage caused by *S. marcescens* ingestion, we found that the midgut epithelium is severely harmed during the first 6-9 hours of infection. The *Drosophila* intestinal epithelium is able to recover with an amazing speed, within the following 6 to 18 hours, leading to a completely repaired epithelium at 24 hours post infection.

Six hour-long regeneration of the *Drosophila melanogaster* midgut following its partial degradation by ingested *Serratia marcescens*

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SUMMARY

Potent virulence factors have been selected in microbial pathogens as they can inflict severe damage to host cells and thus contribute to a successful infection. The intestinal epithelium represents a major frontier of any animal with ingested microbes as well as the endogenous microflora. We have developed an intestinal infection model in *Drosophila melanogaster* in which flies feed on the Gram-negative entomopathogenic bacterium *Serratia marcescens*[1]. Here, we find that ingested bacteria cause extensive morphological and functional damage to the midgut epithelium very early in the infection, within 6 hours. Strikingly, the damaged epithelium seems to be fully regenerated within the next 6 hours. We demonstrate that a secreted cytolysin, *S. marcescens* hemolysin, causes the damage and lyses some enterocytes. The repair process involves, at least partly, the rapid division of intestinal stem cells (ISCs) that subsequently differentiate into hemolysin-resistant enterocytes. Genetically, the regeneration process does not require pathways that have been reported to be important in the control of stem cell proliferation and differentiation in noninfected flies such as the Notch and Wingless pathways. In contrast, regeneration is hampered in mutants that affect JAK-STAT signaling in ISCs. Our findings imply that the intestinal epithelial homeostatic repair mechanisms are much more potent and rapid than previously thought and thus leads to a shift in our understanding of mucosal infections.

RESULTS

Db11 *S. marcescens* is a potent pathogen that kills the fly within 24 hours when injected [1]. Flies feeding continuously on this bacterium succumb only in six days, even though the bacterium is able to cross the digestive tract barrier rapidly and to penetrate the hemocoel [1]. Two major defense mechanisms protect the fly to some extent: i) a local NF-kB-mediated induction of antimicrobial peptide (AMP) genes in the midgut epithelium; ii) Eater-mediated phagocytosis of bacteria that have escaped into the hemocoele, which effectively prevents septicemia [1, 2]. During later stages of the infection, enterocytes undergo massive apoptosis [3]. Yet, the integrity of the epithelium is maintained by the compensatory proliferation of ISCs under the control of the JAK-STAT pathway [3]. Indeed, the midgut epithelium appears to be normal during most of the infection, although a progressive and slow degradation of the epithelium had been documented in other genetic backgrounds. Of note, in the earliest time point examined in the previous study [2], that is 24 hours after the beginning of feeding on *S. marcescens*, we failed to detect any major anomaly in the structure of the infected gut. Fig. 1a shows that, while the intestine of flies three hours post beginning of feeding (pbf) appeared unaffected, it was severely damaged by six hours pbf at 29°C. Namely, the brush border, as visualized by phalloidin staining appeared to be inexistent in some places, or to lie closer to the basal lamina, indicating a much thinner epithelium. Electron microscopy confirmed that the epithelial cells were flattened and a lesser number of large nuclei were observed (Fig. 1b). Strikingly, the strong DNA staining of polyploid enterocytes was reduced to a few nuclei and in many cases had altogether vanished, while small nuclei were still present. The circular and longitudinal muscles that unsheath the midgut appeared to be unaffected (data not shown). It is likely that the few enterocytes that were still present spread out and thus maintained a degree of epithelium integrity. The midgut was still severely affected at 9 hours pbf although in some cases some large nuclei were

observed (Fig. 1b). Thus, there was some variability in the exact timing of the regeneration process depending on the experiments. To assess whether the degradation of the midgut had a functional impact, we determined whether the copper cells that lie in the middle region of the midgut (stomach region) were still able to acidify the luminal content in this region. Bromophenol blue remained blue throughout the lumen at 6 h pbf; yet, at 12 hours, the stomach content was as yellow as in uninfected controls, indicating that the regenerated copper cells had regained their function (data not shown).

We next asked how the midgut regeneration process was taking place. A population of ISCs has been recently described and is characterized by small nuclei in a basal position in the pseudostratified epithelium [4, 5]. These cells, as well as daughter enteroblasts, express the escargot-GFP reporter transgene. As shown on Fig. 2a, esg-GFP positive cells did not disappear during the degradation phase 6-9 h pbf, suggesting that ISCs are not degraded during *S. marcescens* infections. We next determined whether ISCs proliferate to compensate enterocyte loss. Using positively marked mosaic lineage clones (PMML), we observed a significant proliferation of ISCs starting 8 hours pbf (Fig. 2b, white arrows). (Note that not all enterocytes are labeled by this technique). Taken together, these data suggest that at least some of the regenerated enterocytes originated from *de novo* differentiation from proliferating ISCs in a 6 hour time span.

We failed to detect any apoptosis using caspase3 or TUNEL staining during the early degradation phase (data not shown). As a major identified virulence factor of *S. marcescens* is hemolysin, a potent cytolysin able to lyse some cell types by necrosis [6], we tested the 21C4 *S. marcescens* strain, in which hemolysinB is mutated, thus preventing hemolysin A secretion and activation [7]. Strikingly, we no longer observed any early degradation of the midgut epithelium (Fig. 3a and 3b). To determine whether hemolysin is sufficient to induce the observed degradation, we fed flies on transgenic *Escherichia coli*, which had been induced to

express hemolysin A and B (Fig. 3c). As shown in Fig. 3d (upper right panel), midgut epithelial cells were severely damaged as indicated by the loss of large nuclei that characterize differentiated enterocytes. Interestingly, in a few guts, we were able to observe the apparent lysis of the enterocyte nuclei (Fig. 3d, upper left panel). Because we detect apoptosis of enterocytes and not apparent lysis at later stages (from 24 hours pbf onwards), we wondered whether hemolysin was still acting 24 hours pbf. To this end, we fed flies with *S. marcescens* for 24 hours, and then switched the flies to a novel batch of freshly grown *S. marcescens*. Whereas naive flies fed on these fresh bacteria displayed the characteristic degradation of the intestinal epithelium 6 hours pbf, we observed that flies with a regenerated midgut were resistant to the challenge with fresh *S. marcescens* (Fig. 3e). In the converse experiment, feeding naive flies on 24 hour-old bacteria on which other flies had been feeding, we observed no altered midgut epithelia (data not shown) suggesting that hemolysin secretion also decreases with time spent in the feeding solution. These data establish that hemolysin is necessary and, to a large degree, sufficient to cause enterocyte lysis *in vivo*. The subsequently regenerated epithelium appears to be immune to further hemolysin attacks by an unknown defense mechanism, most probably by blocking hemolysin secretion or activation. Since the survival of flies to 21C4 oral infections is similar to that of wild-type *S. marcescens* Db11 (Fig. 3f), we conclude that the regeneration process following the hemolysin dependent degradation is efficient as it does not have an impact on fly survival.

The genetic control of ISC proliferation has been extensively documented, both in normal and stress/pathologic conditions [3, 5, 8-15]. Basal ISC proliferation is controlled by Wingless (Wg) signaling originating from the overlying muscles [15] while differentiation is driven by Notch (N) signaling in enteroblasts induced by Delta expression in the daughter ISCs [4, 5, 12]. We tested *wg* and N thermosensitive mutants, that disrupt embryonic development when grown at the restrictive temperature (data not shown). As shown in Fig.

4a, while midgut epithelial damage appeared somewhat lessened at 6h pbf in *wg* and *N* thermosensitive mutants, we noted a normal regeneration of the epithelium by 12-24 h pbf (Fig. 4a). Surprisingly, these data suggest that differentiation of ISCs can proceed under reduced Notch activity. Previous studies have revealed the important role of the JAK-STAT pathway in driving ISC compensatory proliferation in either stressed, apoptotic, or infected midgut epithelia [3, 8, 13]. We found that the UPD-GFP and UPD3-GFP reporter transgenes, which allow to monitor the expression of two of the three known JAK-STAT receptor ligands in *Drosophila*, were induced during the early phase of the infection (6-9 hours pbf). We did not observe any difference for the 10xSTAT-GFP reporter [16] (data not shown). This could mean that the number of stem cells is not affected, although the kinetics of division would be increased. We next tested mutations that affect the positive or negative regulation of the JAK-STAT pathway. We observed in some experiments a delayed degradation when the JAK-STAT pathway was impaired (Fig. 4d). Thus, while an involvement of the JAK-STAT pathway appears likely in to mediate the compensatory proliferation of ISCs, that mediate, at least partially, the regeneration, we cannot exclude the participation of other regulatory pathway(s).

Our results demonstrate the surprising regenerative properties of the *Drosophila* midgut epithelium, which is able to repair extensive damages in about 6 hours. A previous study documented a 48 hours recovery period following the induction of apoptosis in enterocytes [13].This underscores the remarkable speed of proliferation and differentiation of ISCs, in which multiple rounds of cell division as well as endoreplication are required to generate the desired number of differentiated enterocytes. It also begs the question how the overall number of epithelial cells is regulated. It would be interesting to understand how such rapid cell cycles are molecularly regulated. Finally, it will be worth determining whether mammalian intestines display the same rapid repair properties that may have been overlooked

thus far. A conservation of such a fast regeneration process would change our current understanding of epithelial homeostasis in barrier epithelia, during infections and in cancer.

METHODS

Flies

Stocks were raised on standard cornmeal-agar medium at 25°C. The UAS-PP1-RNAi line was obtained from the Vienna Drosophila RNAi Center (VDRC; https://stockcenter.vdrc.at). The *kenny* (*key*) mutant and *UAS-Diptericin* (M. Ashburner, in *Drosophila. A Laboratory Handbook.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989, p. 1331.), NP1-GAL4[1, 2], HML-GAL4 [9, 17], UAS-PIAS [7], UAS-DomeDN [18], *upd*-GFP [19], *upd3*-GFP [20], *stat92E*-GFP [16] and escargot (esg)-GAL4[4] lines have all been previously described. We incorporated a tubulinGAL80^{ts} transgene onto the HSP70, NP1, and Esg-GAL4 lines. Wg^{ts} and Notch^{ts} [21].

Bacterial strains

S. marcescens Db11, Db11-red and 21C4 are described in (Nehme et al., 2007). The *E. coli* wild-type strain is BL21 (Rosetta), and the *E. coli* BL21-pES14 strain is a kind gift from Professor Volkmar Braun (Max-Planck-Institute for Developmental Biology). The latter is BL21 transformed with the plasmid pES14 that carries the two hemolysin subunits shlA and shlB under the control of the T7 promoter (derived from the vector pT7-5). For expression, an induction of the T7 DNA polymerase gene is done by addition of IPTG [22].

Oral infections

Infection experiments with wild-type flies were all performed at 25°C. Db11 infection protocol is described in [1]. In order to induce hemolysin expression, 1mM IPTG was added to the BL21-pES14 bacterial solution before feeding the flies. As a control, IPTG was also added to the Db11-red and BL21 bacterial solutions before infection.

Wg^{ts} and $Notch^{ts}$ hatched flies were kept for 5 to 7 days at 18°C, then were infected with Db11-red at 29°C.

Immunostainings, fluorescent microscopy and imaging

Dissected midguts were fixed 15 min in 100 mM glutamic acid, 25 mM KCl, 20 mM MgSO4, 4 mM sodium phosphate, 1 mM MgCl2, and 4% paraformaldehyde. Midguts were then permeabilized and blocked for 2 hours in PBS + 0,1% triton X100 + 2% BSA (PBST+BSA) at room temperature, and incubated overnight with primary antibodies in PBST+BSA and then incubated for 1h with secondary antibodies. In some experiments, midguts were then incubated in 10µM FITC-labeled phalloidin (Sigma) in PBST+BSA for 2 h. Midguts were rinced in PBST+BSA at each step. Anti-Phospho-Histone H3 (Ser10) (6G3) antibodies (Cell Signaling Technology) primary antibodies were used in proliferation assays.

Guts were mounted in Vectashield containing DAPI (Vector), and observed under an inverted Zeiss Axiovert 100 M microscope equipped with the LSM510 laser scanning confocal module Images were processed with LSM510 (version 2.5) and ImageJ (version 1.37h) software.

Transmission electron microscopy

Fly midguts were dissected in phosphate buffer 0.1 M (pH 7.2) and fixed with 4% glutaraldehyde for 30 min at room temperature. Samples were post-fixed for 4 h with 1% osmium tetroxide in the same buffer at 4 °C, rinsed, dehydrated through a graded ethanol

series, and embedded in Epon/araldite resin. Ultra-thin sections were contrasted with uranyl acetate and lead citrate. Sections were observed at 60 kV on a Hitachi 7500 transmission electron microscope.

Determination of proliferation

The positively marked mosaic lineage (PPML) strategy was used to induce mitotic clones, as previously described [23]. Briefly, the mitotic clones were induced by two consecutive heat shocks (37°C 20 min – 18°C 30 min – 37°C 20 min – recovery at 25°C during one hour minimum) followed by *Serratia* infection.

DNA synthesis in proliferating cells was detected using 5-ethynyl-2'deoxyuridine (EdU). Briefly, the EdU Click-iT^{TM} EdU Alexa Fluor® 488HSC Assay (Invitrogen) was used to stain the replicating DNA. A 69nL volume of a 0,5mM EdU solution in PBS was injected in the fly hemolymph with Nanoject II (Drumond Scientific). 3 hours after EdU injection, guts were dissected, fixed and stained as described by the manufacturer.

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FIGURE LEGENDS

Figure 1: *Serratia* oral infection causes a rapid degradation of the midgut epithelium

a. DAPI stainings (left panels), phalloidin stainings (middle panels) and merge (right panels, DAPI in blue and phalloidin in green) of A5001 dissected midguts after 6 hours feeding on sucrose (upper panels) or on *Serratia marcescens* Db11 (for 3, 6, 9, 12, and 24h, lower panels). This experiment was performed at 29°C. Scale bar is 50 µm.

b. Transmission electron microscopy pictures of A5001 dissected midguts after 6 hours feeding on sucrose (upper panel) or on *Serratia marcescens* Db11 for 6 hours (lower middle) and for 9 hours (lower panel). The epithelial cell thickness (black double-arrows) and enteroblast nuclei (white arrowheads) are shown. This experiment was performed at 29°C. Scale bar is 20 µm.

Figure 2: Regeneration of the midgut by proliferation of ISCs

a. escargot(esg)-GFP (left panels), DAPI staining (middle panels), and merge (right panels, DAPI in blue and esg-GFP in green) of dissected midguts of esg-GFP expressing flies after 6 hours feeding on sucrose (upper panels) or on *Serratia marcescens* Db11 (for 6, 9, 12, and 24h, lower panels) at 29°C. Scale bar is 50 µm.

b. Mitotic clones were induced (see methods) and observed 8h (left panels) or 20h (right panels) after feeding on sucrose (upper panels) or on *Serratia marcescens* Db11 (lower panels) at 25°C. Scale bar is 100 µm.

Figure 3: Hemolysin is responsible for the early midgut degradation

a. Phalloidin stainings (in green) of A5001 dissected midguts after 6 hours feeding on sucrose (control), on hemolysin mutants *Serratia marcescens* 21C4 (H- S. m), or on wild-type *Serratia marcescens* Db11 (WT S. m). This experiment was performed at 25°C. Scale bar is 50 µm.

b. TEM pictures of A5001 dissected midguts after 6 hours feeding on hemolysin mutants *Serratia marcescens* 21C4 (upper panels) or on wild-type *Serratia marcescens* Db11 (lower panels). This experiment was performed at 29°C. Scale bar is 20 µm.

c. 40µl of supernatants from *Serratia* Db11 (S.m), *E. coli* (E.c), and *E. coli* BL21-pES14 (E.c H+) cultures (OD = 0,1) incubated for 4h with 1mM IPTG, or without IPTG (-IPTG) were

deposited and migrated on a 12% SDS-PAGE gel. The active form of hemolysin (shlA) (160kDa) is shown (black arrow). IPTG induces the expression of the hemolysin active form by the plasmid PES14 (see methods).

d. Merged pictures of DAPI (blue) and phalloidin (green) stainings of A5001 dissected midguts after 6 hours feeding on *E. coli* expressing hemolysin (*E. coli* H+, upper panels), *E. coli* or *Serratia marcescens* Db11 (lower panels). This experiment was performed at 25°C. Scale bar is 50 µm.

e. Phalloidin stainings (in green) of A5001 dissected midguts after 24 hours feeding on wild-type *Serratia marcescens* Db11, followed by 6 hours feeding on fresh wild-type *Serratia marcescens* Db11. This experiment was performed at 25°C. Scale bar is 50 µm.

f. Survival rate of A5001 and *key* mutants, after feeding with Db11 (OD= 0,1) and 21C4 (OD= 0,1), was monitored and expressed in percentage of surviving flies. Infections were performed at 25°C. A5001 die at the same rate when infected either with Db11 or the hemolysin mutant, 21C4 solutions. *kenny* (*key*)/IKKγ mutants are mutants of the IMD pathway.

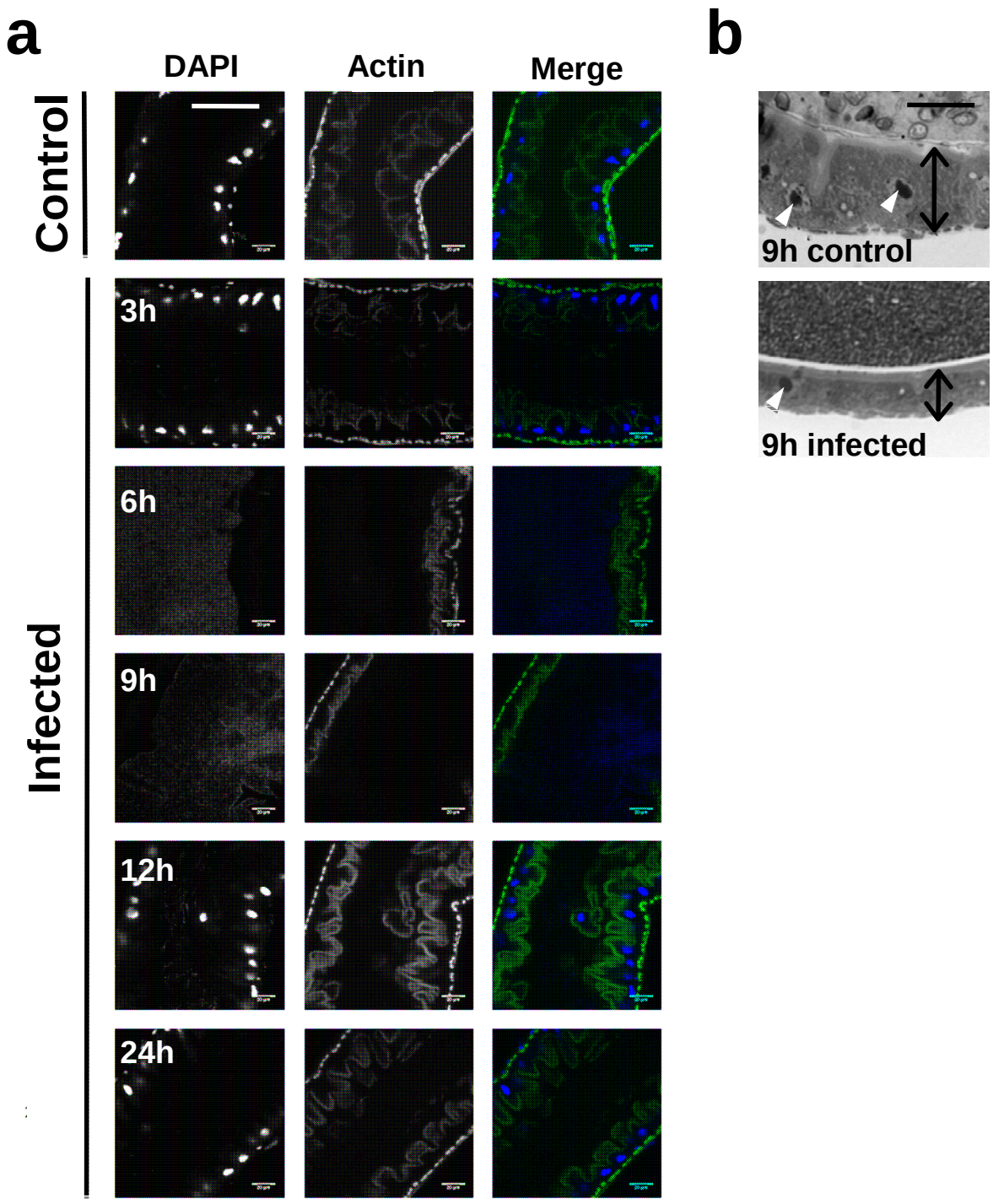
Figure 4: Genetic regulation of the early degradation and regeneration

a. Merged pictures showing DAPI (blue) and phalloidin (green) stainings, dissected midguts after 6h (left panels), 9h (middle panels), 12h (middle panels) and 24h (right panels) feeding on *Serratia marcescens* Db11-red. Flies are A5001 (upper panels), $Notch^{ts}$ (middle panels), and Wg^{ts} (lower panels). In some pictures, bacteria appear red. This experiment was performed at 29°C Scale bar is 50 µm.

b. Dissected midguts of upd3-GFP expressing flies after 6 hours feeding on sucrose (upper pannel) or on *Serratia marcescens* Db11 (for 6, 9, and 24h, lower pannels). This experiment was performed at 25°C. Scale bar is 300 µm.

c. Higher magnification (x63) on upd3-GFP dissected midguts after 6 hours feeding on *Serratia marcescens* Db11 (zone in "b", limited by white bars, second pannel). DAPI stainings (left panels), upd3-GFP stainings (middle panels) and merge (right panels, DAPI in blue and upd3-GFP in green). This experiment was performed at 25°C. Scale bar is 50 µm.

d. The JAK-STAT pathway activation is increasing the kinetics of regeneration: DAPI staining after 6h (left panels), 24h (middle panels) and 120h (right panels) of *Serratia marcescens* Db11 feeding in NP1-DomeDN (upper panels) or NP1-PP1-RNAi (lower panels), showing a delayed degradation when the JAK-STAT pathway is impaired. Scale bar is 50 µm.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3h
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b
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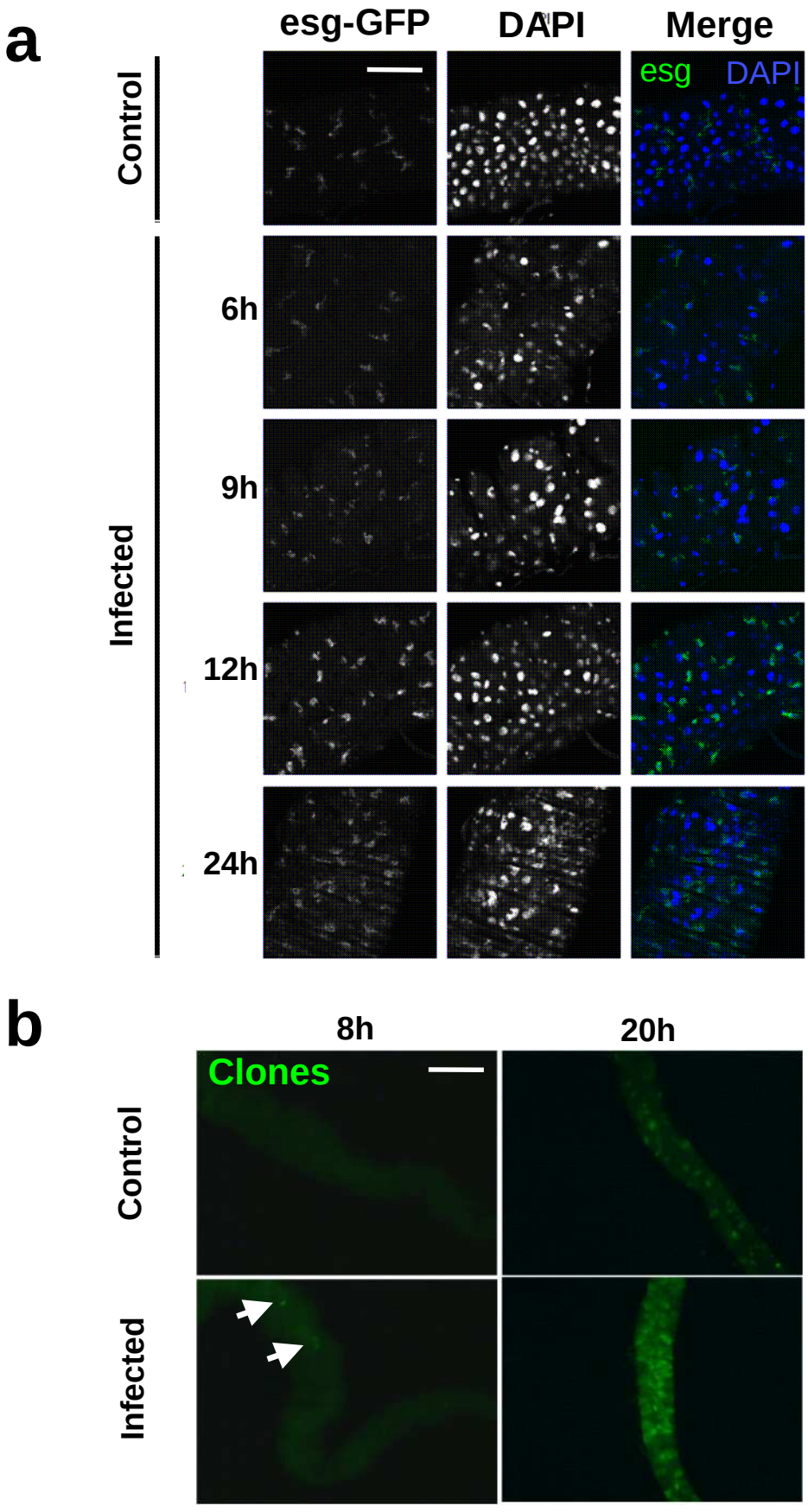
Figure 1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8h
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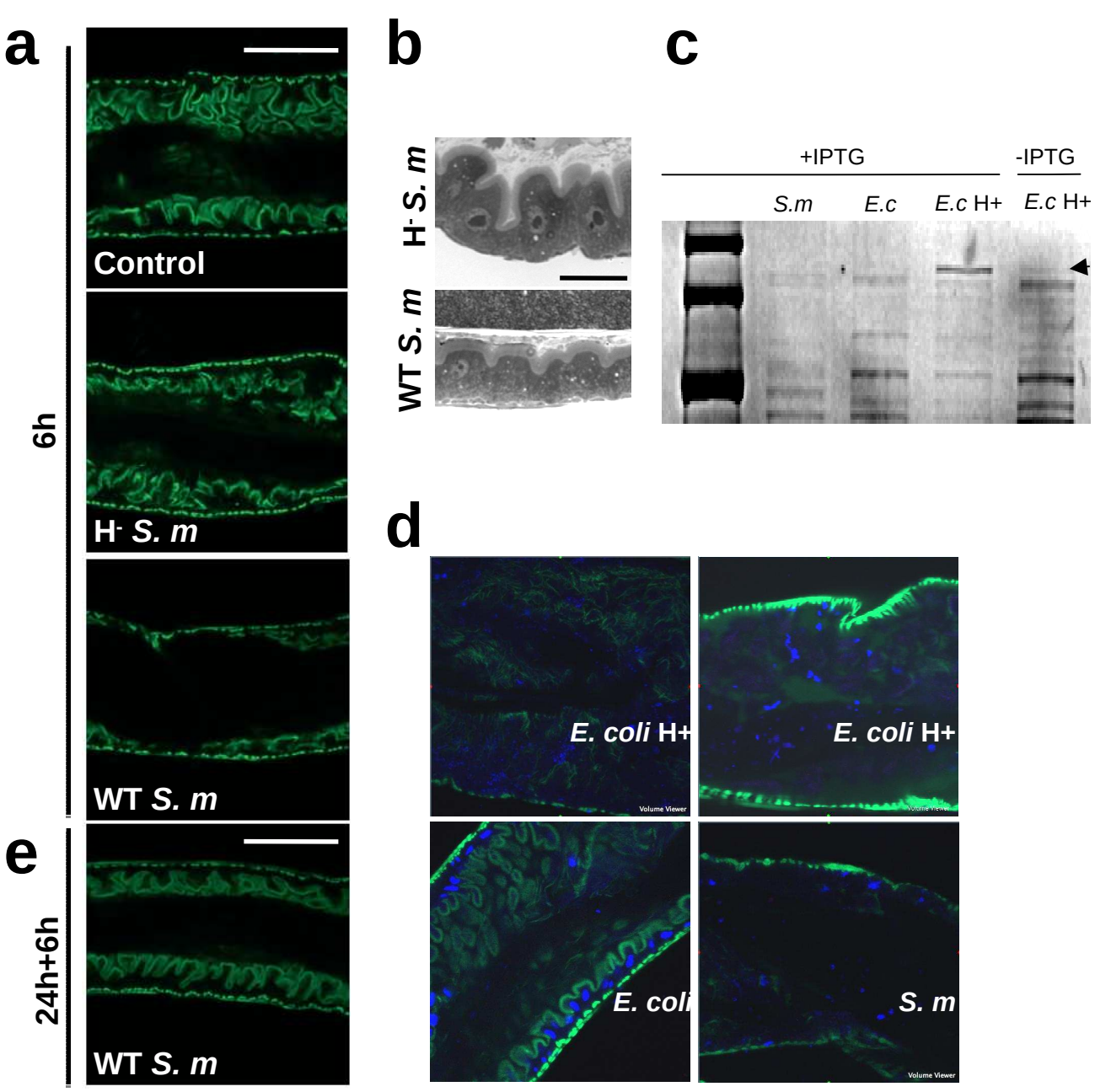
Clones
Control
Infected

Figure 2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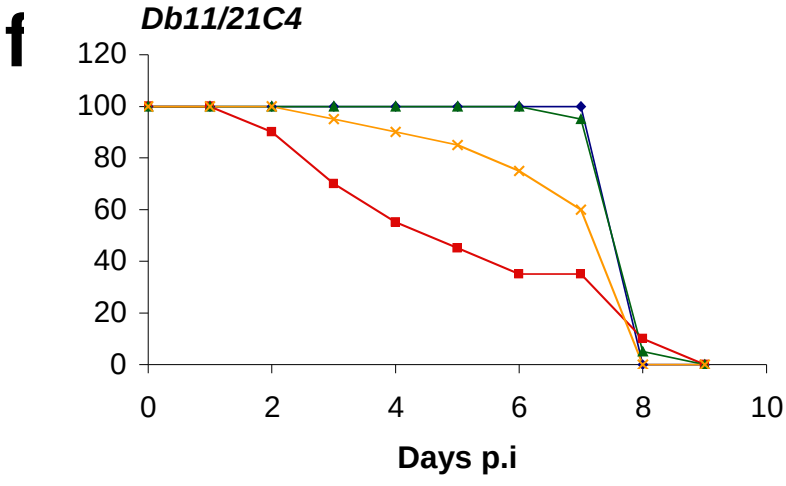
H- S. m
WT S. m
e
24h+6h
WT S. m
b
H- S. m
WT S. m
c
+IPTG
-IPTG
S.m
E.c
E.c H+
E.c H+
d
E. coli H+
E. coli H+
E. coli
S. m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Db11/21C4
120
100
80
60
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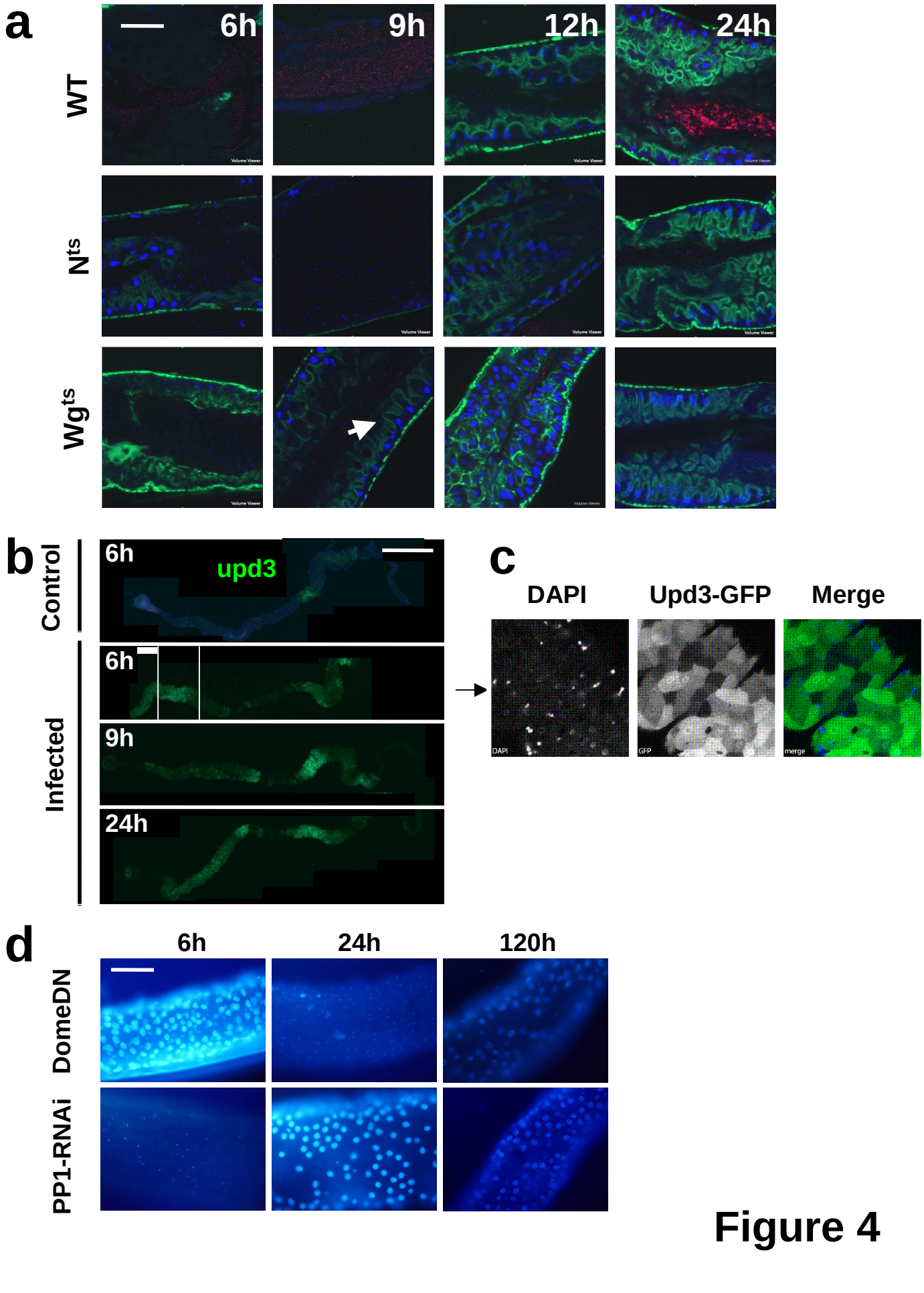
Days p.i
wt 21C4
key 21C4
wt Db11
key Db11

Figure 3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WT
N^ts
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Control
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24h
c
DAPI
Upd3-GFP
Merge
DAPI
GFP
merge
d
6h
24h
120h
DomeDN
PP1-RNAi

Figure 4

Discussion

The *Serratia* hemolysin is a very potent cytolysin. The hemolytic ShlA is secreted and activated with the help of ShlB (see introduction). ShlA then integrates into eukaryotic membranes. This integration is highly dependent on phosphatidylserine, a component of the eukaryotic membrane. Phosphatidylserine, which is absent in prokaryotic membranes has been suggested to be the major feature that allows ShlA to incorporate into eukaryotic and not into prokaryotic membranes (Hertle 2002). The regenerated *Drosophila* midgut epithelium seems to be resistant to the action of hemolysin. To interfere with ShlA-dependent lysis the intestinal cells would need to get rid of the phosphatidylserine in their membranes, which is unlikely, or have to block ShlA activation, likely by secreted proteins. The mechanisms underlying the hemolysin resistance remain unidentified. Further studies will be needed to solve the question of how enterocytes become resistant to lysis by hemolysin.

Another question to be answered is how the rapid regeneration of the intestinal epithelium takes place. There might be an implication of the JAK/STAT pathway (see article). However, it does not seem to be sufficient for epithelial regeneration since midguts in which the JAK/STAT pathway is downregulated in enterocytes using the NP1 driver are still able to recover from the damage inflicted by *S. marcescens*. It would be important to also test flies with misregulated JAK/STAT signaling in ISCs. To repair the gut epithelium completely within a couple of hours the ISCs need to proliferate at an amazing speed, faster than the usual cycling that is needed to renew the gut epithelium under normal conditions, which takes around 8 days (Ohlstein *et al.* 2006). Following *P. entomophila* infection the *Drosophila* intestinal epithelium is able to recover from the damage caused by *P. entomophila* within 2-3 days (Jiang *et al.* 2009). Interestingly, comparable renewal took more than 3 weeks in noninfected flies. Another group found that high doses of *P. entomophila* (OD_{600}=100) fed to the flies block intestinal renewal. Epithelial renewal could be observed when lower doses of the bacterium (OD_{600}=5 or lower) were fed to the flies (Buchon *et al.* 2009a). This suggests that *P. entomophila* is able to interfere with epithelial renewal, probably to promote the infectious process. It has been suggested that two bacterial pathogens of the human digestive tract, *Helicobacter pylori* and

Shigella flexneri, interfere with the intestinal epithelium renewal to exert their pathological effects (Iwai *et al.* 2007; Mimuro *et al.* 2007). The regeneration we observed in the *S. marcescens* oral infection model is even faster than the one observed after *P. entomophila* ingestion and the two are much faster than the epithelial renewal in noninfected flies. Therefore the question arises why the epithelium renewal is very fast in some cases and not in others. As stated before, the activation of ISC proliferation depends on several signaling pathways, including the JAK/STAT pathway, that are triggered by several means. We do not understand completely how the proliferation is triggered in different situations. There might be signals or combinations of signals, probably also bacteria-derived, that lead to a very fast regeneration and others that promote slow renewal. With all the different models that can be used to investigate epithelial renewal of the intestine, ranging from genetic ablation of enterocytes to infection, it will be possible to further investigate the mechanisms underlying fast or slow compensatory proliferation. To figure out whether bacterial signals are involved it will be important to work also with bacterial mutants to decipher the interactions between the gut epithelium and the pathogen.

3 *Pseudomonas aeruginosa* infections

3.1 *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model

Introduction

Drosophila oral infection with *S. marcescens* helped us to identify hundreds of genes involved in antimicrobial defense. The use of tissue specific drivers to express the different RNAi constructs made it possible to identify the tissues in which the different genes are needed during the infectious process. The genes that function specifically in the gut gave us insights into the mechanisms that lead to epithelial resistance and endurance to oral infection. We were able to show that the JAK/STAT pathway plays an important role in dealing with damage caused by ingested bacteria. Functional JAK/STAT signaling is crucial for maintaining gut homeostasis. Unfortunately, methods to genetically manipulate *S. marcescens* are limited. Therefore we were not able to have a dual perspective, from the host side and from the bacterial side, on host-pathogen interactions in this infection model.

For this reason, we decided to use another Gram(-) bacterium, *Pseudomonas aeruginosa*, which has been intensively studied (see Introduction and Annex). Therefore, genetic manipulation of *P. aeruginosa* is well established. We developed an oral infection model that is based on the *Serratia* model, albeit with some differences. To have sufficient difference in survival between *key* mutant flies and wild-type flies without prolonging the overall survival too much, we had to increase the bacterial concentration to $3*10^8$ bacteria per mL (corresponding to an optical density (OD) at 600nm of 0.25), instead of a concentration of $1.7*10^8$ bacteria/mL (OD=0.1) used for *Serratia* infections. In addition, *P. aeruginosa* PA14 was grown in Brain Heart Infusion Broth (BHB) instead of Luria Broth (LB, used for *S. marcescens*). In this model wild-type flies succumb to the infection within 8-10 days. Flies susceptible to the infection, like IMD and Toll pathway mutants (see article below), succumb significantly earlier to ingested *P. aeruginosa* PA14.

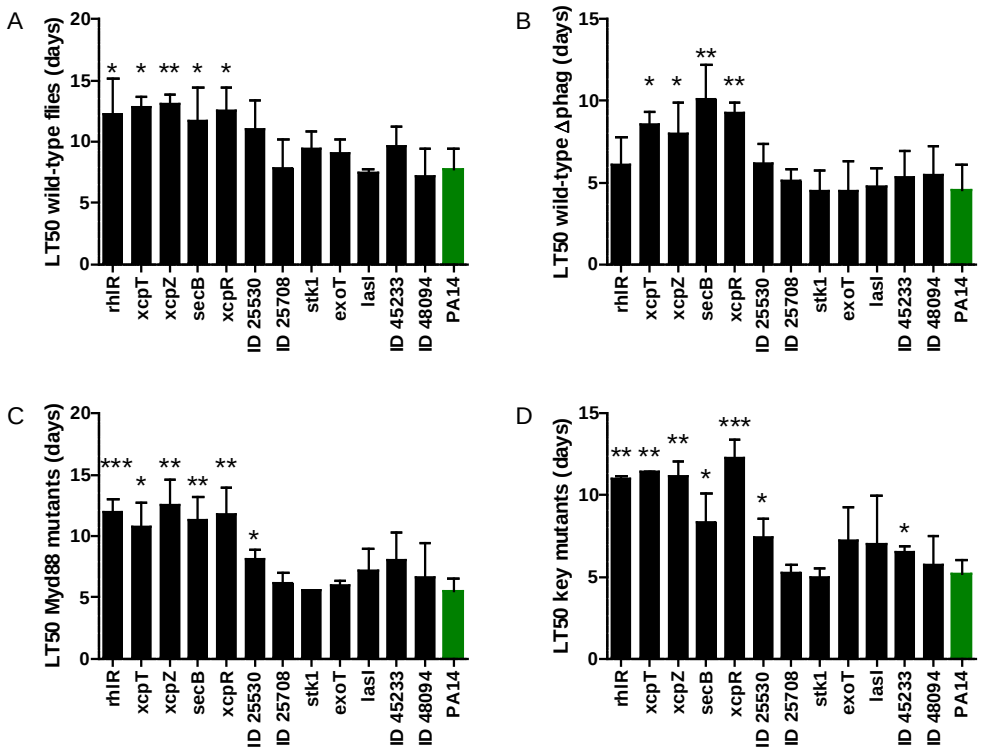

Figure 19: **Survival of wild-type and mutant flies infected with different PA14 mutants**. Wild-type flies (**A**), Latex-bead injected flies (**B**), Toll mutant flies (*Myd88*, **C**) and IMD mutant flies (*key*, **D**) were infected with different PA14 transposon insertion mutants. Survival data are shown as LT50 values plus standard deviation. The LT50 value represents the time at which 50% of the flies had succumbed to the infection. * indicate statistical significance compared to the same fly genotype infected with wild-type PA14; *p<0,05; **p<0,01; ***p<0,001; n=2-3. n: number of independent experiments (20 flies used for each experiment).

Toll pathway mutants, IMD pathway mutants, and flies in which phagocytosis had been blocked through injection of non-degradable Latex-beads succumbed significantly faster to oral infection with the *P. aeruginosa* wild-type strain PA14 (Figure 19). We had access to the transposon insertion library created in the laboratory of Frederick Ausubel (Liberati *et al.* 2006), from whom we received a sublibrary of mutants that showed altered virulence in a *C. elegans* infection model. Of these mutants (~400), we first tested those that have transposon inserted in genes involved in quorum sensing or secretion (Table 2). Toll pathway mutants, IMD pathway mutants, and Latex-bead injected flies were infected with the selected bacterial mutants in parallel to wild-type flies (Figure 19). As mentioned before *Myd88* (p=0.0001, n=22), *key* (p=0.00005, n=22) and Latex-bead injected ($p=8x10^{-7}$, n=9, n: number of independent experiments using 20 flies for each) flies succumbed to infection with wild-type PA14 significantly faster than wild-type flies. Four of the PA14

mutants tested were less virulent in wild-type and all immunosuppressed fly backgrounds (*xcpT*, *xcpZ*, *xcpR*, *and secB*; Figure 19, Table 2). These four mutants have an altered type II secretion system (general secretory pathway; reviewed in (Filloux 2004)). Unexpectedly, a mutant for another putative component of this secretion system (mutant ID 25530) displayed an altered virulence in IMD and Toll pathway mutants, but not in wild-type or Latex-bead injected flies.

Interestingly mutants for *rhlR*, a major component of the Rhl quorum sensing system, displayed an altered virulence in wild-type, IMD and Toll mutant flies, but normal virulence in Latex-bead injected flies. This suggested that the Rhl quorum sensing system is needed to overcome the cellular immune response since *rhlR* mutants regain their virulence in the absence of the cellular immune response. The Las quorum sensing system has been suggested to stand above the Rhl system in the quorum sensing hierarchy (Latifi *et al.* 1996), yet *lasI* mutant bacteria do not show a phenotype in the oral infection model (Figure 19, Table 2). Since the *rhlR* and *lasI* phenotypes are very interesting, we decided to further investigate the role of quorum sensing in the *Drosophila* oral infection model. To this end, we needed first to understand the specific features of this novel oral infection model.

Table 2: **Summary of the phenotypes of the bacterial mutants that were previously associated with quorum sensing or secretion.** The phenotypes in different fly backgrounds are listed. *: p<0.05; **p<0,01; ***p<0,001; ns: not significant (compared to wild-type PA14 in the same fly background)

		wild-type	wild-type Δphag	*Myd88*	*key*
rhlR	acylhomoserine lactone dependent transcriptional regulator	*	ns	***	**
xcpT	general secretory pathway protein G	*	*	*	**
xcpZ	general secretory pathway protein M	**	*	**	**
secB	secretion protein SecB	*	**	**	*
xcpR	general secretory pathway protein E	*	**	**	***
ID 25530	putative type II secretion system protein	ns	ns	*	*
ID 25708	conserved hypothetical protein	ns	ns	ns	ns
stk1	serine-threonine kinase Stk1	ns	ns	ns	ns
exoT	exoenzyme T	ns	ns	ns	ns
lasI	autoinducer synthesis protein LasI	ns	ns	ns	ns
ID 45233		ns	ns	ns	*
ID 48094	putative ClpA/B-type chaperone	ns	ns	ns	ns

Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model

Pseudomonas aeruginosa *RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model*

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ABSTRACT

An in-depth mechanistic understanding of microbial infections necessitates a molecular dissection of host-pathogen relationships. *Drosophila melanogaster* and *Pseudomonas aeruginosa* have both been intensively studied. Here, we analyze infection of *D. melanogaster* by *P. aeruginosa* by using mutants in both host and pathogen. We show that orally ingested *P. aeruginosa* crosses the intestinal barrier and that it proliferates in the hemolymph. Unlike other oral infection models in *Drosophila*, flies succumb to systemic *P. aeruginosa* infection, *i.e.*, bacteremia. Host defenses against ingested *P. aeruginosa* include an Immune deficiency (IMD) response that takes places in the intestinal epithelium, systemic Toll and IMD pathway responses, and phagocytosis of bacteria in the hemocoel by hemocytes. While phagocytosis and the intestinal immune response presumably act throughout the infection, there was a late onset of the systemic IMD and Toll responses. *P. aeruginosa* PA14 does not require its type III secretion system or other well-studied virulence factors such as the two-component response regulator GacA or the protease AprA for virulence in this oral infection model. In contrast, the quorum sensing transcription factor RhlR, but surprisingly not LasR, plays a key role in counteracting the cellular immune response against PA14, possibly at an early stage when only a few bacteria are present in the hemocoel. These results illustrate the power of studying infection from the dual perspective of host and pathogen by revealing that RhlR plays a more complex role during pathogenesis than previously appreciated.

INTRODUCTION

A thorough understanding of microbial infection of complex hosts requires insights obtained from the dual perspective of both host and pathogen. Thanks to its powerful genetics and the lack of an adaptive immune response, the fruitfly *Drosophila melanogaster* is an ideal host in which to study many evolutionarily conserved features of host-pathogen relationships (1). The *Drosophila* host defense response in a septic injury model (in which pathogen cells are delivered directly into the body cavity) relies on the rapid activation of immune defenses including coagulation and melanization, phagocytosis of invading microorganisms mediated by hemocytes, and a potent systemic humoral response involving the production of antimicrobial peptides by the fat body, the insect equivalent of the mammalian liver (1, 2). In the case of bacterial infections, **P**attern **R**ecognition **R**eceptors (PRR) discriminate between two types of peptidoglycan (PGN). Diaminopimelic acid PGN, which is found in the inner cell wall of Gram-negative bacteria (and some Gram-positive bacilli) triggers the **Im**mune **D**eficiency (IMD) pathway in the fat body. The antibacterial action of the IMD pathway is mediated in part by antimicrobial peptides (AMPs) including Diptericin, which is active against Gram-negative bacteria. In contrast, lysine-type PGN, which is found in the outer cell walls of some Gram positive bacteria, leads to the systemic activation of the Toll pathway, another NF-kappaB pathway that functions in parallel to the IMD pathway to activate the expression of a partially overlapping set of immune effectors, including the AMP Drosomycin.

The *Drosophila* defense against infection is not limited to immunity in the body cavity (hemocoel). For instance, intestinal infection models have revealed a role for the IMD pathway in barrier epithelia including the midgut epithelium (3-6). Previously, we developed a *Drosophila* oral infection model with the potent entomopathogenic bacterium *Serratia marcescens*, which is able to cross the intestinal barrier (4). Interestingly, *S. marcescens* loses

virulence in the hemocoel and is controlled by phagocytosis. We identified about 900 *Drosophila* genes that are involved in defense against ingested *S. marcescens* (7). The well-studied human opportunistic pathogen *Pseudomonas aeruginosa* is also a potent *Drosophila* pathogen (8). Because of the extensive genetic tools available for *P. aeruginosa*, including a nonredundant transposon mutation library (9), and the multifaceted nature of *P. aeruginosa* virulence (10, 11), we and others have used *Drosophila* - *P. aeruginosa* oral feeding models to study evolutionarily conserved mechanisms underlying infectious disease (12-16). Here, we address pathogenesis from the dual perspective of host and pathogen by using mutants in both organisms. We find that ingested *P. aeruginosa* strain PA14 traverses the gut barrier and kills its host through a systemic infection. The *P. aeruginosa* quorum sensing regulator RhlR is required for virulence, apparently by allowing *P. aeruginosa* to circumvent the hemocyte-mediated cellular immune response.

RESULTS

Ingested P. aeruginosa *kills the flies by bacteremia in the hemocoel*

We first assessed various parameters that influenced the life span of adult flies fed on a sugar solution (supplemented, or not, with bacterial growth medium) containing *P. aeruginosa* strain PA14. The severity of infection ranged from asymptomatic (PA14 in sucrose only solution) to severe (PA14 in sucrose solution supplemented with bacterial growth medium) (Fig.1A-B & S1). Typically, infected flies died much more rapidly after a septic injury (48 hours; Fig. S2) (8, 14, 17, 18) than they did when *P. aeruginosa* was incorporated into their food. Interestingly, under feeding conditions in which ingested *P. aeruginosa* killed the flies (supplemented with bacterial growth medium), the survival curve declined rapidly after about day 7 with 70% of the flies dying then within two days (Fig. 1A). *P. aeruginosa* PA14 did not appear to persistently colonize the fly intestine despite the

presence of a stable steady-state number of viable bacteria in the intestine when flies were continuously feeding on the pathogen (Fig. S3). Indeed, flies that were fed on *P. aeruginosa* for up to three days did not succumb to the infection when transferred to vials containing only a sterile sucrose solution (Fig. 1C) and actually cleared the bacteria from the gut (Fig. S3). In contrast, flies transferred to the sterile sucrose medium after feeding on PA14 for four days died with similarly rapid kinetics as flies fed continuously on the pathogen, except that killing occurred about a day later, even though the pathogen was cleared from the digestive tract (Fig. 1C & S3). In contrast to an earlier study in which flies were also fed *P. aeruginosa* strain PA14 (15), we observed no significant degradation of the intestinal epithelium nor an increase in intestinal stem cell proliferation even at late stages of infection (one day before the flies started to die) (Fig. S4 and Supplementary text).

P. aeruginosa PA14 was able to cross the intestinal epithelium, although bacteria were barely detectable in the hemolymph after the first day of feeding on PA14 unless phagocytosis was blocked (Fig. 1D). Afterwards, the titer of PA14 increased slowly in the hemolymph of wild-type flies or behaved somewhat erratically in flies with impaired host defense (see below) during the first three-four days while remaining at an absolute level of less than 100 bacteria per fly in the hemolymph. When we injected a similar number of bacteria in the body cavity (septic injury model), 50% of the flies succumbed within 48 hours (Fig. S2). In contrast, a similar number of bacteria is found in the hemolymph of orally infected wild-type flies around day four, yet flies succumb only starting from days seven-eight (Fig. 1A, D). Thus, *P. aeruginosa* in the hemocoel appeared to be initially less virulent than in the septic injury model. Likewise, *S. marcescens*, another Gram-negative opportunistic pathogen able to orally infect *Drosophila*, is much more virulent following a septic injury. *S. marcescens* also crosses the intestinal barrier, but in contrast to *P. aeruginosa*, does not proliferate in the hemocoel (4). Taken together with the lack of persistent colonization of the intestine by *P.*

aeruginosa PA14, the steadily increasing bacterial titer in the hemolymph suggests that orally infected flies die from bacteremia.

***Both the systemic and the cellular immune response are required in the host defense against ingested* P. aeruginosa**

Next, we investigated the different facets of host defense in the *P. aeruginosa* oral infection model. The *Drosophila* IMD pathway responds to diaminopimelic acid PGN released during growth and proliferation from the inner cell wall of Gram-negative bacteria. As expected, IMD pathway mutants succumbed significantly earlier than wild-type flies of the same genetic background (Fig. 1A). The IMD pathway has been implicated in the local immune response that takes place in barrier epithelia as well as in the systemic immune response (3, 5, 6, 19). Indeed, we observed the induction of a *Diptericin-LacZ* reporter transgene in the proventriculus from day one onwards (the proventriculus is the valve-like structure that connects the foregut to the midgut; Fig. 2B-D)(20). In contrast, the expression of the *Diptericin-LacZ* reporter was induced only from day five onwards in fat body lobules in which the systemic immune response takes place (Fig. 2E-G). The bulk of endogenous *Diptericin* mRNA started accumulating at days four-five, suggesting that the majority of *Diptericin* expression occurs in the fat body (Fig. 2A). As expected, *Diptericin* expression was not induced in flies in which the IMD pathway gene *kenny* (*key*) is mutated, but was induced in *Myd88* mutant flies in which the Toll pathway is abrogated (Fig. 2A) (21, 22).

To determine which tissues are functionally relevant to the IMD defense against ingested PA14, we expressed a transgenic wild-type copy of *imd* either in the midgut, in hemocytes, or in the fat body of an *imd* mutant (rescue experiment) or of wild-type flies (overexpression experiment) using the UAS-Gal4 expression system (23). We then monitored survival in multiple independent experiments. To assess the degree of susceptibility to

infection, we computed for each survival experiment the time required to kill 50% of the flies (LT50). The overexpression of the transgene in a wild-type background did not significantly enhance protection against PA14. In contrast, the *imd* susceptibility phenotype was rescued by expressing the wild-type gene in either the midgut, hemocytes, or fat body, suggesting that the IMD pathway can control defense responses in at least three different immune tissues.

Typically, the Toll pathway is not strongly activated by Gram-negative bacteria, but rather by lysine-type PGN found in the cell wall of some Gram-positive bacteria, or by secreted proteases (2) Even though *P. aeruginosa* is a Gram-negative bacterium, it has been shown to induce the Toll pathway, which is required for defense against *P. aeruginosa* in a septic injury model (17). Indeed, consistent with these previous studies, Toll pathway mutant flies such as *spätzle* and *Myd88* were more sensitive to oral *P. aeruginosa* infection (Fig. 1A). In keeping with these survival experiments, a *Drosomycin-GFP* reporter transgene, a Toll pathway activation read-out (24), was expressed in the fat body from day five onwards, whereas it was not expressed at all in the gut (Fig. 3B and data not shown). Similarly to *Diptericin*, the expression of endogenous *Drosomycin* as measured by RT-qPCR also became significant only from day five onwards (Fig. 3A). The expression of a wild-type copy of *Myd88* in hemocytes but not in the midgut was sufficient to rescue the *P. aeruginosa* susceptibility phenotype of a *Myd88* mutant (Fig. 3D). Similar to *imd* overexpression, transgene-mediated activation of the Toll pathway using UAS-$Toll^{10b}$ (encoding a constitutively-active form of the receptor) or UAS-$Myd88^{+}$ in the midgut or the hemocytes prior to *P. aeruginosa* ingestion did not provide enhanced protection against the infection in a wild-type background (Fig. 3D). We could not use the fat body-specific driver *yolk-Gal4* for these experiments since overexpression of *Myd88* or $Toll^{10b}$ was lethal, whether in a wild-type or a mutant background. Taken together, the expression data and the genetic experiments

suggest that in the late stages of the infection process the Toll pathway acts through the systemic immune response to impede *P. aeruginosa* infection.

Phagocytosis constitutes an important arm of host defense in several infection models (13, 14, 25-27). We therefore asked whether the cellular immune response mediated by hemocytes plays an important role in our *P. aeruginosa* PA14 feeding model. We blocked phagocytosis either by injecting nondegradable latex beads (28) or by use of mutant flies deficient for the phagocytic receptor Eater (25, 28). In both cases, we observed a significantly reduced resistance to ingested *P. aeruginosa* (Fig. 1B). Indeed, when using conditions in which ingested *P. aeruginosa* does not kill wild-type flies (sucrose only), we found that *eater* mutant flies succumb to the infection (Fig. 4E-F). Flies in which phagocytosis was blocked displayed a higher bacterial titer than wild-type flies during the second phase of the infection after day 3 (Figs. 1D; 4B). We therefore investigated the possibility that PA14 impairs the phagocytic machinery of hemocytes. Even during the final phase of the infection, however, hemocytes were still able to ingest fluorescein-labeled *Escherichia coli* (Fig. S5), suggesting that hemocytes are present and not impaired in their ability to phagocytose bacterial particles.

Taken together, our data suggest that different host defenses become relevant at distinct stages of the infection. Whereas the local IMD immune response was induced early in the intestine and may limit the crossing of bacteria through the gut epithelial barrier as found for *S. marcescens* (4), the systemic IMD and Toll responses appeared to be triggered by sustained bacterial proliferation and thus were activated only late in the infection. In contrast, phagocytosis restricts bacterial multiplication throughout the course of the infection.

The RhlR but not the LasR acylhomoserine lactone quorum sensing transcription factor is required for the virulence of orally ingested* P. aeruginosa *PA14

To determine which bacterial factors influence the virulence of PA14 in the oral infection model, we challenged wild-type flies with bacteria defective for the type III secretion system (T3SS) (*pscD*) or one of its effectors (*exoT*), the GacA virulence regulator, the AprA alkaline protease, which is important for *P. entomophila* virulence in an oral infection model of *Drosophila* (6), the LasR N-3-(oxododecanoyl)homoserine lactone quorum sensing regulator, the LasB elastase, and the low phosphate response regulator PhoB. All of these bacterial mutants displayed normal virulence (Fig. 4A). In contrast, several independent PA14 *rhlR* mutants, which are deficient for the C4-acylhomoserine lactone-dependent quorum sensing regulator RhlR, the regulator of a second acylhomoserine lactone quorum sensing system in *P. aeruginosa* (29), were severely impaired in virulence and killed the flies three to four days later than wild-type PA14 (Fig. 4A, C-D). Interestingly, flies infected with *rhlR* mutants did not succumb as synchronously as flies infected with wild-type PA14. The survival curve was significantly shallower as quantified by the Hill coefficient, which measures the steepness of a sigmoid curve (Fig. S6). Consistent with their reduced ability to kill flies, the titer of the *rhlR* mutants in the hemolymph reached a maximum of around 100 bacteria per fly at day 6 of infection, and usually was less than 10 per flies at other stages of the infection (Fig. 4B). Thus, *rhlR* bacteria appear to be cleared from the hemolymph. In keeping with these data, the systemic immune response as measured by the accumulation of *Drosomycin* and *Diptericin* mRNAs was hardly induced (Fig. S7A).

RhlR is required to circumvent the cellular immune defense of* P. aeruginosa *orally-infected flies

To distinguish the possibilities that RhlR is required to counteract or elude either or both the systemic humoral immune response or the cellular arm of host defense, we first infected wild-type or *key* or *Myd88* mutant flies with either wild-type *P. aeruginosa* or an isogenic *rhlR* mutant. We found that *rhlR* mutant bacteria killed *key* or *MyD88* mutant flies at

the same low rate as that measured in wild-type flies (Fig. 4C). Regardless of the fact that *key* or *MyD88* mutants did not appear to suppress the avirulent phenotype of the *rhlR* mutant, the titer of PA14 *rhlR* was somewhat higher in the *key* or *Myd88* mutants than in wild-type flies, presumably because the bacteria were not cleared as efficiently from the hemolymph (Fig. S7B). As expected, PA14 *rhlR* mutants did not induce *Diptericin* in *key* mutants, but interestingly, *Drosomycin* was also induced to somewhat lower levels by PA14 *rhlR* in the *key* mutant than in wild-type flies (Fig. S7A). Conversely, PA14 *rhlR* did not induce *Drosomycin* in *MyD88* mutants and *Diptericin* was induced to significantly lower levels than those measured after an oral challenge with wild-type *P. aeruginosa* (Fig. S7A).

Next, we neutralized the cellular arm by the injection of latex beads prior to feeding the flies on *rhlR* bacteria. In striking contrast to *Myd88* or *key* mutants, latex bead-injected flies succumbed almost as rapidly as controls fed with wild-type *P. aeruginosa* (Fig. 4D). In other words, the *rhlR* mutant is highly virulent when the cellular immune response is impaired. In keeping with this finding, *rhlR* mutant bacteria fed to flies in a medium that lacked bacterial broth (sucrose only) killed *eater* mutant flies at the same rate as wild-type PA14 (Fig. 4E). Moreover, the *rhlR* bacterial titer measured in latex bead-treated flies was as high as that of PA14 in wild-type flies (Fig. 4B). These results suggest that RhlR's role in virulence in this infection model is to circumvent the cellular arm of immunity. Interestingly, whether the *rhlR* bacteria were virulent or not in various immuno-deficient flies or in latex-bead injected flies, we noted that the slopes of survival curves were shallower than with wild-type *P. aeruginosa*, indicating that RhlR's requirement in synchronizing the rate of death among infected flies is independent of host defenses (Fig. 4C-D, S5). We also assessed the role or RhlR in a septic injury model. Consistent with the oral feeding model, *rhlR* mutants were significantly less virulent in wild type flies, but not in phagocytosis-deficient, latex

bead-injected flies (Fig. S8), further supporting the idea that RhlR is involved in counteracting phagocyte-mediated defense responses.

Unexpectedly, we found that LasR was required for virulence in flies defective for phagocytosis (Fig. 4F). Thus, *lasR* mutants present a phenotype that is the inverse of *rhlR* mutants: normal virulence in wild-type flies but attenuated virulence in phagocytosis-deficient flies.

DISCUSSION

A fly model of generalized bacteremia following gastrointestinal infection

Drosophila has been widely used as a model host to study *P. aeruginosa* pathogenesis, *e.g.*, (12, 14-18, 30, 31). Here, we used an oral infection model to investigate in detail the interplay between bacterial virulence mechanisms and the host response by using both host and pathogen mutants defective in immunity or virulence, respectively. In previously described *Drosophila* oral infection models using *P. aeruginosa*, it may be assumed that flies die because of local damage to the intestine, *e.g.*, (12, 16). This inference appears to be supported by a recent study that reported extensive damage to the *Drosophila* midgut following feeding on *P. aeruginosa* strain PA14, the same bacterial strain used in our study (12, 15, 16) (see also Supplementary Text for a discussion of the distinction between different feeding models and bacterial damage to the gut). In contrast to this previous study, however, in our study, which utilized different *Drosophila* strains, we did not observe any extensive damage to intestinal epithelial cells. Thus, in the particular model described in this paper, it is unlikely that flies succumb to intestinal damage. Rather, our results show that some ingested *P. aeruginosa* can cross the peritrophic matrix and the underlying intestinal epithelium (a conclusion that can also be drawn from the study reported in Ref (14)) and cause a systemic infection as evidenced by the high bacterial titer measured in the hemolymph prior to death

(Fig. 1), akin to human infections caused by foodborne pathogens (32, 33). In support of this conclusion is our finding that flies succumb to a bacteremia when they are fed on *P. aeruginosa* for four days and are then transferred to sterile feeding solution, even though the bacteria are quickly cleared from the gut after being transferred (Fig. 1C & S3). Thus, this oral infection model in *Drosophila* provides a paradigm in which to study intestinal infections that can lead to bacteremia.

Two phases of infection and a switch in the virulence program controlled by the RhlR virulence regulator?

Several studies with *Drosophila* and other insects have shown that very low numbers of *P. aeruginosa* cells (as few as 1 to 10) introduced into the body cavity by microinjection or pricking are able to rapidly multiply, causing a lethal bacteremia over the course of about two days (14, 17, 18, 34). The behavior of *P. aeruginosa* in our oral feeding model is markedly different. The bacterial titer in the hemolymph remains low during the first phase of the infection (Fig. 1D, S6B)). This may reflect a low virulence state of the bacteria that cross the gut barrier as described previously for *S. marcescens* (4), or it may reflect the ability of humoral or cellular immune defenses to initially cope with the invading bacteria. Consistent with the observation that *P. aeruginosa* do not proliferate to high numbers in the hemolymph during the first few days of the oral infection model, a systemic immune response is significantly induced only at day five of feeding, when the bacterial titer in the hemolymph has increased significantly (Fig. 2,3). We note that a systemic immune response is induced earlier in the infection in immunodeficient flies (Fig. 2,3), in which case the bacterial titer also increases more rapidly (Fig. 1D).

Because, DAP-PGN is not exposed on the surface of Gram-negative bacteria, they cannot be detected by *Drosophila*'s PRRs unless the bacteria proliferate and release small

PGN fragments generated during cell wall remodelling (4). One explanation of our data showing that a systemic immune response only occurs after five days of feeding (Figs. 2, 3) is that the *P. aeruginosa* cells that initially cross the epithelial barrier into the hemolymph are in a relatively avirulent state but eventually switch to high state of virulence (see Fig. 1D and 4B). Alternatively, or concomitantly, the late onset of systemic immunity may reflect the gradual influx of bacteria through the gut into the hemolymph until they reach sufficiently high numbers to overcome local and phagocytic defenses. Finally, it is possible that the *P. aeruginosa* cells in the intestine or the few that translocate into the hemocoel actively suppress the systemic immune response as has been observed in a septic injury model with *P. aeruginosa* PA14 (31).This latter hypothesis may appear somewhat unlikely given the low number of bacteria retrieved in the hemolymph during the early phase of the infection. The food transfer experiments, in which flies succumb to infection only if they have been kept on a PA14 solution for at least four days (Fig. 1C), are consistent with either of these models. Because adult hemocytes are phagocytically active throughout the course of the infection and because the systemic immune response is not activated in the first phase of the infection, phagocytosis may be the main active defense during the early phase. In summary, there seems to be two phases in the infection. In the early phase, bacteria cross the gut barrier and are most likely controlled efficiently in the hemocoel by phagocytosis. In the late phase, *P. aeruginosa* PA14 is able to resist at least partially the cellular immune response and then starts proliferating rapidly in the hemolymph, thus activating a systemic immune response, which in turn slows the infection process.

Genetic analysis of host-pathogen interactions yield novel insights into the in vivo roles of* P. aeruginosa *quorum sensing virulence regulators

By employing genetic mutants in both partners of an infectious host-pathogen relationship, we have been able to reveal unexpected *in vivo* roles for two global regulators of

P. aeruginosa virulence, the transcription factors RhlR and LasR. RhlR is the major regulator of C4-homoserine lactone quorum sensing, one of two quorum sensing systems in *P. aeruginosa*, which is itself under environmental control (29, 35). Our data show that RhlR plays a key role in the oral infection model as *rhlR* mutants display strongly attenuated virulence (Fig. 4C). However, RhlR is unlikely to be required for passage through the intestinal barrier because *rhlR* mutants kill phagocytosis-deficient flies as rapidly as wild-type PA14 (Fig. 4D-E).

It has been proposed that *P. aeruginosa* partially inhibits the systemic AMP response induced in the septic injury model (31). We do not think, that RhlR is responsible for this virulence function: RhlR mutants displayed an attenuated virulence phenotype in both IMD and Toll pathway mutant flies (*key* and *MyD88* mutants; Fig. 4C), arguing that NFkappaB-independent defense mechanisms contain the infection in these cases. Furthermore, we did not detect enhanced induction of AMP gene expression when wild-type flies were infected with *rhlR* mutant bacteria (Figure S6A), suggesting that RhlR is not involved in suppressing the AMP responses. Rather, the decreased induction of the AMP genes likely reflects the reduced ability of *rhlR* bacteria to proliferate *in vivo* (Fig. 4B).

In contrast to the results obtained with IMD and TOLL pathway mutants, RhlR function is dispensible when the cellular immune response is impaired (Fig. D-E). These data suggest that an essential *in vivo* function of RhlR is to circumvent phagocytosis of *P. aeruginosa* by professional phagocytes. However, in contrast to the *P. aeruginosa* toxins secreted by the type III secretion system, such as ExoS, RhlR is not required to impair the general phagocytic activity of hemocytes since heat-killed *E. coli* appeared to be ingested normally (Fig. S5). In light of the three explanations delineated above for why *P. aeruginosa* initially fails to proliferate in the hemolymph and fails to activate a systemic immune response, it is possible that RhlR is required at a critical period during the infection to protect

P. aeruginosa bacteria from phagocytocytic clearance by an unknown mechanism. Interestingly, RhlR function appears to be required at a relatively early time in the infection process when the bacterial titer in the hemolymph is rather low (about 100 per fly) (compare Fig. 4B to Fig. 4D). Classical quorum sensing would not be expected to be activated at this low bacterial concentration.

When either wild-type flies or flies with an impaired immune function were infected with a *P. aeruginosa rhlR* mutant, they exhibited a shallow survival curve. In contrast, flies that had ingested wild-type PA14 died in a more synchronized manner. Thus, RhlR seems to play an important role in the coordinated onset of pathology in the population of infected flies as a whole, which may be related to its classical role in quorum sensing. In the absence of RhlR, bacteria may be behaving in a more erratic manner because of a lack of coordination of bacterial virulence properties through quorum sensing.

In contrast to RhlR, the transcriptional regulator LasR, which controls 3-oxo-C12-homoserine lactones, is not required for virulence in wild-type flies (Fig. 4A and 4F). *lasR* mutants display a phenotype that is the opposite of *rhlR*; *i.e., lasR* mutants, unlike *rhlR* mutants, are attenuated in phagocytosis-impaired flies (Fig. 4E-F). Classically, it has been proposed that the two acylhomoserine lactone quorum sensing systems of *P. aeruginosa* function in a hierachical order, with the LasR system on top of the RhlR regulon (29). However, it has recently been shown that RhlR can control the expression of LasR-specific factors independently of LasR and conversely (35). Furthermore, quorum-sensing systems themselves have been shown to also be under environmental control (36). Thus, our study underscores the necessity to functionally dissect the role of virulence factors *in vivo*, in both, immunocompetent and immunocompromised hosts to obtain insights into their complex regulatory roles in pathogenesis. Indeed, the finding that LasR is required for virulence in phagocytosis-deficient flies reveals a subtler LasR function that may be masked in wild-type

flies. Thus, bacterial screens for avirulent mutants in host sensitized background are likely to yield novel insights that may not been gained using just wild-type host organisms. This, and many other features of this study, highlight the usefulness of model organisms to study infectious disease.

MATERIAL AND METHODS

Survival experiments: An overnight culture of bacteria was centrifuged (4000 rpm, 10min, 4°C) and diluted in fresh Brain-Heart-Infusion Broth (BHB) to obtain a solution of OD_{600}=2.5. This solution was then diluted 10 times with a sterile 50 mM sucrose solution to OD_{600}=0.25. Two absorbant pads (Millipore AP1003700) were placed at the bottom of clean medium-size vials (3.5 cm diameter) and 2 mL of bacterial solution were added to the filters prior to the introduction of about 20 flies, which had been feeding on a 50mM sucrose solution for two days at 25°C. Survival experiments were performed at 25°C and 50% humidity and the number of surviving flies was monitored. For overexpression and rescue experiments, flies were first incubated at 29°C for 48h (on flyfood (37)) prior to infection, to inactivate Gal80 and allow for strong Gal4 activity.

For experiments using the oral infection model under conditions in which wild-type flies are not killed (Fig. 4E, F and Fig. S1), bacteria were centrifuged and washed in phosphate-buffered saline. The pellet was then diluted with 5% sterile sucrose solution to an OD_{600} of 0.1 and 7 mL aliquots of this medium were pipetted onto sterile cotton balls placed at the bottom of empty fly culture vials.

For statistical analysis, because each of the survival experiments described in this paper have been performed multiple times and because LogRank analysis can compare only two survival curves at a time in the same experiment, we decided instead to compute the LT50 (see below) and then perform statistical analysis on the LT50s using the Student t test.

As the slope of survival curves is reproducible from experiment to experiment (Fig. 4E and Fig. S6B), it is legitimate to use this approach.

Calculation of Hill coefficient and LT50 values: Hill coefficient (HillSlope) and LT50 (logEC50) were calculated with GraphPad Prism® 5 software. Values shown are absolute values. Statistical analysis was performed using the Student t test.

Further descriptions of Materials and Methods may be found online in Supplementary Material.

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FIGURE LEGENDS

Figure 1: **Systemic and cellular immune responses contribute to host defense against orally ingested *P. aeruginosa* PA14**

A-**B**: Survival following PA14 oral infection. IMD pathway mutants (*imd* (p=0.0003, n=8), *key* (p=0.00005, n=22) and Toll pathway mutants (*Myd88* (p=0.0001, n=22), *spätzle* (*spz*) (p=0.01, n=4)) succumbed faster to the infection than wild-type (wt) flies (A). Flies defective for phagocytosis (*eater* (p=0.01, n=3), latex bead-injected flies: wtΔphag ($p=8x10^{-7}$, n=9)) also died faster than wt (B). **C:** Flies were either fed continuously or fed for the indicated period on the bacterial solution and then fed on a sterile sucrose solution that was changed daily; survival data are shown. At least four consecutive days of feeding were required to develop a lethal infection. **D**: Bacterial titers measured in the hemolymph collected from

batches of 10 flies in seven independent experiments is shown on a logarithmic scale. The values shown correspond to the bacteria titer per fly. Error bars are standard deviation.

Figure 2: **An early-activated local IMD response and a late systemic IMD response both contribute to host defense against orally ingested *P. aeruginosa* PA14**

A: qRT-PCR analysis of the induction of *Diptericin*, a classic IMD pathway readout, in infected flies. Results are expressed as a percentage of the induction measured six hours after a septic injury challenge with *E. coli*. p-values (*) refer to the comparison between infected and non-infected flies of the same genotype: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n=7. p-values (°) refer to the comparison between mutant and wild-type flies at the same day of infection: ° $p<0.05$; n=7. **B-G**: ß-galactosidase staining of *Diptericin-LacZ* flies. *Diptericin* is induced in the proventriculus (arrows) throughout the infection (B-D), whereas systemic *Diptericin* induction in the fat body (arrowheads) of the fly occurs in later stages of the infection (E-G). **H**: Rescue of the *imd* PA14 susceptibility phenotype by overexpression of a UAS-imd^{+} transgene (>IMD) with a gut (NPG4G80), a hemocyte (hmlG4G80), or a fat body (ylkG4)-specific driver as documented by the average time it takes to kill 50% of the flies (LT50). Note that AMPs synthesized in hemocytes and the fat body are secreted into the hemocoel. In this series of experiments, wild-type flies succumbed somewhat earlier than usual. p-values compared to *imd* mutant flies * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n=5. Error bars are standard deviation. Scale bars: B-C: 200µm; E-G: 250µm

Figure 3: **Late Toll pathway activation contributes to systemic host defense against orally ingested *P. aeruginosa* PA14**.

A: qRT-PCR analysis of the induction of *Drosomycin*, a classical read-out of Toll pathway activation, in infected flies. Results are expressed as a percentage of the induction measured

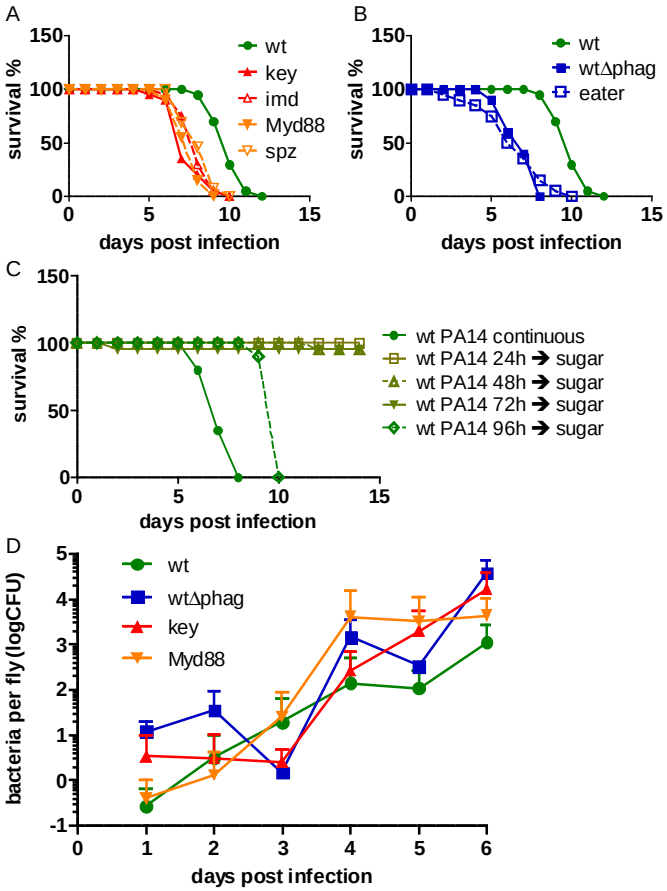
24 hours after a septic injury challenge with *M. luteus.* p-values (*) refer to the comparison between infected and non-infected flies of the same genotype: * p<0.05; ** p<0.01; *** p<0.001; n=6. No significant difference was observed between wt and *key* flies with respect to *Drosomycin* expression levels. **B-C**: *Drosomycin-GFP* reporter induction in the fat body upon infection. **D**: Overactivation of the Toll pathway and rescue of the *Myd88* susceptibility phenotype by overexpression of a UAS-$MyD88^{+}$ transgene (>MyD88) with a gut (NPG4G80) or a hemocyte (hmlG4 or hmlG4G80)-specific driver, as documented by the average time it takes to kill 50% of the flies (LT50). Rescue was observed by overactivation of the Toll pathway in hemocytes, but not in the gut. Note that AMPs synthesized in hemocytes are secreted into the hemocoel. The UAS-$Toll^{10B}$ transgene (>Toll10B) expresses a gene encoding a constitutively active form of the Toll receptor. In this series of experiments, wild-type flies succumbed somewhat earlier than usual. p-values compared to *Myd88* mutant flies * p<0.05; ** p<0.01; n=5. Error bars are standard deviation.

Figure 4: **RhlR, but not LasR, is required to counteract the cellular immune response against *P. aeruginosa* PA14**

A: Survival experiments in wild type *Drosophila* to analyze virulence of *P. aeruginosa* mutants in known virulence factors. The average time it takes to kill 50% of flies (LT50) is plotted. Two *rhlR* transposon insertion mutants (*rhlR* 37943 (referred to as *rhlR*) and *rhlR 34255*) and a deletion *(ΔrhlR*) displayed the same attenuated virulence phenotype, whereas other mutants were not significantly less virulent than wild-type (wt) PA14. *pscD* is a mutation that affects the secretion machinery and thus prevents the secretion of all T3SS effectors, including ExoT. *exoT* mutant bacteria were tested in independent experiments using flies of a different genetic background and also did not show a phenotype (n=3). * p<0.05;

p<0.01;*p<0.001, n=3 or 4 depending on the mutant tested. **B-F:** In the legends to Figures, the first caption refers to the genotype of the host (wild-type (wt) or mutant flies) whereas the second refers to the genotype of the pathogen (PA14 refers to wild-type PA14) **B**: Bacterial counts per fly measured in the hemolymph collected from PA14 and *RhlR* infected wild-type (wt) and latex bead-injected flies (wtΔphag) expressed on a logarithmic scale (n=3). **C**: Survival experiments using wild-type PA14 and *rhlR* mutant bacteria. *rhlR* mutants are less virulent (p-values PA14 vs. *rhlR* in wild-type flies: p=0.0017, n=7; *key* flies: p=0.0020, n=6; *Myd88* flies: p=0.0001, n=7). **D:** *rhlR* mutant bacteria killed phagocytosis-deficient latex bead-injected flies as rapidly as wild-type bacteria (p>0.05, n=6). **E:** *rhlR* (p>0.05, n=3), n=3), mutants display the same level of virulence as wild-type PA14 in phagocytosis-deficient *eater* mutant flies under conditions in which wild-type flies do not succumb to the oral infection (sucrose only medium). **F:** *lasR* mutants (p=0.001) are less virulent in *eater* mutant flies. Error bars in **A** and **B** are standard deviations.

Figure 1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survival %
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100
50
0
0
5
10
15
days post infection
wt
key
imd
Myd88
spz
B
survival %
days post infection
wt
wtΔphag
eater
C
survival %
days post infection
wt PA14 continuous
wt PA14 24h → sugar
wt PA14 48h → sugar
wt PA14 72h → sugar
wt PA14 96h → sugar
D
bacteria per fly (logCFU)
5
4
3
2
1
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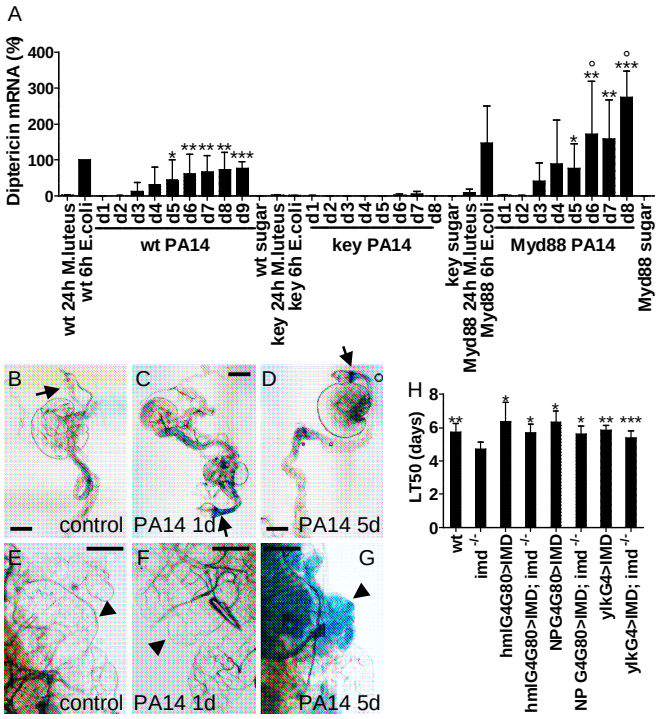
0
1
2
3
4
5
6
days post infection
wt
wtΔphag
key
Myd88

Figure 2


A
Diptericin mRNA (%)
wt 24h M.luteus
wt 6h E.coli
wt PA14
wt sugar
key 24h M.luteus
key 6h E.coli
key PA14
key sugar
Myd88 24h M.luteus
Myd88 6h E.coli
Myd88 PA14
Myd88 sugar
B
C
D
control
PA14 1d
PA14 5d
E
F
G
control
PA14 1d
PA14 5d
H
LT50 (days)
wt
imd -/-
hmlG4G80>IMD
hmlG4G80>IMD; imd -/-
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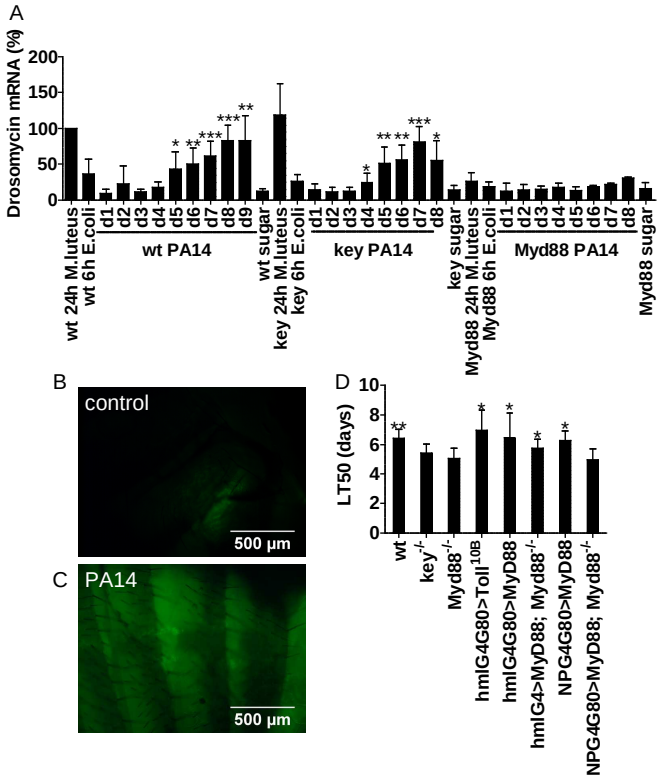
NP G4G80>IMD; imd -/-
ylkG4>IMD
ylkG4>IMD; imd -/-

Figure 3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Drosomycin mRNA (%)
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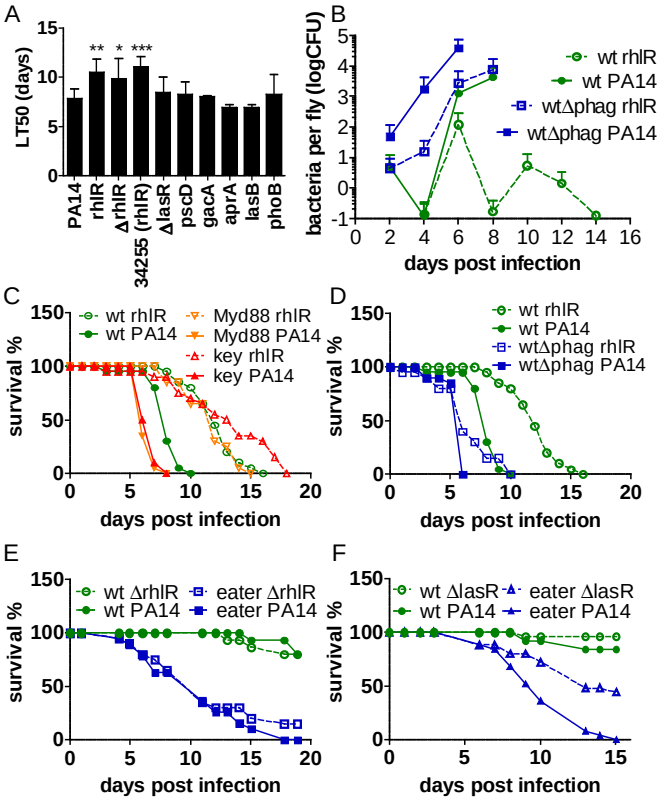
150
100
50
0
wt 24h M.luteus
wt 6h E.coli
d1
d2
d3
d4
d5
d6
d7
d8
d9
wt PA14
wt sugar
key 24h M.luteus
key 6h E.coli
key PA14
key sugar
Myd88 24h M.luteus
Myd88 6h E.coli
Myd88 PA14
Myd88 sugar
B
control
500 µm
C
PA14
500 µm
D
LT50 (days)
10
8
6
4
2
0
wt
key-/-
Myd88-/-
hmlG4G80>Toll10B
hmlG4G80>MyD88
hmlG4>MyD88; Myd88-/-
NPG4G80>MyD88
NPG4G80>MyD88; Myd88-/-

Figure 4


A
LT50 (days)
PA14
rhlR
ΔrhlR
34255 (rhlR)
ΔlasR
pscD
gacA
aprA
lasB
phoB
B
bacteria per fly (logCFU)
days post infection
wt rhlR
wt PA14
wtΔphag rhlR
wtΔphag PA14
C
survival %
days post infection
wt rhlR
wt PA14
Myd88 rhlR
Myd88 PA14
key rhlR
key PA14
D
survival %
days post infection
wt rhlR
wt PA14
wtΔphag rhlR
wtΔphag PA14
E
survival %
days post infection
wt ΔrhlR
wt PA14
eater ΔrhlR
eater PA14
F
survival %
days post infection
wt ΔlasR
wt PA14
eater ΔlasR
eater PA14

Supplementary Text

In a recently described oral feeding model using *D. melanogaster* strain *esg-Gal4*, ingested *P. aeruginosa* PA14 was reported to cause apoptosis of enterocytes and a subsequent compensatory proliferation of intestinal stem cells (ISCs), thus maintaining the homeostasis of the posterior (*caudal*-expression region) midgut epithelium (1). In contrast, under our conditions using *D. melanogaster* strain A5001, the morphology of the midgut epithelium appeared normal throughout the infection (Fig. S4), even on the last day of the infection when most of the flies were dead. To indirectly monitor the damage inflicted to the gut by ingested PA14, we measured the rate of division of ISCs because compensatory proliferation of these stem cells has been shown to be required for the homeostasis of the midgut epithelium (1, 2). We assessed the mitotic rate by phosphohistone H3 staining and by following the incorporation of the labeled nucleotide EdU in the distal midgut region of the infected A5001 flies (Fig. S4). With both techniques, we failed to observe proliferation beyond normal background levels. Because the JAK-STAT pathway has been shown to be required to control ISC proliferation during infection, we also monitored the expression of Unpaired3, which stimulates the JAK-STAT pathway (Fig. S4) (2-4). We observed a weak induction of an *Unpaired3-GFP* reporter transgene in a few enterocytes. We also observed some expression in intestinal muscle cells and in some undefined epithelial cells of a *GFP* reporter transgene that is activated by STAT92E binding to 10 copies of its DNA-binding site (Fig. S4). However, we were unable to detect any expression of this reporter the 10xSTAT92E reporter in ISCs, in contrast to the previously published *S. marcescens* oral infection model from our lab (2). Because we failed to observe any significant degradation of the intestinal epithelium during *P. aeruginosa* PA14 oral infection, even at the end of the infection (Fig. S4), and because flies transferred to a sterile medium are eventually killed in the absence of any detectable PA14 in the gut (Fig. 1C & S3), we conclude that death in our infection model is unlikely to be caused by gut damage. The discrepancy with previously published results may

be due to differences in *Drosophila* genetic background, fly husbandry, or other factors as further discussed in the next section.

Supplementary Discussion

On the absence of major gut damage after orally feeding of flies with* P. aeruginosa *PA14

A previous study has documented the effect of ingested *P. aeruginosa* PA14 on the intestinal epithelium (1). As stated above, we did not observe damage to the gut epithelium and concomitant increased proliferation of ISCs as reported in reference (1). Several reasons may account for this discrepancy including differences in the infection protocol, differing fly husbandry conditions such as food and microbiota, or asymptomatic viral infection of stocks. Another important variable may be the interplay of these parameters with the genetic background of the particular flies used in the different laboratories. In this regard, we noticed that our wild-type OregonR flies as well as the *escargot*-Gal4, UAS-*GFP* stock were somewhat more prone to *P. aeruginosa* PA14-induced intestinal epithelial damage, as inferred from the compensatory proliferation of ISCs, than the other *Drosophila* wild type strains used in this study (*DD1 cn bw, w* A5001*)*, although the damage was less pronounced than described in reference (1).

Apidianakis *et al.* (1) reported increased susceptibility to feeding on *P. aeruginosa* PA14 in fly mutants in which the integrity of the intestinal epithelium could not be maintained, a consequence perhaps of increased translocation of bacteria across intestinal epithelial cells and/or earlier activation of a virulence switch after bacteria have gained access to the hemocoel. The increased susceptibility to feeding on *P. aeruginosa* of IMD pathway mutants caused by a defective activation of the pathway may be explained in a similar manner by a defective control of bacterial translocation at the level of the intestinal epithelium. Indeed, we were able to rescue the susceptibility phenotype of *imd* mutant flies by overexpressing a wild-type copy of *imd* in the gut epithelium (Fig. 2H), Interestingly, a similar level of rescue was obtained in hemocytes and in the fat body, indicating that all three

immune tissues can participate in the immune defense (Fig. 2H). Thus, the IMD pathway seems to play multiple roles in the host defense against ingested *P. aeruginosa*, locally in the gut epithelium likely by controlling at least partially the rate of passage through the gut (5) and systematically in the fat body or hemocytes, which also secrete AMPs into the hemolymph.

Models of **P. aeruginosa** ***infection in insects*** **and their usefulness to address different aspects of bacterial pathogenesis and host defense**

Here, we first discuss the different protocols that have been used to orally infect *Drosophila* adult flies with *P. aeruginosa* in several studies. Next, we compare the specifics of the *Drosophila* feeding models in comparison with the insights gained into *P. aeruginosa* pathogenesis using other insect systems.

We have used two methods to orally infect *Drosophila* in this study, in which adult, unstarved flies were fed *P. aeruginosa* PA14 in the presence or absence of bacterial growth medium (BHB or LB) (Fig. S1). In both cases, bacteria are found mostly in the digestive tube and to a much lesser extent in the crop, a storage diverticulum in which the bacteria accumulate when flies are fed after prior starvation. In contrast, bacteria accumulate in the crop in a third oral feeding infection protocol (not used in our study) (5, 6). In this third model flies were starved for food and water for 3-5h prior to infection and then fed on a concentrated bacterial solution placed on a filter on top of sucrose agar (5, 6). It has not yet been determined whether flies succumb to a systemic infection in this latter oral feeding model. It will be interesting to measure the bacterial titer in the hemolymph and to assess the susceptibility of mutants that affect host defense in this third model of infection to determine whether flies also succumb to bacteremia or whether flies succumb to damage inflicted to the crop , as suggested by the authors (5, 6).

P. aeruginosa has been reported to kill different species of insects in different models

of infection. For instance, it was found to be a major cause of death in laboratory cultures of grasshoppers (7). Infection by feeding led to the demise of insects, which harbored a titer of 10^9 bacteria per insect. Interestingly, even though the bacteria had been rapidly cleared from the gut, they ultimately were able to proliferate to high titers in the hemolymph. Thus, this infection model displays similarities to the one we are using in *Drosophila*.

Larvae of the Greater Wax Moth *Galleria mellonella* have been used to identify *P. aeruginosa* PA14 virulence factors (8, 9). Interestingly, it was found that the T3SS system and ExoT, which is a toxin secreted through the T3SS, were required for virulence. In contrast to these findings, we did not observe any requirement for the T3SS in our *Drosophila* oral infection model. We note however that another T3SS effector, ExoS, which is present in *P. aeruginosa* strain PAK but lacking in strain PA14, blocks phagocytosis in *Drosophila* by regulating the small Rho GTPase family member Rac2 (10, 11). As regards *P. aeruginosa* PA14, the discrepancy between observations in *Galleria* and *Drosophila* may be due to the evolutionary divergence between these two species that last shared a common ancestor about 340 million years ago. Alternatively, the difference may reside in the infection route (oral vs. septic injury) or in the developmental stage (adult vs. larva).

Another difference between our *Drosophila* oral feeding model and other insect infection models is the role of GacA in virulence. GacA belongs to a two component system that regulates bacterial virulence in plants and animals and controls the expression of the AprA protease in *P. entomophila*, a major virulence factor in an oral infection model (12). GacA is also important for virulence in the *G. mellonella* infection model (8). In contrast, we observed normal virulence of *gacA* mutants in our *Drosophila* oral infection model.

These observations underscore the importance of the particular infection model used for assessing virulence factor contribution to pathogenesis, and illustrate that opportunistic bacteria can resort to multiple virulence strategies depending on the context (13).

Supplementary Material and Methods

Fly strains:

Stocks were raised on standard cornmeal-agar medium at 25 °C. Different wild-type strains were used: Oregon R, w^- A5001 and *yw P[ry*$^+$*, Dipt::LacZ = pDipt-LacZ], P[w*$^+$*mC Drom::GFP = pDrom-GFP S65T]*; *cn bw* (*yw*DD1-*cn bw*) (14, 15). The wild-type flies all behaved in the same manner. In our experiments whenever possible, we used the wild-type strain corresponding to the background in which the mutants were generated as controls. Thus, "wild-type" in different Figures may correspond to different genetic backgrounds. Mutants in the A5001 background: key^{c02831}, $Myd88^{c03881}$ (Gottar, Gobert & Ferrandon, unpublished)(16); mutants in the *yw*DD1-*cn bw* background: imd^{shadok} (17). IMD overexpression and rescue experiments: *hml-Gal4,tub-Gal80/+;UAS-imd*$^+$*/+*. *hml-Gal4,tub-Gal80,* $imd^{shadok}/imd^{shadok}$*; UAS-imd*$^+$*/+*. *NP3084-Gal4,tub-Gal80/+; UAS-imd*$^+$*/+*. *NP3084-Gal4,tub-Gal80,*$imd^{shadok}/imd^{shadok}$*; UAS-imd*$^+$*/+*. *ylk-Gal4/UAS-imd*$^+$*.* $imd^{shadok}/imd^{shadok}$*; ylk-Gal4/UAS-imd*$^+$ (18-20). Toll pathway overstimulation and rescue: *UAS-MyD88*$^+$*/+; hml-Gal4,tub-Gal80/+*. *UAS-MyD88*$^+$*/+;* *hml-Gal4,* $Myd88^{c03881}/Myd88^{c03881}$*. UAS-MyD88*$^+$*/+;NP3084-Gal4,tub-Gal80/+*. *UAS-MyD88*$^+$*/+; NP3084-Gal4,tub-Gal80,* $Myd88^{c03881}$*/* $Myd88^{c03881}$ (16).

JAK-STAT reporter transgenes: *upd3-GFP* (21), *10xStat92E-binding site-GFP* (22).

eater Df(3R)D605/*Df(3R)Tl-I* (23).

Bacterial strains and growth conditions:

Wild-type strain: *P. aeruginosa* PA14 (24); *rhlR* mutants: deletion (*ΔrhlR*) and two transposon insertions: GID3229 ID#37943 (referred to as *rhlR*), GID3229 ID#34255 (referred to as 34255 (*rhlR*)) (25); *lasR* mutant: deletion (*ΔlasR*); *pscD* mutant: deletion (*ΔpscD*); *gacA* mutant: deletion (*ΔgacA*) (24); *aprA*, *lasB* and *phoB* mutants are transposon insertions: *aprA* GID865 ID#23768, *lasB* GID759 ID#45691 and *phoB* GID3473 ID#48234 (25).

All bacteria were grown in Brain-Heart-Infusion Broth (BHB) over night with shaking at 37°C. We observed similar survival curves of infected flies when PA14 was grown and incubated with Luria Broth (LB). *E. coli* and *M. luteus* for qRT-PCR controls were grown in LB overnight with shaking at 37°C.

Bacterial counts in the hemolymph:

Bacterial counts were measured as previously described (26). Hemolymph solutions were plated on LB plates containing 10µg/ml Rifampicin and incubated at 37°C for 16h. We checked that hemolymph counts were representative of the degree of infection of the body cavity by dissecting away the digestive tract after the fly's hemolymph had been collected and plating an extract of the carcass at different stages of the infection.

β-Galactosidase staining and p*Drom*-GFP observation:

Flies were infected and their abdomens dissected. *pDrom*-GFP abdomens were mounted in glycerol and observed. *pDipt*-lacZ abdomens were fixed in 1% glutaraldehyde for 10min, washed and stained for 30min in coloration solution at room temperature (8.4mM Na_2HPO_4, 1.6mM Na_2HPO4, 0.15M NaCl, 1mM $MgCl_2$, 3.5mM K_3FeCN_6, 3.5mM K_4FeCN_6, 0.15% X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)), washed again, mounted in glycerol and observed using a Zeiss Axioskope 2 fluorescence microscope. Images were processed using ImageJ 1.41o.

Injection of latex beads:

Flies were injected with latex beads as previously described (26).

Quantitative reverse-transcription PCR :

This analysis was done as previously described (27).

In vivo* phagocytosis assay and injection of FITC-labeled *E.coli:

69nl of FITC-labeled *E.coli* (33µg/µl) (Invitrogen Bioparticles) were injected into the flies' thorax with PA14. Flies were kept at room temperature, and one hour later 2x 69nl of Trypan Blue were injected to quench the fluorescence of non-ingested FITC-*E. coli*. 10min later, the abdomens were dissected, mounted in glycerol and observed using a Zeiss Axioskope 2 fluorescence microscope. Images were processed using ImageJ 1.41o. This experiment was performed for each day of the infection.

Intestinal Colonization assay:

Flies were first fed on a *P. aeruginosa* PA14 solution (OD_{600}=0.5) for different times and transferred after a given incubation period to vials containing a sterile 50mM sucrose solution. After one hour on sucrose solution the flies were again transferred to a new sterile vial. After this, flies were transferred to new vials every day to avoid contamination of the sugar solution by the flies' feces. We were not able to detect more than 10-50 bacteria on the filters after the first two changes of sterile vials; most of the bacteria were cleared from or killed in the gut in the first hour as determined by plating gut extracts (crop included). Survival at 25°C was monitored every day.

Immunostainings:

Primary antibodies: Rb α pHH3 (1:1000; Millipore), M α GFP (1:100; Roche). Secondary antibodies conjugated to Alexa Fluor-488 (Invitrogen) were used at a 1000x dilution. Standard immunohistochemical methods were used. Texas Red®-X phalloidin (stock solution (6,6µM) was diluted 8x (0,825µM), Invitrogen) was added to the secondary antibodies or used alone after 2h blocking at room temperature. Guts were mounted in Vectashield with

DAPI (Vector laboratories, Inc.) and observed using the inverted confocal microscope Zeiss LSM 510. Images were processed using ImageJ 1.41o.

Septic injury assay:

An overnight culture of bacteria was centrifuged (4000 rpm, 10min, 4°C) and the pellet was diluted in sterile PBS to a concentration of 10 bacteria per nl. Of this solution 9.2nl were injected into the thorax of the flies. Afterward, flies were put on a 50mM sucrose solution. Survival at 25°C was observed after 24h (and 36h for wt flies) and then surviving flies were counted each hour.

EdU incorporation:

69nl of a 0.5mM solution of EdU in PBS was injected into the flies' thoraces. Following incubation for 3h at 25°C guts were dissected and stained following the suppliers instructions (Click-iT® EdU Alexa Fluor® 488, Invitrogen). Guts were mounted in Vectashield with DAPI (Vector laboratories, Inc.) and observed using a Zeiss LSM 510 inverted confocal microscope. Images were processed using ImageJ 1.41o.

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Legends to supplementary figures

Figure S1: **Influence of the presence of bacterial broth in the medium on PA14 virulence in a *Drosophila* oral infection model**.

Wild-type flies feeding on *P. aeruginosa* PA14 in 5% sucrose did not succumb to oral infection, while flies feeding on *P. aeruginosa* PA14 in 80% bacterial broth (LB or BHB)/4% sucrose succumbed rapidly to infection. Note that the bacterial concentration in the feeding medium hardly influences survival. Data are representative of five independent repeats.

Figure S2: **Flies die from bacteremia in the septic injury model.**

About 100 bacteria were introduced in the hemocoel of flies by septic injury. Survival was monitored (left-hand y-axis: black curve) and the bacterial titer in the hemolymph was measured (right-hand logarithmic scale, gray curve). This experiment is representative of three independent experiments.

Figure S3 : ***P. aeruginosa* PA14 does not colonize the digestive tract of *Drososphila***

When feeding continuously on *P.aeruginosa* PA14, flies display a constant bacterial load of around 20,000 bacteria per intestine (PA14 4d and 5d). The intestines of flies that have been transferred to a sterile sucrose solution after 4 days of feeding (PA14 4d + 24h sugar) have hardly any detectable bacteria in their intestine ($p<0.05$), n=3.

Figure S4: **Orally fed *P. aeruginosa* PA14 does not severely damage the gut epithelium**.

Guts were dissected every day until one day prior to death of the orally-infected flies (strain A5001 unless indicated otherwise). Control flies were fed on a sucrose solution. The data

shown here are from the last time point we examined. First row: After fixation, some guts were stained with Texas-Red phalloidin, which binds to actin filaments and thus mainly stains the brush border and intestinal muscles. Phalloidin staining indicates no difference in gut epithelium integrity. Second row: To monitor the proliferation of intestinal stem cells, phosphohistone H3 staining (pHH3: green) was performed. There was no detectable difference in proliferation. Third row: Alternatively, EdU was injected into flies three hours prior to fixation and staining with an EdU-specific fluorescent azide (green). No difference in EdU signal was detected. Fourth and fifth row: To investigate JAK-STAT pathway activation, *upd3-GFP* and 10xSTAT92E binding-site-GFP transgenic flies were used. JAK-STAT pathway activation by *P. aeruginosa* PA14 in the latter line appeared to be restricted to intestinal muscles, while the upd3 signal was only weak and restricted to a few intestinal epithelial cells.

Figure S5: **The hemocytes of *P. aeruginosa* PA14 infected flies do not loose their ability to phagocytose bacterial particles**.

Phagocytosis of injected fluorescein (FITC)-labeled *E.coli* particles (Invitrogen) at day 8 of infection with PA14 as observed under epifluorescent illumination. The fluorescence from free, non-phagocytosed bacteria was quenched with trypan blue. The region of the fly body corresponding to the area from which high magnification microscopy pictures were taken is indicated in the upper panel (dashed lines). The fluorescent signal corresponds to FITC-*E. coli* phagocytosed by sessile hemocytes lining the dorsal vessel (white arrows). Flies infected with *P. aeruginosa* PA14 and non-infected control flies displayed the same ability to phagocytose FITC-*E. coli*. No phagocytosed FITC-*E.coli* were found in latex bead-injected flies (control; right lower panel).

Figure S6: **RhlR is required for the synchronized killing of flies that have ingested *P. aeruginosa* PA14**

A: Hill coefficient (absolute value) of survival curves (some of which are shown in Fig. 4) using wild-type or immunosuppressed flies infected with wild-type PA14 or *rhlR* mutant bacteria. The Hill coefficient measures the slope of sigmoid curves. The higher absolute values of the Hill coefficient correspond to steeper curves as illustrated in Fig. 4 C&D; **p<0.005, n=3 **B**: Hill coefficient (absolute values) of survival curves (mean LT50 values shown in Fig. 4A) of wild-type flies infected either by wild-type PA14, a *rhlR* deletion mutant (*ΔrhlR*), or a *rhlR* transposon insertion mutant (*34255(rhlR*)). These two *rhlR* mutants are distinct from the *rhlR* mutant (37943) that was used for most of the experiments (see Supplementary Material and Methods). * p<0.05; **p<0.005, n=3. Error bars are standard deviation.

Figure S7: ***rhlR* bacteria fail to establish a strong, systemic infection in wild-type or humoral immune response mutant flies.**

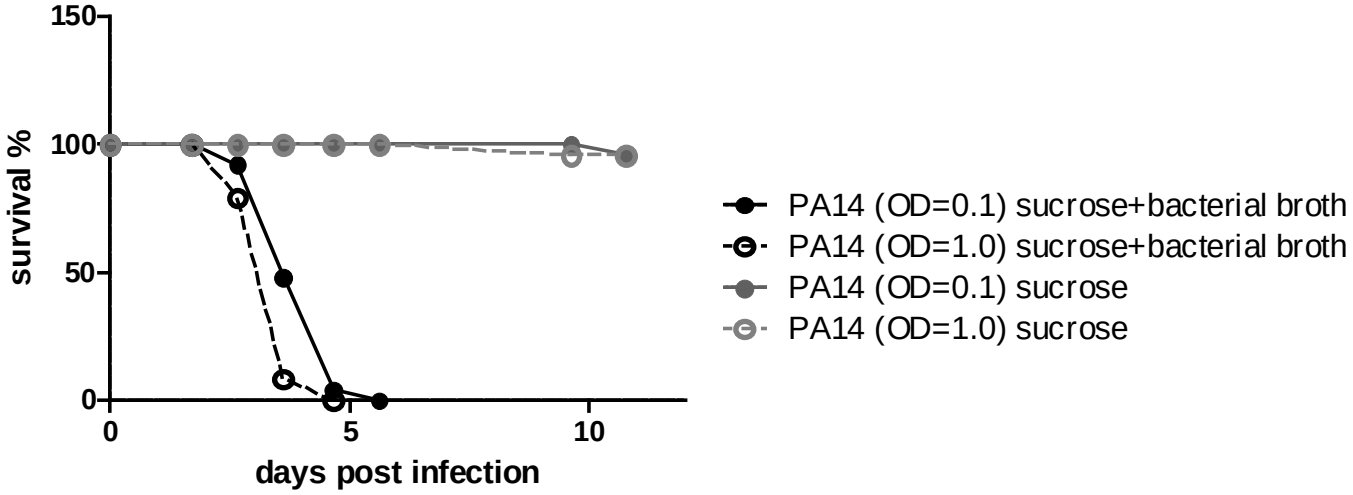
A: qRT-PCR analysis of the expression of *Diptericin* and *Drosomycin* of wild-type, IMD mutant (*key*), and Toll mutant (*Myd88)* flies after infection with PA14 or *rhlR* mutant bacteria (mean of three independent experiments). Statistically significant differences between flies fed on *P. aeruginosa* and flies of the same genotype fed on sucrose solution are indicated by *: *p<0.05; ** p<0.01; *** p<0.001. The signal measured in wild-type and *Myd88* flies infected with *rhlR* is significantly weaker than that induced by PA14, as indicated by °: °p<0.05; °°p<0.01. **B**: Bacterial numbers in the hemolymph of wild-type (wt), Toll mutants, and IMD mutants (each data point is the mean of three independent experiments). This number per fly is represented with a logarithmic scale. The bacterial titer in *key* and *Myd88* mutants is higher than that measured in wild-type flies also orally challenged with *rhlR*. Note that half of the *rhlR*-infected flies have succumbed by day 12 and that the lower titer may be

measured in flies that have been less severely infected and as a result die more slowly, consistent with the shallow curve observed in survival experiments. Error bars are standard deviation.

Figure S8: ***rhlR* mutant bacteria are less virulent in a septic injury model, unless the cellular immune response is impaired**.

Septic injury survival experiment using wild-type and phagocytosis-deficient latex bead-injected flies. *rhlR* mutant bacteria kill wild-type flies significantly slower than *P. aeruginosa* PA14: p=0.012, n=2. The results of one of two experiments is shown.

S1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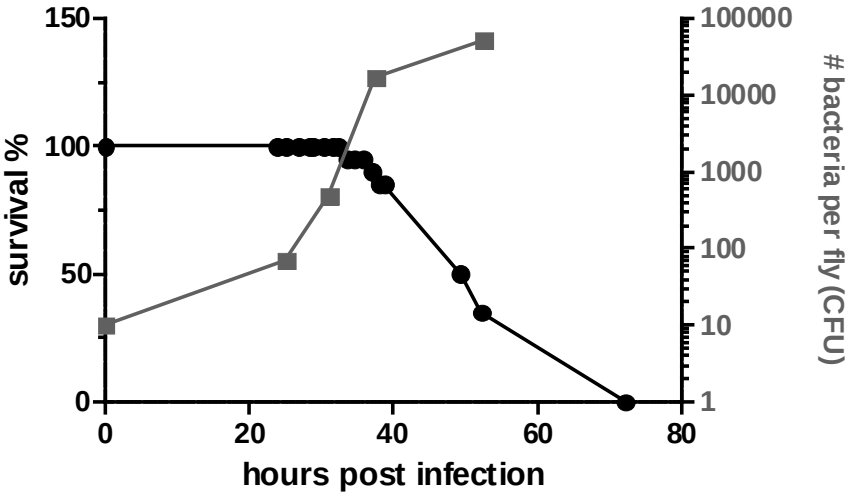
survival %
0
5
10
days post infection
PA14 (OD=0.1) sucrose+bacterial broth
PA14 (OD=1.0) sucrose+bacterial broth
PA14 (OD=0.1) sucrose
PA14 (OD=1.0) sucrose

S2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10000
1000
100
10
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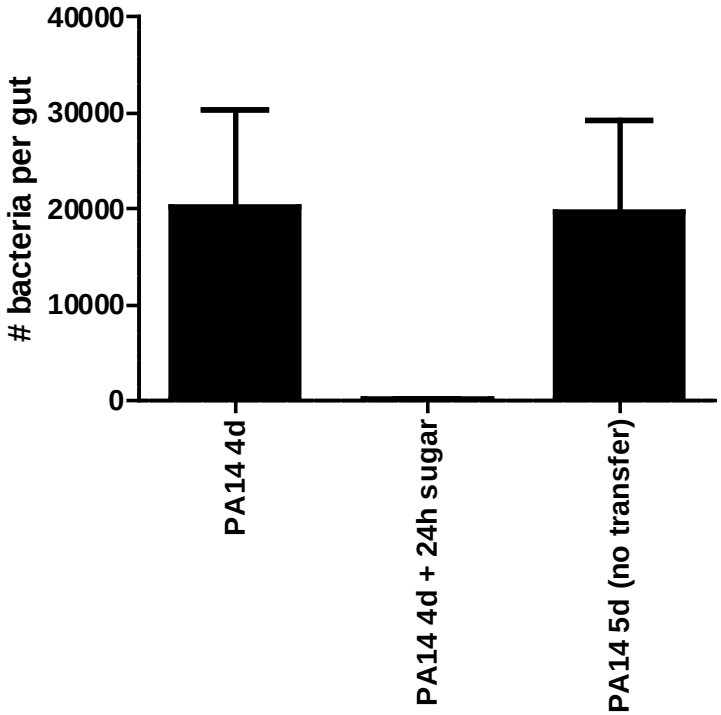
0
20
40
60
80
hours post infection

S3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30000
20000
10000
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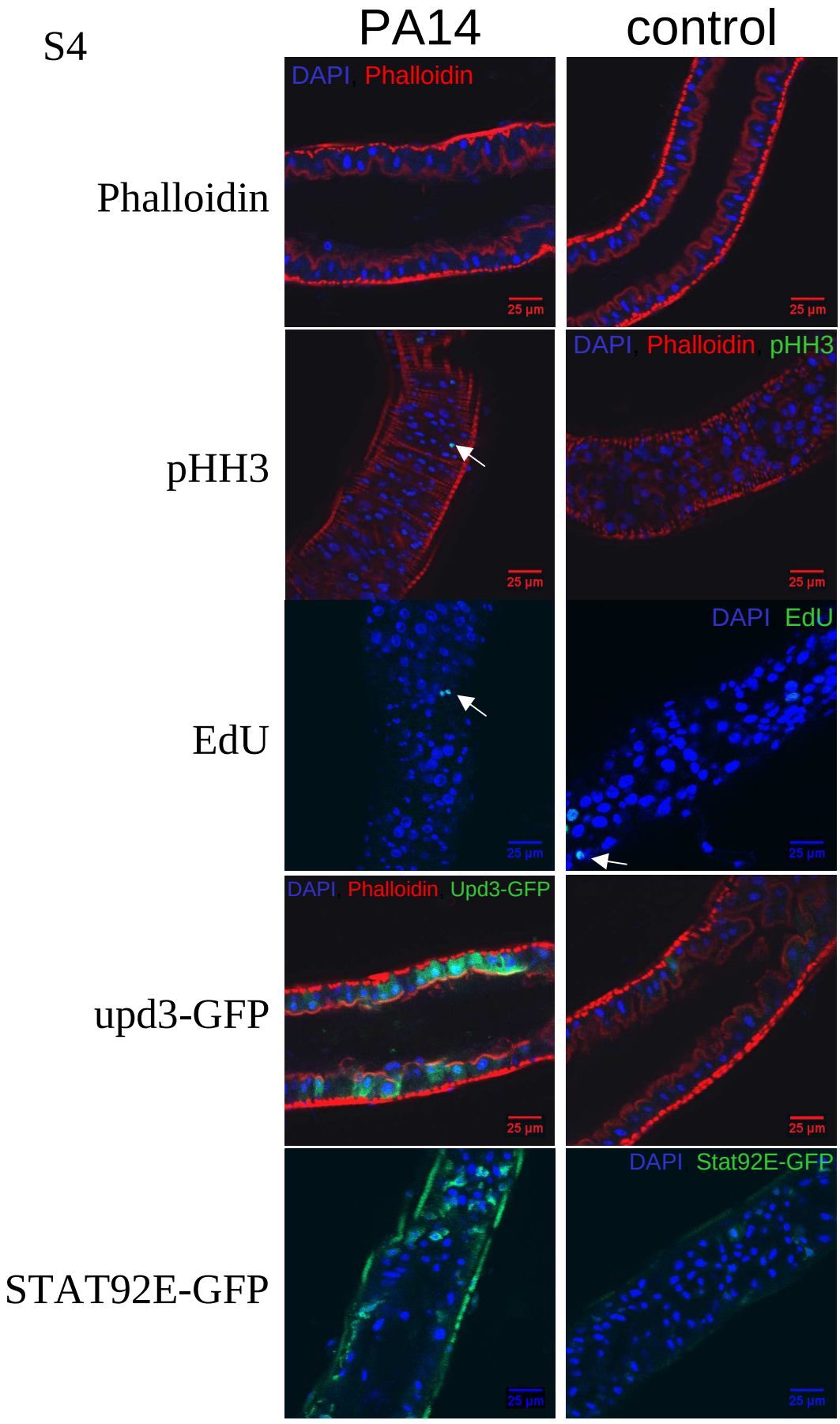
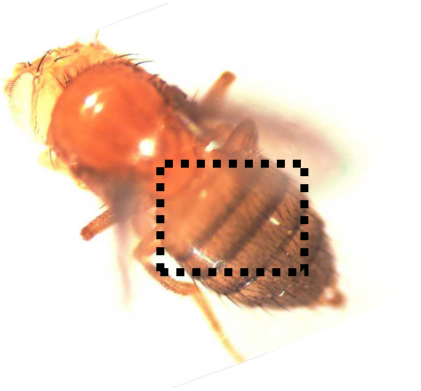
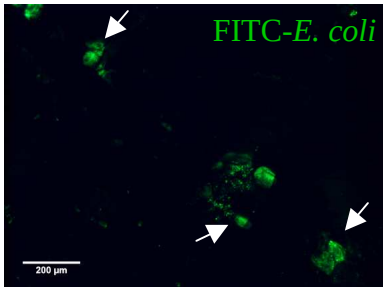
PA14 4d
PA14 4d + 24h sugar
PA14 5d (no transfer)

S4

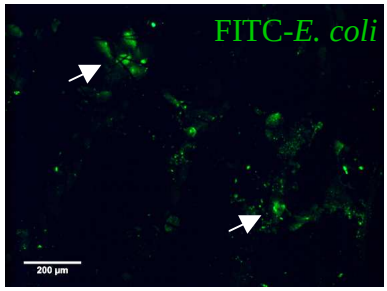

PA14
control
Phalloidin
pHH3
EdU
upd3-GFP
STAT92E-GFP
DAPI, Phalloidin
DAPI, Phalloidin, pHH3
DAPI, EdU
DAPI, Phalloidin, Upd3-GFP
DAPI, Stat92E-GFP
25 µm

S5

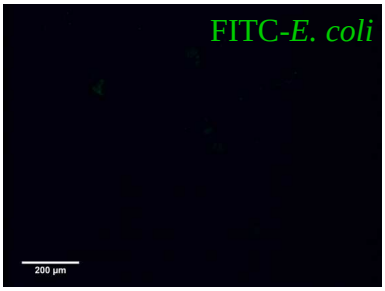




d8 PA14

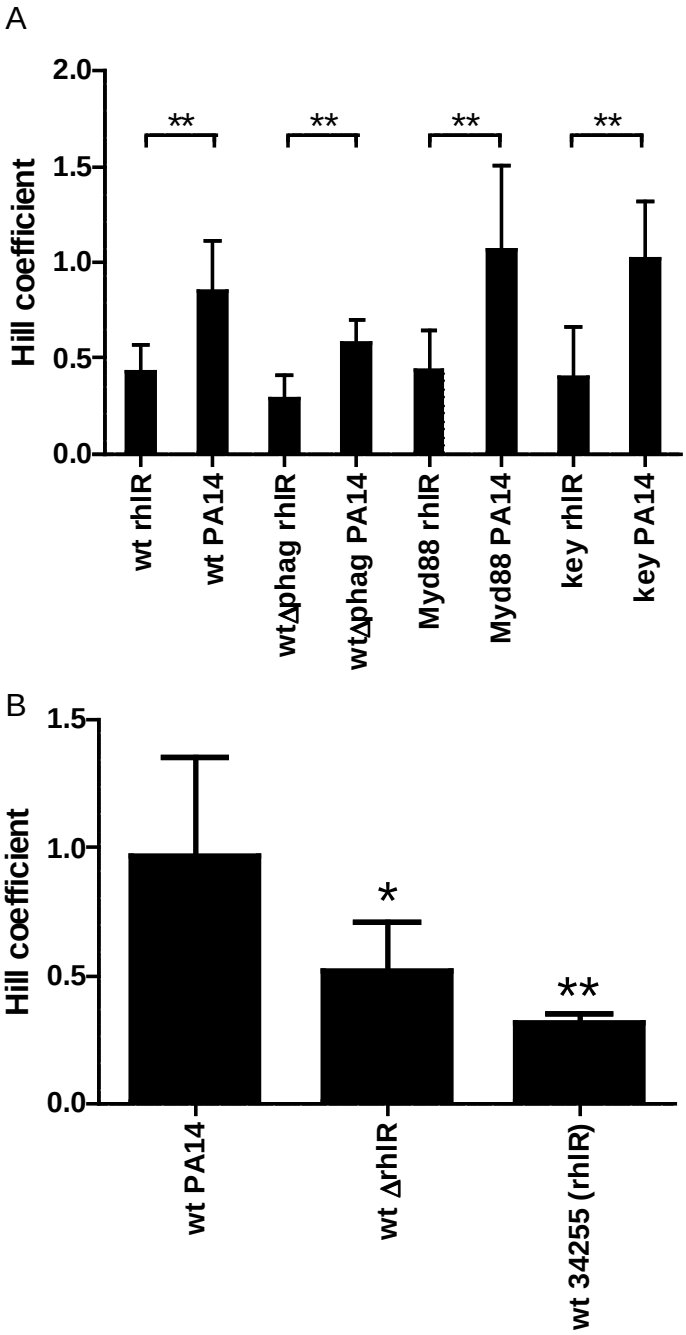


control



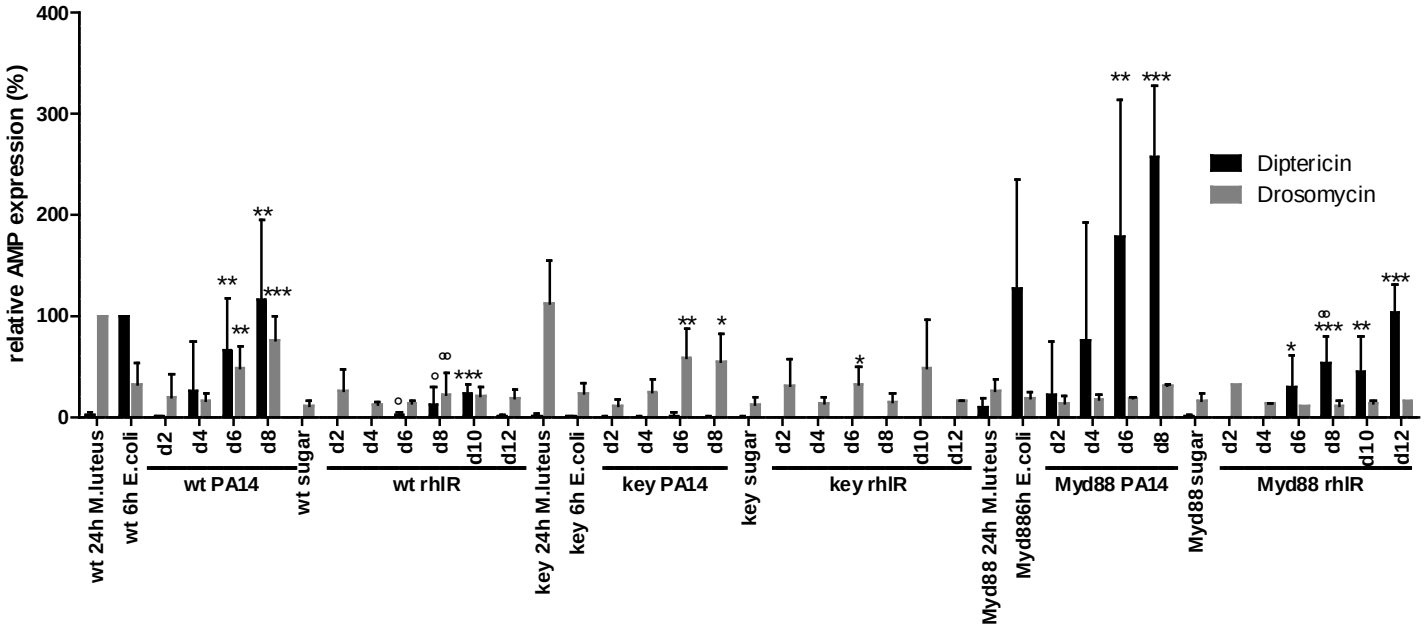
latex beads

S6

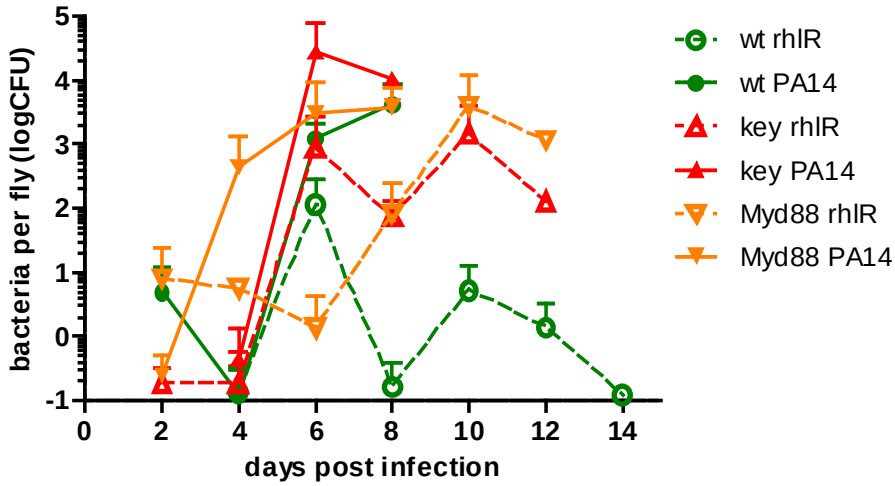

A
Hill coefficient
2.0
1.5
1.0
0.5
0.0
**
**
**
**
wt rhlR
wt PA14
wtΔphag rhlR
wtΔphag PA14
Myd88 rhlR
Myd88 PA14
key rhlR
key PA14
B
Hill coefficient
1.5
1.0
0.5
0.0
*
**
wt PA14
wt ΔrhlR
wt 34255 (rhlR)

S7

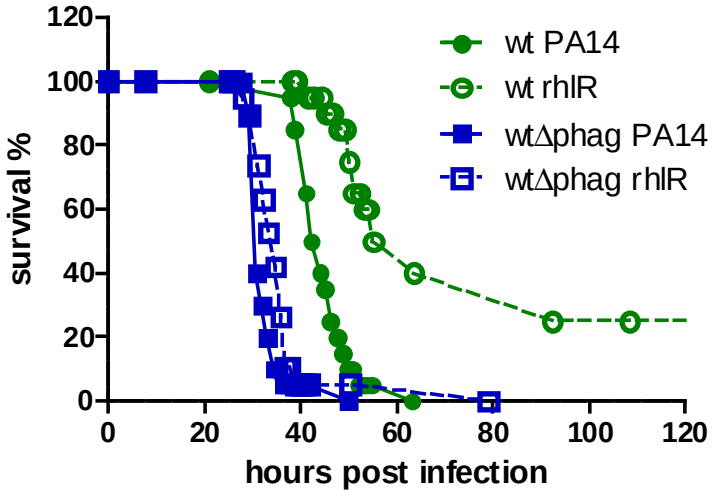
A


relative AMP expression (%)
400
300
200
100
0
Diptericin
Drosomycin
wt 24h M.luteus
wt 6h E.coli
d2
d4
d6
d8
wt PA14
wt sugar
d10
d12
wt rhlR
key 24h M.luteus
key 6h E.coli
key PA14
key sugar
key rhlR
Myd88 24h M.luteus
Myd886h E.coli
Myd88 PA14
Myd88 sugar
Myd88 rhlR

B


bacteria per fly (logCFU)
5
4
3
2
1
0
-1
0
2
4
6
8
10
12
14
days post infection
wt rhlR
wt PA14
key rhlR
key PA14
Myd88 rhlR
Myd88 PA14

S8


120
100
80
60
40
20
0
survival %
0
20
40
60
80
100
120
hours post infection
wt PA14
wt rhlR
wtΔphag PA14
wtΔphag rhlR

Further characterization of the oral infection by *P. aeruginosa* and discussion

In this study, we gained some insights into the mechanisms of *P. aeruginosa* oral infection, but many questions remained open:

- How does *P. aeruginosa* manage to cross the gut epithelium?
- What triggers *P. aeruginosa* proliferation in the hemolymph if there is indeed a switch in its virulence?
- Are the distinct host defense responses independent of each other and if yes, how does *P. aeruginosa* trigger the Toll pathway since it is a Gram(-) bacterium, which are not optimal inducers of this pathway?
- Is the role of RhlR during infection quorum sensing dependent?
- What is the role of the T2SS?

How does *P. aeruginosa* manage to cross the gut epithelium?

Ingested *P. aeruginosa* do not colonize the intestinal tract of the fly, but still cross the epithelial barrier and cause a systemic infection. It is not clear how the bacteria are able to pass the gut epithelium. Pathogens have been reported to cross epithelia either intracellularly or by moving between the epithelial cells (reviewed in (Sousa *et al.* 2005)). To investigate whether *P. aeruginosa* can be found inside the epithelial cells or between them, electron microscopic analysis of infected guts needs to be performed. Yet, in the case of *S. marcescens* infections, intracellular bacteria were very difficult to visualize by electron microscopy, as passage through the gut may be a rare event with respect to the thinness of the sections. An efficient strategy was to use IMD mutant flies to increase the rate of bacterial passage.

Some bacteria, *e.g.*, enteropathogenic *E. coli* (EPEC) and *Salmonella*, promote disruption of intestinal barrier function and/or their uptake into the host cell by injecting effectors directly into its cytoplasm often by using a T3SS (reviewed in (Reis *et al.* 2010)). In *P. aeruginosa* oral infection of *Drosophila*, the T3SS does not seem to be important since *P. aeruginosa* mutant for PscD, a major component of the T3SS machinery that is indispensable for secretion through this channel, do not display altered virulence (see article). Yet, the bacterial titer in the hemolymph needs to be directly measured to assess the possible existence of a weak effect not

detectable in survival experiments. Other bacteria (*e.g. Vibrio cholerae*) secrete proteins into the extracellular space that are taken up by the host cells and interact with cell-cell junctions thereby perturbing epithelial barrier function (Feng *et al.* 2004). The T2SS of *P. aeruginosa* is important for virulence in *Drosophila*. Nevertheless, it is unlikely that *P. aeruginosa* relies only on T2SS effectors to cross the gut epithelium since T2SS mutant bacteria are still found in the hemolymph of the fly (see below).

Experiments using *Drosophila* S2 cells indicate that *P. aeruginosa* is phagocytosed by *Drosophila* cells and can survive intracellularly at least for some time (Figure 20). Interestingly, *P. aeruginosa* is also found inside S2 cells unable to phagocytose because their actin network has been experimentally blocked by incubation with cytochalasin D. *E. coli* were not found inside cytochalasin D-treated S2 cells. These data indicate therefore that *P. aeruginosa* is able to actively invade host cells in an actin independent manner. This S2 cell assay may be helpful to find the mechanism of active or passive uptake that *P. aeruginosa* may use to invade epithelial cells. This would require the screening of mutant bacteria to be combined later on with the functional analysis of host genes through RNA interference.

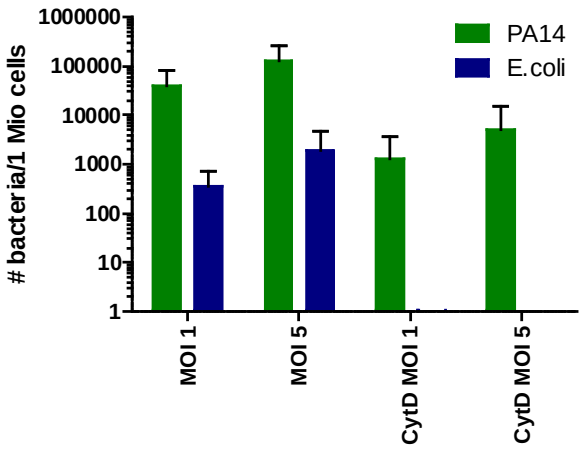


Figure 20: ***P. aeruginosa* is able to enter S2 cells by an actin-independent mechanism**. *Drosophila* S2 cells were infected with *P. aeruginosa* PA14 (multiplicity of infection (MOI) 1 or 5) for one hour at room temperature. After the incubation 100µg/ml gentamycin was added followed by another one hour-incubation at room temperature. The S2 cells were washed several times and then lysed by sonication (15 min) and subsequent centrifugation at 4000 rpm. The pelleted bacteria were resuspended in buffer and plated on LB agar plates containing 10µg/ml rifampicin. As control, S2 cells were infected with ampicilin-resistant *E. coli* following the same procedure. These bacteria were plated on ampicilin-containing LB plates. For some samples Cytochalasin D (CytD, 10µg/ml / 19.7µM) was added to the S2 cells one hour before infection to block the actin network of the cells, which is needed for phagocytosis. *P. aeruginosa* as well as *E. coli* are phagocytosed by S2 cells. In contrast to *E. coli*, *P. aeruginosa* seems to be able to enter S2 cells actively in an actin-independent manner. Of note, actin dependent processes also contribute to the internalization of PA14. n=5. n: number of independent experiments.

A similar assay has been used to identify host genes implicated in the actin-dependent uptake of *P. aeruginosa* PAK in S2 cells (Pielage *et al.* 2008). Several genes involved in actin cytoskeleton regulation were tested for their implication in *P. aeruginosa* engulfment. *P. aeruginosa* uses the Abl-kinase pathway for entering host cells. Pielage et al. also found that the T3SS effectors ExoS and ExoT interfere with this pathway. *P. aeruginosa* PA14 does not express ExoS, and ExoT does not seem to be important for pathogenesis in vivo in the *Drosophila* oral infection model. Therefore, it remains to be established whether PA14 uses the same mechanism for uptake into host cells as PAK and whether the same interactions occur inside the host as in S2 cells. Since the type II and type III secretion systems do not seem to play an essential role in the passage of the epithelial barrier, it would also be important to analyze in the oral infection model bacterial mutants for other secretion systems (Type I, V, and VI) since invasion of and passage through epithelia usually depend on the secretion of effector proteins (reviewed in (Sousa *et al.* 2005; Reis *et al.* 2010)).

What triggers a switch to virulence in the hemolymph?

In the *S. marcescens* model, bacteria in the hemolymph never trigger the systemic immune response, likely because they do not proliferate at a sufficiently high rate. It is very likely that the IMD pathway is induced by PGN fragments that are released by bacteria during the cell wall remodeling that occurs during growth and division (Bou Aoun *et al*, submitted; see also discussion of the article). While *S. marcescens* is a potent pathogen in the septic injury model, it appears to lose its virulence or repress its virulence programs after passage through the intestinal tract in the oral infection model.

As the IMD pathway is not activated before day 5 of the infection in the *P. aeruginosa* PA14 infection model, bacteria do not appear to proliferate strongly in the hemolymph during the early days of the infection. Therefore, the bacteria retrieved in the hemolymph before this time likely originate from the gut compartment. Bacteria present in the body cavity appear to change their behavior midway through the infection and may either start to proliferate or become able to circumvent phagocytosis, which appears to be the major host defense acting in the hemocoel during the early phase of the infection. Is this apparent switch triggered by a change in the virulence and transcription profile of bacteria in the feeding solution or of

bacteria inside the fly? Preliminary data suggest that the bacteria in the feeding solution do not change their virulence profile. Indeed, naïve flies infected with bacteria on which other flies had been feeding for five days before died at about the same rate as when infected with fresh bacteria (Figure 21). Therefore, the change likely occurs inside the fly. Since the bacterial numbers in the fly hemolymph at day four of the infection are rather low (around or under 100, see article) their density might not be high enough to activate quorum sensing and thereby proliferation. In addition, Las quorum sensing system mutants do not show a virulence phenotype. Rhl system mutants still seem to be able to proliferate inside the fly, at least when phagocytosis is blocked (see article). We also tested bacteria mutant for *pqsR* (also called *mvfr*), the receptor for PQS, and found that they did not show altered virulence. Therefore, it remains to be established what triggers the proliferation of *P. aeruginosa* inside the body cavity of the fly, which ultimately leads to bacteremia. The alternative interpretation is that bacteria do not change their virulence properties throughout the infection but progressively and cumulatively circumvent phagocytosis by acting on hemocytes.

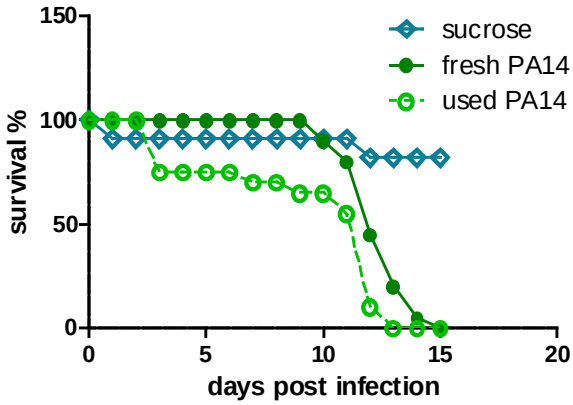


Figure 21: **The virulence of the bacteria in the feeding solution does not change during the infection**. Wild-type flies infected with fresh PA14 die about the same rate as flies infected with used bacteria. Used bacteria were bacteria in a feeding solution on which other flies had been feeding for five days. Naïve flies were transferred onto the used bacterial solution and their survival was monitored. Note that in this survival experiment, bacteria have been killing slower than usual.

Are the host defense responses independent of each other and if yes, how does *P. aeruginosa* trigger the Toll pathway?

The humoral IMD and Toll dependent immune reaction as well as the cellular immune response act against *P. aeruginosa* PA14 infection. To determine whether the different arms of the immune response act independently against *P. aeruginosa* infection, I constructed IMD, Toll pathway double mutants (*Myd88, key*) and injected IMD mutants, Toll mutants, and the double mutants in addition with Latex-beads to functionally block phagocytosis. Thus, I was able to impair either both arms of the humoral response (*Myd88, key*), one arm of the humoral response and the cellular response (*Myd88* Δphag or *key* Δphag), or all responses (*Myd88, key* Δphag). When infected orally with *P. aeruginosa, Myd88, key* double mutant flies succumb significantly earlier to the infection than *key* (p=0.0009; n=3) or *Myd88* (p=0.0074; n=3) single mutant flies, suggesting that the IMD and the Toll pathway act independently of each other (Figure 22). When injected prior to infection with Latex-beads, *key* (key Δphag; p=0.0072; n=3) as well as *Myd88* (Myd88 Δphag; p=0,0307; n=3) mutant flies succumb significantly faster than nontreated *key* or *Myd88* mutant flies. Even *Myd88, key* double mutants seem to succumb faster when phagocytosis is blocked compared to *Myd88, key* with functional phagocytosis (p=0.0157; n=2) (Figure 22). These data suggest that the IMD and Toll pathways are acting independent of each other, and also independently of the cellular immune response since the phenotypes appear to be somewhat additive. In addition, flies in which all these defenses are inactivated still succumb more slowly than in the septic injury model. Interestingly, there does not seem to be a difference in survival between *key* mutant flies in which phagocytosis is blocked and *Myd88, key* double mutants in which phagocytosis is blocked. To explain this observation further experiments will be needed.

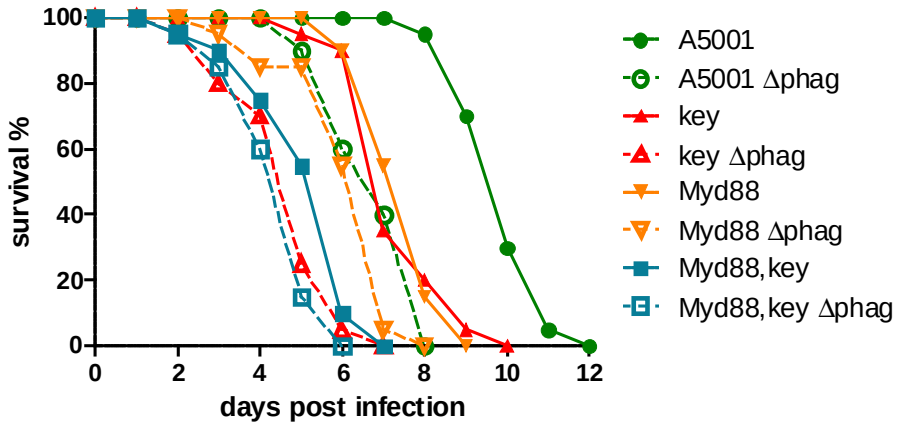


Figure 22: **Additive effects of the different components of the immune response**. The IMD and the Toll pathways act in parallel to fight *P. aeruginosa* infection, since *Myd88, key* double mutant flies die faster than the single mutant flies. In addition, the cellular and the humoral immune response are independently active against invading *P. aeruginosa*. *key*, *Myd88* or *Myd88, key* double mutants treated with Latex-beads (Δphag) succumb faster to the infection than when phagocytosis is functional. Survivals shown are representative of 3 experiments (2 for *Myd88, key* Δphag; 20 flies used for each experiment).

While the IMD pathway is triggered by DAP-type PGN fragments, the Toll pathway is mainly activated by Lys-type PGN (see introduction), which is lacking in the cell wall of *P. aeruginosa* (Heilmann 1972; Heilmann 1974). Yet, the *Drosomycin* expression from day 5 of oral infection onwards is Toll pathway dependent, since it is absent in *Myd88* mutant flies while present in *key* mutant flies (see article above). Therefore the question arises as to how the Toll pathway is activated. The Toll pathway can, apart from Lys-type PGN, be triggered by bacterial and fungal proteases via the Persephone serine protease (Ligoxygakis *et al.* 2002; Gottar *et al.* 2006; El Chamy *et al.* 2008). Thus, it is possible that *P. aeruginosa* infection is sensed through PSH. qRT-PCR analysis of *psh* mutant flies shows that *Drosomycin* induction is not altered when PSH is absent (Figure 23). It still remains to be established by what means the Toll pathway is activated during *P. aeruginosa* infection. One would need to test PGRP-SA, PGRP-SD and GNBP1 mutants, as DAP-type PGN is a weak inducer of the Toll pathway through these PRRs. It is possible that bacterial proteases activate the serine protease cascade via another protease that acts in parallel or downstream of PSH. To decipher the mechanism of Toll pathway activation, one could test bacterial mutants, *e.g.*, mutants affecting proteases for their ability to activate the Toll pathway. Alternatively or additionally, fly mutants infected with wild-type *P. aeruginosa* can be tested for their susceptibility to infection and subsequently, if susceptible, for activation of the Toll pathway.

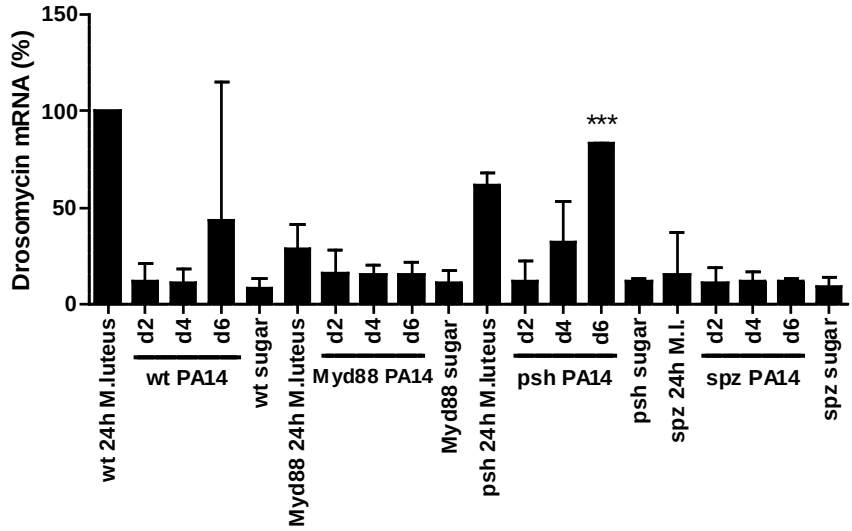


Figure 23: **Drosomycin induction upon oral infection with *P. aeruginosa***. *Drosomycin* induction is dependent on the Toll pathway, since it is absent in *Myd88* and *spz* mutant flies. In contrast, *Drosomycin* is still induced in *psh* mutant flies, indicating that *P. aeruginosa* is not sensed via PSH. *Drosomycin* induction in *psh* mutants at day 6 compared to sucrose-fed controls: *** p<0,0001; n=2. n: number of independent experiments (2x 5 flies used for each experiment).

Mutants of *S. marcescens* that have a mutation affecting the LPS biosynthesis kill wild-type flies slower than wild-type *S. marcescens* (Kurz *et al.* 2003; Nehme *et al.* 2007). The LPS-O-antigen seems to protect the bacteria from the action of the IMD pathway. We found that three *P. aeruginosa* strains affecting different genes of the LPS-O-antigen export pathway that thus lack the LPS-O-antigen display an interesting phenotype ((Liberati *et al.* 2006), Figure 24). These mutant bacteria kill wild-type and *key* mutant flies at the same rate as when infected with wild-type PA14, that is, they are as virulent. In contrast, *Myd88* mutant flies infected with LPS-O-antigen mutant bacteria die more slowly than expected as they succumb at the same rate as wild-type flies and do not display the enhanced sensitivity of *Myd88* mutants. Thus, LPS-O-antigen mutants are less virulent in a Toll mutant background.

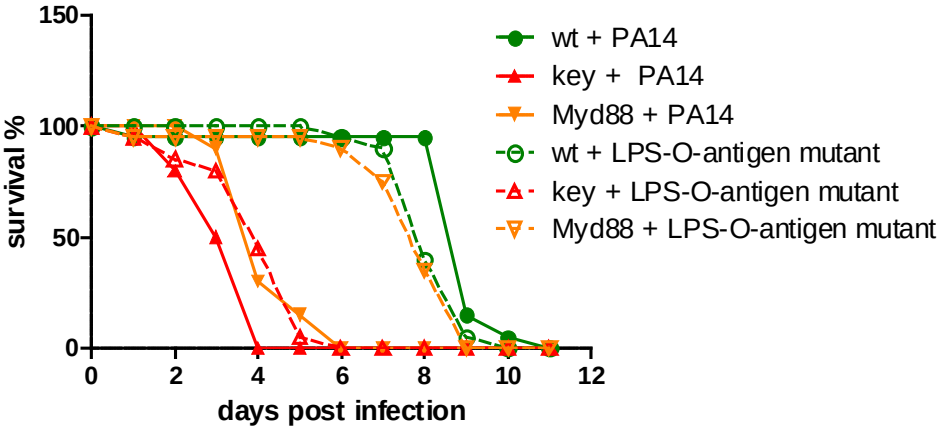


Figure 24: **LPS-O-antigen mutant PA14 kill *Myd88* mutant flies less efficiently than wild-type PA14**. LPS-O-antigen mutants kill wild-type and *key* mutant flies as wild-type PA14. In contrast, while wild-type PA14 kill *Myd88* mutant flies faster than wild-type flies, LPS-O-antigen mutants kill *Myd88* mutant flies at the same rate as wild-type flies. The difference in the survival curves of *key* mutant flies infected with wild-type PA14 or LPS-O-antigen mutant PA14 is not reproducible. The same is true for wild-type flies infected with the two different bacteria. n=3. n: number of independent experiments (20 flies used for each experiment).

This is a puzzling result at first sight. We developed a theory that could explain this surprising result (Figure 25). There might be a LPS-O-antigen-dependent virulence mechanism that is sensed and/or counteracted in a Myd88 (Toll pathway?)-dependent manner. In *Myd88* mutants infected with wild-type bacteria, this virulence device would be active but not counteracted and thus lead to a premature death of *Myd88* flies. In LPS-O-antigen mutant bacteria, the LPS-O-antigen-dependent virulence mechanism is not active, but other virulence factors of *P. aeruginosa* suffice to kill the flies since wild-type flies are killed by LPS-deficient mutants at the same rate as when infected with wild-type bacteria. For infection with these LPS-deficient bacteria MyD88 appears to be dispensable since the LPS-O-antigen-dependent virulence that should be counteracted is not there, leading to a survival comparable to that of wild-type flies. It would be important to test other Toll pathway mutant flies to figure out whether the observed phenotype is an effect of the Toll pathway or of MyD88 alone. In addition, it would be important to monitor the induction of the Toll pathway effector *Drosomycin* in wild-type flies infected with LPS-O-antigen mutant bacteria. If the Toll pathway is activated by sensing the LPS-O-antigen or an effect of the LPS-O-antigen dependent virulence, LPS-O-antigen mutant bacteria should not

trigger the Toll pathway. We will need to perform more experiments to exactly understand how the Toll pathway is activated during *P. aeruginosa* oral infection.

Flies also induce *Diptericin* and *Drosomycin* when *P. aeruginosa* is directly introduced into the body cavity of the fly by pricking with a tungsten needle previously dipped into a *P. aeruginosa* culture. Interestingly, preliminary data suggest that in this case the *Drosomycin* induction is IMD dependent since it is absent in *key* mutant flies (Figure 26). It has been previously shown that *Drosomycin* expression can be regulated by the IMD pathway in a local epithelial immune reaction (Ferrandon *et al.* 1998). The finding that *Drosomycin* expression in the septic injury model seems to be IMD dependent and the underlying processes need further investigation. A possible synergy with the Toll pathway also needs to be investigated (Tanji *et al.* 2007; Tanji *et al.* 2010).

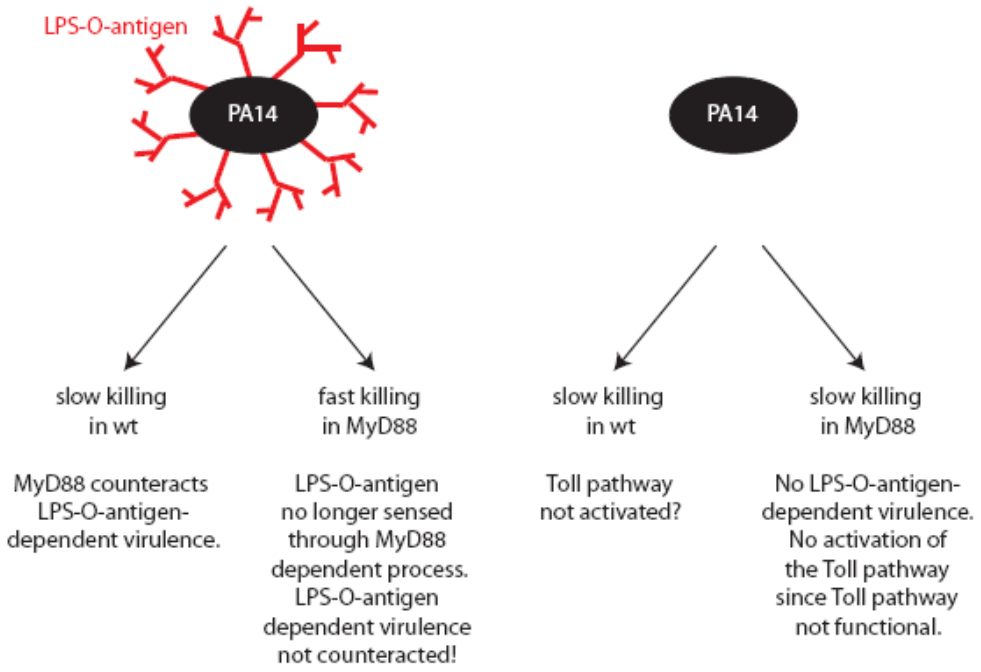


Figure 25: **Model for the role of the LPS-O-antigen.**

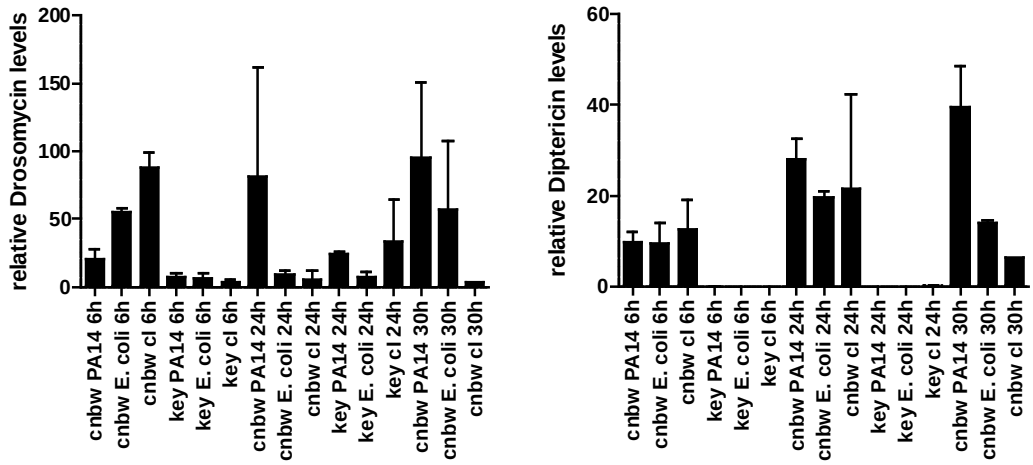


Figure 26: ***Drosomycin* and *Diptericin* induction after a septic injury with *P. aeruginosa***. *Drosomycin* (left panel) and *Diptericin* (right panel) induction in wild-type flies pricked with a needle previously dipped into a *P. aeruginosa* solution of an OD_{600} of 0.1. *Drosomycin* and *Diptericin* induction seem to be IMD-dependent since they are absent in *key* mutant flies. Control flies have been pricked either with a needle previously dipped into a concentrated pellet of *E. coli* or with a sterile needle (clean injury, cI); n=2. n: number of independent experiments (2x 5 flies used for each experiment).

Is the role of RhlR during infection quorum sensing dependent?

RhlR seems to confer at least partial resistance to phagocytosis (see article). At the time of infection at which RhlR appears to counteract phagocytosis the hemolymph bacterial titer is very low (about 100 bacteria). Quorum sensing is usually triggered when bacteria reach a rather high density. RhlR has an effect on the virulence of the bacteria since *rhlR* mutant bacteria kill wild-type, IMD mutant and Toll mutant flies significantly slower than wild-type PA14 (see article). In addition, RhlR is important to synchronize the infection, leading to a uniform death of the flies that translates into a steep survival curve at the time of death (see article). This latter function seems to be independent of phagocytosis since also Latex-bead injected flies that are infected with *rhlR* mutant bacteria die less synchronously than when infected with wild-type bacteria. To figure out whether these functions of RhlR are quorum sensing dependent, we performed survival experiments with several different *rhlI* transposon insertion mutants since RhlI is required to synthesize the C4-HSL that activates RhlR (Figure 13 and 27A). We infected wild-type flies with three different transposon insertion mutants of *rhlI*. Two of the mutants showed a phenotype comparable to *rhlR* mutants, while one showed intermediate virulence (Figure 28). All three transposons are inserted in the coding region of the gene: two have the same

orientation as the open reading frame (ORF) (ID # 34355 and 39424), while the last one is oriented in the opposite direction (ID # 33961) (Figure 27A). The mutants having the transposon insertions with the same orientation as the ORF displayed a reduced virulence comparable to that of *rhlR* mutants (ID # 34355 and 39424). The third transposon mutant exhibited an intermediate phenotype (ID # 33961). Since the different mutants did not show the same phenotype, it is necessary to construct a deletion mutant of *rhlI* to be sure of the contribution of RhlI to PA14 virulence. It is likely that a deletion mutant will show altered virulence, since it is not probable that two transposon insertions cause a phenotype independent of the gene of insertion. Note that also one of the *rhlR* transposon insertion mutants does not display a phenotype (ID # 54300, Figure 28). This transposon has the same orientation as the ORF. Since the transposon contains a transcription start site (Liberati *et al.* 2006), it is a possibility that a truncated, partially functional, protein is formed. The two other transposon insertion *rhlR* mutants display the same phenotype as the *rhlR* deletion mutant (Δ*rhlR*, Figure 28), therefore the observed effects of RhlR are likely to be real. To establish that circumventing the cellular immune response is quorum sensing dependent, it will be necessary to reproduce the regained virulence of *rhlR* mutants in Latex-bead injected flies also with *rhlI* mutants. In addition, supplementation of *rhlI* mutants with C_4-HSL should rescue the altered virulence phenotype (Stoltz *et al.* 2008). C_4-HSL could be added to the feeding solution or directly injected into the flies body cavity at different time points of the infection. These experiments are important experiments that need to be performed to show that the RhlR effect is quorum sensing dependent. It would also be interesting to use paraoxonase (Stoltz *et al.* 2008) expressing flies. Human paraoxonase is able to degrade 3-oxo-C_{12}-HSL. If the Las system is really not required during oral infection with *P. aeruginosa*, the paraoxonase-expressing flies should behave as wild-type flies.

Interestingly, *lasR* mutant bacteria did not exhibit a phenotype (see article and Figure 28). In addition, to the *lasI* transposon (ID # 39292) insertion mutant tested in the beginning (Figure 19), wild-type flies were infected with another *lasI* transposon (ID # 37259) insertion mutant (Figure 27B and 29). The two *lasI* mutants did not display a decreased virulence phenotype like the *lasR* deletion mutant. As mentioned above, the Las quorum sensing system is supposed to act hierarchically above the Rhl system. Therefore, the effect of RhlR is either independent of quorum sensing, or the normal hierarchy of quorum sensing systems is not valid in the *Drosophila* oral

infection model. As mentioned in the introduction (Dekimpe *et al.* 2009) recently showed, that the Rhl system can be activated independently of LasR in liquid culture. In wild-type bacteria the Las system is activated in the exponential phase and triggers the subsequent activation of the Rhl system. The activation occurs earlier than the LasR independent activation of the Rhl system in *lasR* mutants, but the Rhl system can clearly be activated in absence of LasR (Dekimpe *et al.* 2009). Moreover, RhlR can regulate LasR dependent genes in a *lasR* mutant background. The authors hypothesize that the Rhl system might be triggered by either the basal expression of *rhlR* and *rhlI*, which would lead to the slow accumulation of the signaling molecule, or by environmental factors. The activation might also be a result of a mixture of the two. Therefore, it is possible that in the *Drosophila* oral infection model, environmental factors, in this case probably fly-derived, trigger independently of LasR the Rhl system, which is then needed to overcome the cellular immune defense of the host. To assess this possibility the characterization of *rhlR lasR* double mutants as well as the study of Rhl effector expression in different mutant backgrounds will be interesting.

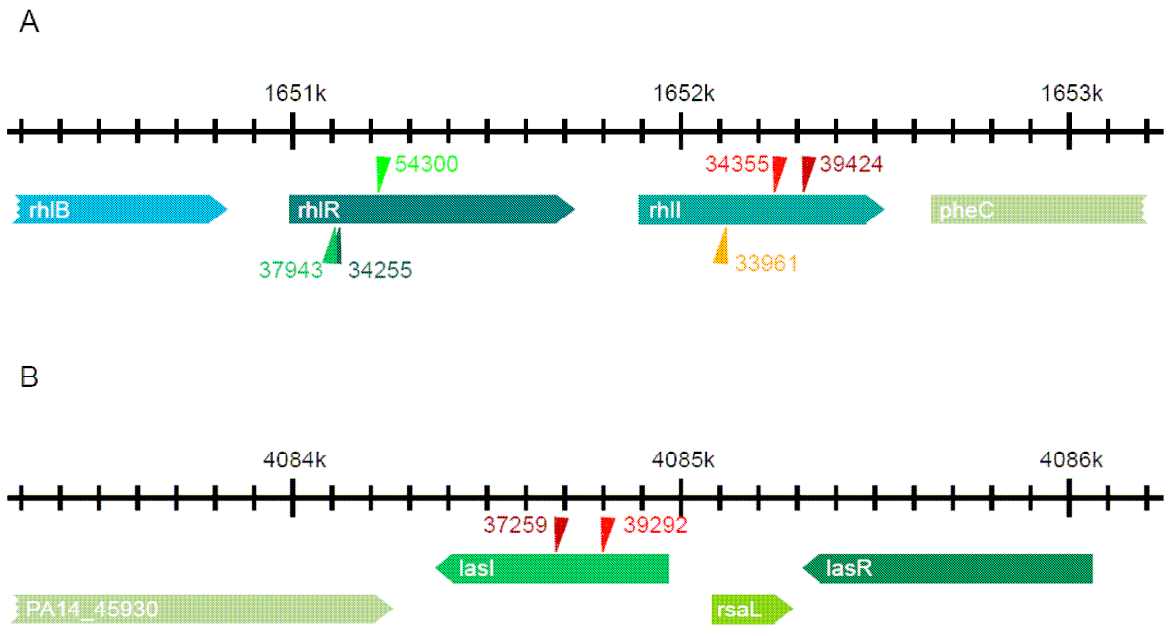


Figure 27: **Transposon insertion sites in the *rhlR*, *rhlI* and *lasI* genes**. **A**: transposon insertion sites in the *rhlR* and *rhlI* genes. **B**: transposon insertion sites in the *lasI* gene. No transposon insertions are available for *lasR*.

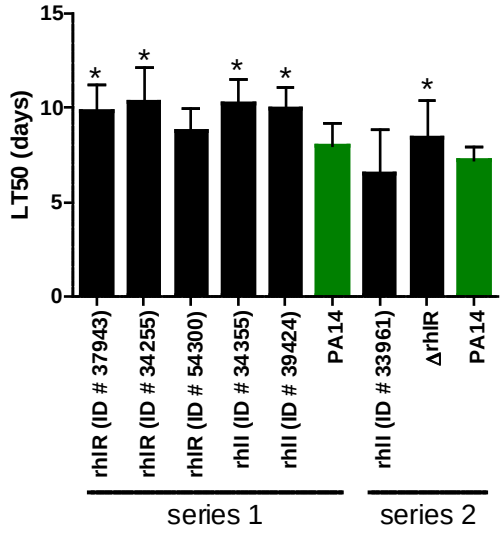


Figure 28: **Survival of wild-type flies infected with different *rhlI* and *rhlR* transposon insertion mutants**. Wild-type flies were infected with different bacterial mutants. The survival experiments have been performed in two different experimental series. Mean LT50 values are shown. Statistical analysis has been performed using the PA14 infected flies as control. *p<0,05, n=5. n: number of independent experiments (20 flies used for each experiment).

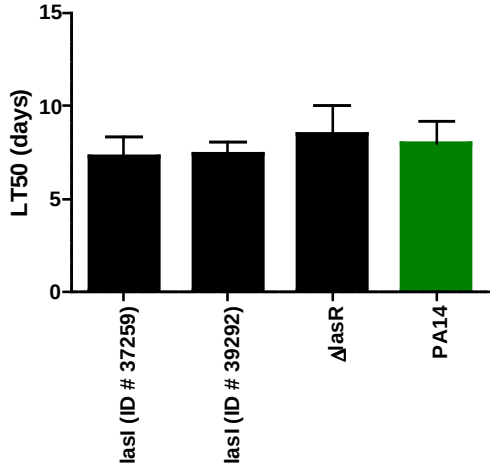


Figure 29: **Survival of wild-type flies infected with *lasI* transposon insertion mutants and a *lasR* deletion mutant.** *lasR* mutant as well as *lasI* mutant bacteria do not show altered virulence compared to wild-type PA14 when infecting wild-type flies. Mean LT50 values are shown. n=5. n: number of independent experiments (20 flies used for each experiment).

What is the role of the T2SS?

As stated above, the type II secretion pathway is important for virulence in all fly backgrounds since T2SS mutants are less virulent in wild-type, IMD pathway mutant, Toll pathway mutant and Latex-bead injected flies (Figure 19). Preliminary data suggests that T2SS mutants are more sensitive to the IMD and Toll dependent humoral immune response than wild-type bacteria. T2SS mutants did not reach high numbers in the hemolymph of wild-type flies, while they were able to achieve high numbers in IMD mutant (*key*) and Toll mutant (*Myd88*) flies (Figure 30). High numbers of bacteria are nevertheless measured later in infections with T2SS mutants than in infections using wild-type bacteria, suggesting that T2SS mutants are susceptible to both IMD and Toll pathway effectors and therefore require more time

to reach high numbers in flies mutant for just one of the two pathways. To assess this possibility it would be important to test T2SS mutant bacteria in flies mutant for both the IMD and the Toll pathway (*Myd88, key*). If the mutant bacteria are susceptible to the action of the humoral immune response they should regain their complete virulence in *Myd88, key* mutant flies and should be able to reach the same titer as fast as wild-type bacteria. The T2SS is needed for the secretion of many virulence factors, including proteases and peptidases, which might be important to degrade AMPs. To assess this possibility one should perform survival experiments using protease and/or peptidase mutant *P. aeruginosa* strains. The difficulty here lies in the likely redundancy of different proteases and peptidases. *P. aeruginosa* mutants that are just depleted for a single protease or peptidase therefore might not display a phenotype. It would be important to generate bacteria with multiple mutations in different protease and/or peptidase encoding genes that are not affecting the T2SS machinery. Generation of such mutants is unfortunately not trivial.

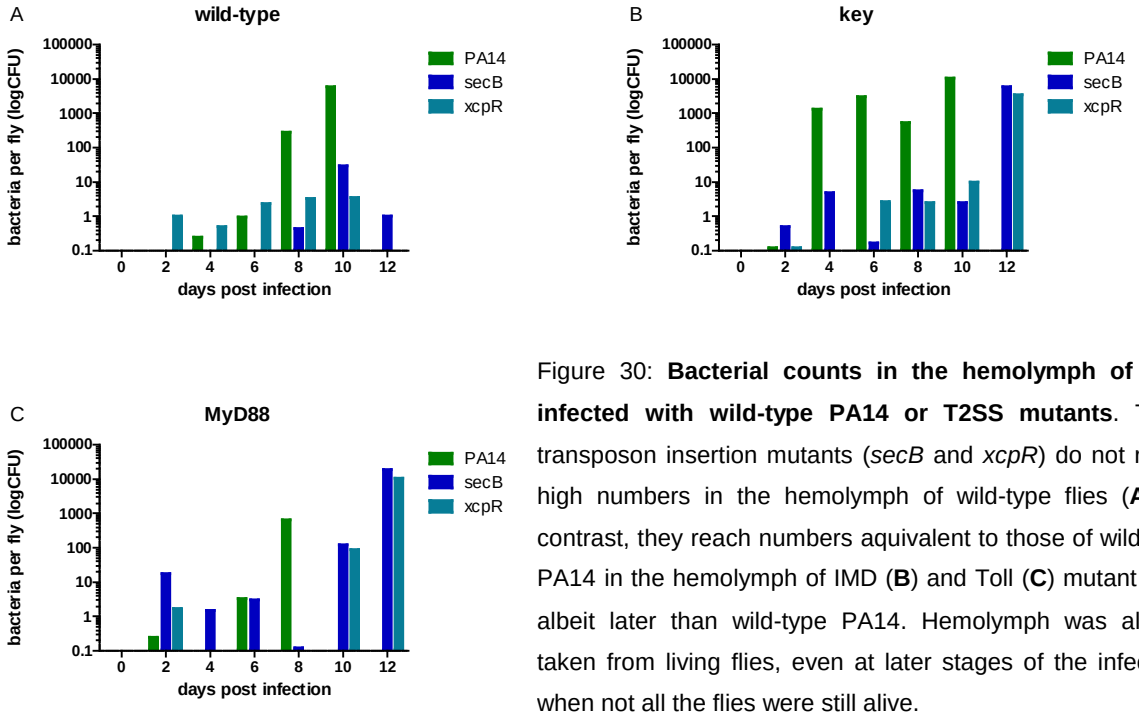


Figure 30: **Bacterial counts in the hemolymph of flies infected with wild-type PA14 or T2SS mutants**. T2SS transposon insertion mutants (*secB* and *xcpR*) do not reach high numbers in the hemolymph of wild-type flies (**A**). In contrast, they reach numbers aquivalent to those of wild-type PA14 in the hemolymph of IMD (**B**) and Toll (**C**) mutant flies, albeit later than wild-type PA14. Hemolymph was always taken from living flies, even at later stages of the infection, when not all the flies were still alive.

Overall, the *P. aeruginosa-D. melanogaster* oral infection model allowed us to gain important insights into *P. aeruginosa* infection. The strong advantage of this system is the possibility to genetically manipulate both the host and the pathogen. Combinations of bacterial and fly mutants allow to decipher in detail host-pathogen interactions. It would not have been possible to find the importance of RhlR in fighting the cellular immune response of the host without infecting Latex-bead injected flies

(or *eater* mutants) with *rhlR* mutant bacteria. In an experimental approach with wild-type flies and bacterial mutants, one would just have been able to state that RhlR is needed for virulence. Equally, the use of wild-type bacteria to infect mutant flies would have just revealed a contribution of phagocytosis. Therefore, to gain an in-depth understanding of infectious processes and the interactions taking place between the pathogen and the host, it is crucial to combine the tools available in the host with those available for manipulating the pathogen.

The *P. aeruginosa* transposon insertion library and the deletion mutants available after decades of research joined with the sophisticated tools available to manipulate *Drosophila* have the potential to be of great use in seeking to understand *P. aeruginosa* infections.

4 Concluding remarks

The model system

Drosophila is a well-studied model organism with great genetic, genomic and molecular tools that have been developed in more than a century of *Drosophila* research. To fight pathogens, insects rely on the innate immune response. They have developed several mechanisms to detect infection and respond to it. The immune system of the fly relies on several effector mechanisms including clotting, melanization, encapsulation, phagocytosis and the NF-κB-dependent inducible production of, for example, AMPs (see Introduction and Annex). The innate immune systems of insects and mammals show a high degree of conservation. One of the most striking examples is that of the discovery of Toll-like receptors. The Toll-like receptors in mammals have been found following the discovery of the Toll pathway as a major component of the *Drosophila* immune response. Of great importance is also the evolutionary conservation of the NF-κB signaling cascades, on which the *Drosophila* humoral immune response depends.

When studying the immune system of the adult, two main infection routes can be used, the inoculation of the bacteria directly into the hemocoel by a septic injury or oral infection. The focus of this study was mainly on host-pathogen interactions during oral infection. In this working model, young flies are constantly feeding on a bacteria-containing sucrose solution. This is of course an artificial system since the fly is not likely to feed on a pure culture of one bacterial species for several days in the wild. Nevertheless, it may be closer to natural infection than the septic injury model since wild fruit flies feed on decaying fruits that are a milieu with high microbial concentrations.

It has recently been shown that polymicrobial infections that are for example often found in the lungs of CF patients can lead to important effects on pathogenicity (Sibley *et al.* 2008). Such effects can probably be caused by feeding on different microbes at the same time or by interactions of ingested microbes with the intestinal microbiota. The microbiota of flies is not as complex as that of mammals (10^{14} microbial cells, 500 species (Savage 1977; Eckburg *et al.* 2005)), but still consists of $3{,}5x10^{5}$ microbial cells belonging to 5-20 different species (Cox *et al.* 2007; Ren *et al.* 2007; Ryu *et al.* 2008), although its exact composition and importance likely varies with the increasing age of the flies. Indeed, aged flies display a gut full of bacteria that actually harm the homeostasis of the intestinal epithelium (Biteau *et al.* 2008). Coinfection with two distinct microorganisms can lead to a faster demise of the fly

than can be accounted for by an additive effect (Sibley *et al.* 2008). This aspect of infections has not been examined in this study.

Intestinal infections

Ingested bacteria, whether in the form of a single strain or that of a complex mixture of strains encounter a hostile environment in the intestine of the fly. Apart from competing commensals that might reside in the intestine, they have to survive the attacks of the gut epithelium, which comprise the production of ROS and AMPs (see Introduction and Annex). In the case of *S. marcescens* (Nehme *et al.* 2007) and *P. aeruginosa*, the IMD-dependent local response seems to be effective. Concerning the ROS response, it would be important to test ROS-response defective flies (*Duox* mutants) for their survival to infection. ROS-sensitive bacterial strains will also be useful. Indeed, a catalase-deficient *S. marcescens* Db11 strain appears to be less virulent in the oral infection model (P. Giammarinaro, A. Ayyaz, unpublished data). The two oral infection models established in our laboratory will help to further decipher the mechanisms underlying the local immune response in the gut.

The intestinal epithelium of flies as well as that of mammals needs to be able to discriminate between commensal microorganism and pathogens. Like pathogens, the commensals produce MAMPs such as LPS or PGN that are in principle capable of provoking a constant host immune response. In barrier epithelia, it is crucial to down-regulate the immune reaction triggered by the sensing of commensal microbes and to activate it just when pathogens need to be fought. Indeed, a constantly activated immune response is energetically very costly and can even be detrimental to the host. Furthermore, it may change the composition of the microbiota and select for an initially minor bacterial strain, which becomes harmful to the fly when selected for by the constitutive activation of the IMD pathway (Ryu *et al.* 2008). It is essential to keep the balance between defense and connivance in such microbe-interacting epithelia. Not surprisingly, chronic inflammatory diseases occur in mammals when this balance is disrupted (Guarner *et al.* 2003; Pasparakis 2009). The molecular mechanisms underlying the distinction between normal microbiota and pathogenic conditions are currently not fully understood, but advances have been made (see Introduction and Annex).

Pathogenic bacteria like *S. marcescens* or *P. aeruginosa* cross the gut epithelium. To do so, they have to overcome several barriers. The *Drosophila* midgut

is lined by a chitinoproteinaceous layer, the peritrophic matrix, which is produced by the proventriculus (cardia) and represents the first physical barrier, followed by the epithelium itself. In addition the gut of the fly has an acidic region, the copper cells that serve as “stomach” (see Introduction). Overall, the gut of the flies is a hostile environment. Some of the bacteria survive the hostile environment and cross the gut epithelium rapidly. To investigate the passage of *P. aeruginosa* in detail, the described oral infection model will be very helpful since in this model it is possible to combine genetic tools of the host and the pathogen to decipher the distinct interactions.

Virulence in the hemocoel

After having crossed the epithelial barrier, *S. marcescens* as well as *P. aeruginosa* do not seem to be as virulent as in the septic injury model. The basis for the reduced virulence after gut passage remains to be established. Here as well, the *P. aeruginosa* model will be of use. In the hemolymph, bacteria are attacked by phagocytic cells (hemocytes) and, at least in the case of *P. aeruginosa* infection, by the effectors of the IMD- and Toll-dependent humoral immune response. Phagocytosis appears to be efficient against infection with both pathogens since in both cases phagocytosis-deficient flies succumb faster to the infection. The phagocytic receptor Eater is involved in the phagocytosis of *S. marcescens* (Kocks *et al.* 2005) and *P. aeruginosa* (see above). *P. aeruginosa* RhlR plays a role in circumventing phagocytosis as was established by the low virulence of *rhlR* mutant bacteria in wild-type and IMD or Toll mutant flies, which was regained in Latex-bead injected or *eater* mutant flies. Further studies with RhlR mutants in diverse fly mutants will help to find the RhlR target in the fly.

Tolerance/endurance, an important mechanism of host resistance

Pathogens have to deal with various attacks from the host side. Successful pathogens have developed mechanisms to withstand the hostile environment inside the host. In addition, pathogens produce factors that attack the host. For instance, ingested *S. marcescens* severely damage enterocytes, either through hemolysin during the early phase or by as yet unidentified virulence factors during later stages of the infection. Therefore, host survival is dependent first on the capacity of its

armamentarium to limit pathogen numbers (resistance), and second on its ability to deal with pathogen- or own immune response-induced damage (tolerance). Resistance and tolerance both contribute to the outcome of the infection, as was nicely illustrated by the case of oral *S. marcescens* infection in *Drosophila*. The flies' capacity to limit bacterial numbers via the IMD-dependent local immune response and phagocytosis adds up with its tolerance towards bacteria-induced damage of the intestinal epithelium, that is dealt with through JAK/STAT dependent compensatory proliferation of ISCs, to prolong fly survival. When either an important resistance or an important tolerance mechanism is affected, flies succumb faster to the infection. This shows that resistance and tolerance are essential complementary dimensions of host defense. Thus, tolerance lies at the intersection between infectiology and physiology. The importance of tolerance has long been disregarded by animal immunologists, who focused on the mechanisms underlying resistance to infection. In contrast, plant biologists already made the distinction between resistance and tolerance in the late 1800s (Cobb 1894). Tolerance has been one focus of work by plant scientists since then (Rausher 2001; Kover *et al.* 2002). Recently immunologists have started to focus also on tolerance mechanisms in animals (Corby-Harris *et al.* 2007; Ayres *et al.* 2008; Raberg *et al.* 2009; Seixas *et al.* 2009). Our study demonstrated the importance of tolerance and the infection models developed in the laboratory will help to further investigate tolerance mechanisms. Because tolerance has a precise meaning in mammalian adaptive immunity (tolerance mechanisms avoid that the immune system attacks its self antigens), we propose to employ the term endurance, which is less heavily connoted.

This study cast some light on different aspects of oral infection. It showed the importance of endurance mechanisms as the JAK-STAT dependent compensatory proliferation during *S. marcescens* oral infection and the need to take these mechanisms in account when aiming to understand infectious processes. It is likely that many of the genes identified through our genome-wide screen on host defense against *Serratia marcescens* infection are involved in endurance mechanisms. Some of the hits we found may play a role in compensatory proliferation of ISCs. However, it is likely that other genes found in the screen will be shown to play a role in other, as yet unidentified, homeostatic mechanisms that allow the fly to withstand bacterial and host induced (*e.g.* ROS) damages.

The oral infection of the fly with *P. aeruginosa* was characterized and a role of *P. aeruginosa* RhlR in counteracting phagocytosis was established. The analysis of the genes found in the genome wide screen will help to shed light on the many aspects of host-pathogen interactions we do not yet understand. One thing that is clear by now is that each infection, which involves a specific microbial strain and its host, is unique, as was illustrated by the differences between *S. marcescens* and *P. aeruginosa* oral infections in *Drosophila*. The host is the same and both pathogens are Gram(-) bacteria, but still the two infections are strikingly different from each other, probably even in the cause of death. Nevertheless, there are parallels, such as the reduced virulence after passage through the gut epithelium, that might be based on general mechanisms.

The stage is now set for a detailed dissection of host-pathogen interactions from the dual perspective of both partners of the infectious process. The contribution of model organisms, both for the host and the pathogen, will be invaluable to understand and act on diseases caused by microbial infections.

5 Annex

Virulence on the fly: *Drosophila melanogaster* as a model genetic organism to decipher host-pathogen interactions

Virulence on the fly: *Drosophila melanogaster* as a model genetic organism to decipher host-pathogen interactions

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Abstract

Drosophila melanogaster is widely used to study host-pathogen interactions. To gain an in-depth understanding of infectious processes one has to understand the specific interactions between the virulence factors of the pathogen and the host defense mechanisms. A deep understanding is crucial for identifying potential new drug targets and developing drugs to which the pathogens might not gain resistance easily. To give an overview over the current knowledge on *Drosophila*-pathogen interactions and the approaches that can be used to study those interactions this review focuses mainly on infections with two pathogens: the well-studied gram-negative bacterium *Pseudomonas aeruginosa* and the yeast *Candida albicans*. We also discuss the usefulness of *Drosophila* infection models for studying specific host-pathogen interactions and high-throughput drug screening.

Keywords: *Candida albicans* virulence, *Drosophila* immunity, endurance, host-pathogen interactions, model organism, *Pseudomonas aeruginosa* virulence

Introduction

Many microbes develop resistance to currently available antibiotics and thus pose a formidable challenge for fighting infections. Ideally, novel strategies need to be implemented that would limit the risk of pathogens evolving resistance to this armamentarium, for instance by targeting virulence factors of microbes rather than essential genes. To this end, we need to obtain an in-depth mechanistic understanding of infections at the molecular level to identify critical genes and processes that should be targeted by pharmacological approaches. At first glance, if one were to use a genetics approach, one might consider that the interactions of any pair of genes, one gene from the host and one from the pathogen, should be investigated in order to determine whether this interaction is specific and relevant to pathogenesis [1]. Given the number of genes present in the host genome of multicellular organisms (usually 14,000 to 30,000) and given that of microbial pathogens (roughly ranging from a few thousand to more than 10,000 genes, viruses excluded), several million interactions should be analyzed, which is not feasible using current technologies. Fortunately, the identification of host defenses on the one hand and virulence genes of the pathogen on the other hand limits the number of interactions to be first investigated. That is, one genome is kept constant while the other is scanned for mutations that respectively alter host defense or pathogen virulence. By doing this, one ignores the specific interactions that may be revealed only by placing a mutant pathogen in the context of a host impaired for a specific host defense targeted by the pathogen. However, even this simplified approach of considering only one genome at a time cannot be easily implemented at present as we are not aware of any infection model in which all host defense genes and all virulence genes of the pathogen have been tested and identified by genetic analysis. Of note, this is a reachable objective in *Drosophila* as a genome-wide screen has already identified genes involved in host defense against ingested *S. marcescens*

[2]. A similar approach on the pathogen side has been made possible with the development of an ordered library of *P. aeruginosa* mutants [3].

For medical purposes, the host of interest is *Homo sapiens sapiens*, who is not well-adapted for experimental research because of obvious ethical reasons. An alternative has been to study infected patients, especially those that appear to be prone to developing specific infections. This makes use of the sophisticated medicine that is available nowadays and that allows for a detailed diagnostic, that is a level of description of the phenotype that can only be gained from medical doctors and is hard to achieve in model animals [4]. One strategy is to sequence candidate genes from the susceptible patients to pinpoint the genetic origin of the deficiency. Often, the candidate genes have been identified from studies performed in model experimental organisms such as *Mus musculus* [5]. Mice provide a convenient alternative as they can be rather extensively genetically manipulated. However, it is still difficult to perform directed or random mutagenesis at a genome-wide scale, even though large-scale screens have been implemented to study the immune response of mice. Furthermore, the golden standard experiment is to perform survival studies in which animals are infected and the lethal outcome of the disease monitored. To be meaningful, these studies should use at least about 10 animals per condition and ought to be repeated in independent experiments, which is rarely performed in mice because of ethical and financial considerations. Finally, a mouse is not a human and even though the last common ancestor we share with mice lived only about 90 million years ago, caution should be exercised in transposing knowledge gained in animal models to humans.

Is there a way to bypass this conundrum? Invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* may provide an interesting alternative because of their powerful genetics, even though the last common ancestor they share with us, Urbilateria, must have lived between 555 million to one billion years ago. However, the

important point is that Urbilateria evolved in a world dominated by microbes and thus must have had a primitive innate immune system from which both the deuterostome and the protostome innate immune systems are derived. The work of the past twenty years has largely emphasized the amazing molecular conservation of pathways and processes involved for instance in development or immunity, despite the long evolutionary divergence between protostomes and deuterostomes [6, 7]. For instance, the discovery of an immune role for the Toll pathway in *Drosophila* led to the characterization of Toll-like Receptors as major Pattern Recognition Receptors in mammals [8, 9].

In this article, we shall first briefly review the advantages and limits of the fly model for the study of host-pathogen interactions. Next, we shall summarize the salient features of host defense in this model organism before dwelling on host-pathogen interactions. As an example of the variety of approaches that have been implemented in *Drosophila*, we shall essentially focus on the study of host-pathogen interactions using *Pseudomonas aeruginosa* as a pathogen. Finally, we shall also review some studies investigating fungal pathogenesis before drawing general conclusions on the usefulness of the *Drosophila* model for the identification of novel drug targets to limit infectious diseases.

Overview of the* Drosophila melanogaster *model

The major advantages that led Charles W. Woodworth and shortly later Thomas Hunt Morgan to choose *Drosophila* as a research system more than a century ago were its small size, its ease of breeding and low price, its short living cycle (about 10 days at 25°C), and its large offspring, which could be bred by millions so as to identify naturally occurring mutations. Indeed, when a vial containing a fly couple is left aside for a month, almost all of the food present in the vial transforms into living flies. However, the most important reason for choosing *Drosophila* as a model system is Morgan's vast scientific legacy, with the

development of powerful and sophisticated genetics throughout the past and present century [10]. Forward genetic screens remain the bread and butter of *Drosophila* research. Saturating genome-wide screens were first introduced some 30 years ago by Christiane Nüsslein-Volhard and Eric Wieschaus to discover the genes involved in the zygotic development of the fly, thus actually performing functional genomics many years before the term was invented [11-14]. While the identification of mutants initially relied on natural events, the introduction of X-ray mutagenesis by Müller, then of chemical mutagenesis by Ed Lewis allowed for a more efficient isolation of mutants. One wealth of *Drosophila* genetics is the obtention of allelic series, with hypomorphic alleles going all the way to the null phenotype and sometimes neomorphic and conditional mutants such as heat-sensitive phenotypes. The mapping of chemically-induced mutations was slow but has now considerably improved with the wealth of tools available, the genome sequence being not the least. Other mutational techniques include the use of transposon insertions, which in some cases can be excised imprecisely, thus generating small deletions. Insertion mutants in about 65% of annotated genes are currently available in public stock centers and some private companies [15]. A powerful novel technique is that of RNA interference using transgenic hairpins designed to target a specific gene, which can be implemented in a spatially and temporally controlled manner by using the UAS-GAL4 expression system (see Box) [16, 17]. This latter system allows the controlled expression of any transgene in a tissue of choice. For instance, it is possible to modulate JAK-STAT signaling specifically in intestinal stem cells to modulate infection-induced compensatory proliferation and thus alter the homeostasis of the epithelium [2, 18]. It is also possible to knock-out gene by directed recombination, although this technique is long and sometimes challenging [19]. These approaches on flies can be complemented by work on cellular cultures [20]. Genome-wide RNAi screens are relatively easy to implement and have

been used in many studies, for instance to identify regulatory genes of signal transduction pathways or to identify the genes required for the growth of intracellular pathogens [21-23].

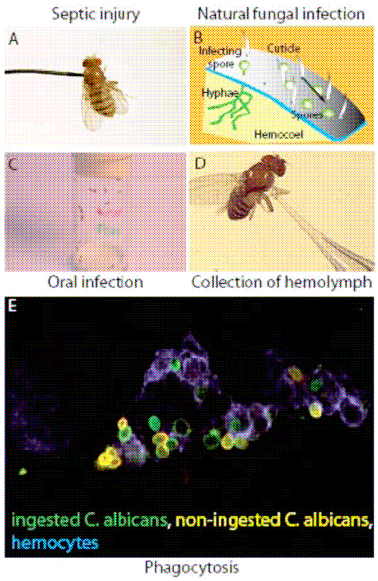
These tools and genomics approaches (microarrays, RNAseq; see box) have been used by the large community of Drosophilists to generate a vast body of knowledge that makes this organism one of the best-understood at present. All *Drosophila*-related information is freely accessible in the FlyBase database (http://flybase.org/). One of the great strengths of the field ever since Morgan developed it is the free exchange of *Drosophila* stocks, which are usually shared after publication and sometimes before. Many problems of modern biology are being tackled in *Drosophila*, ranging from the control of gene transcription and RNA molecules at the nucleotide level to cellular and developmental biology, behavior, study of human diseases (for example, understanding the function of genes involved in Parkinson disease [24]), immunity and host-pathogen relationships, population and evolutionary genetics (the whole genome sequence of 12 *Drosophila* species is available) [25]. Clearly, the ability to move rapidly from a gene to the whole organism is a major benefit of working on the *Drosophila* model.

Whereas *Drosophila* is especially well-suited to study fundamental biological mechanisms, it is obviously limited to study mammalian-specific processes, for instance the function of the cerebellum. One has always to take care to define carefully the function under study and be careful in transposing insights gained in flies to mammals. In the case of host-pathogen relationships, one drawback is that the optimal temperature for raising flies is 25°C. When grown at temperatures higher than 29°C, males will become sterile. When treating flies with drugs, one has to consider that the pharmacokinetics and metabolic processing of the drug may be distinct from that occurring in mammals. For example, flies have no kidneys but Malphigian tubules. Oxygen is directly delivered to the tissues by a network of trachea and the hemolymph does not transport red blood cells and has no major function in respiration.

Also, the small size of flies and the absence of thermoregulation has to be taken into account. Another limitation is that it may be sometimes difficult to identify subtle phenotypes or specific conditions under which phenotypes may arise. Finally, similar to the evolution of HeLa cells, fly stocks that are commonly in use were isolated more than 50 years ago and may well have adapted to laboratory conditions. A classical example of this phenomenon in model organisms is that of the FLO11 gene in the yeast *Saccharomyces cerevisiae*, which is required for flocculation and has been counterselected for in the laboratory, thus allowing liquid culture [26]. Indeed, most flies collected in the wild nowadays contain the P-element transposon, which has invaded most *Drosophila* throughout the world in the second half of last century.

Box: Tools of the trade.

Drosophila melanogaster is one of the best studied organisms to date thanks to the inheritance of a century of genetic work that includes very sophisticated tools: e.g. targeted gene expression to overexpress recombinant proteins, reporter transgenes or RNA interference (RNAi)-hairpin constructs, which can be spatially an temporally controlled by the UAS-Gal4 expression system [16, 17]. In addition, mutant collections, access to the sequenced genome, and a powerful database manage the knowledge accumulated by thousands of researchers (http://flybase.bio.indiana.edu/) and render the fly an ideal organism to model host-pathogen relations.



Several infection models are available and established to study host-pathogen interactions in *Drosophila*: bacteria, fungi, yeasts and viruses can be directly inoculated into the body cavity (of both larvae and adults) with a sharpened needle dipped into concentrated cultures of microorganisms (A) or by injecting microbial suspensions with a micropipette (not shown). Natural infection can be achieved by shaking anesthetized flies on a sporulating fungal culture (B). In addition, feeding on microorganisms leads to oral infection in adults (C, bacteria culture added to filter disks) and larvae. [50, 130].

The pathogenicity of microbes can be monitored by observing survival to infection and quantifying microbial titers in the whole fly, dissected tissues and/or the hemolymph (D, collection of hemolymph with micropipette). In response to infection, *Drosophila* induces systemically or locally antimicrobial peptides (AMPs). The analysis of transcripts by Northern-blot and/or qRT-PCR together with the use of reporter transgenes (e.g. LacZ and GFP) are the most common experimental approaches to monitor AMP expression patterns.

The collection of sufficient material from larvae or adults allow biochemical approaches like matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and two-dimensional (2D) gel electrophoresis [167] to analyze the defense reaction on the protein level.

Several techniques have been developed to visualize and quantify phagocytosis in hemocytes (major plasmatocytes): treatment (via injection) with Indian ink [46, 95], fluorescent beads or fluorescence-labeled microbial particles (e.g. *fluorescein isothiocyanate* (FITC)-labeled *E. coli* [168]) and immuno-fluorescence techniques (E) are well established. Phagocytosis can be functionally blocked by injection of non-degradable Latex beads that saturate phagocytosis [169].

The Drosophila *host defense*

As outlined above, *Drosophila* have been evolving away from humans for hundreds of million years. Yet, major principles of innate immunity have been deciphered in insects, such as the discovery of antimicrobial peptides (AMPs) [27], and have been subsequently validated in mammals, although the reverse is true as well. For instance, the number of laboratories working on NF-κB signaling in vertebrates far outranks that of laboratories working on fly NF-κB signaling. The interactions between these scientific communities allow the cross-fertilization of both domains.

As regards the study of host defense, one major advantage of *Drosophila* is the lack of the adaptive immune response found in "higher" vertebrates, which is based on recombineering receptors that are altogether absent in the fly genome. Thus, it is much easier to correlate a phenotype of susceptibility to infection to a molecular defect since defects in innate immunity cannot be compensated for by the adaptive immune system.

Because of its large size and cheap cost, the use of the greater wax moth *Galleria mellonella* has been proposed to identify rapidly microbial virulence factors [28]. Indeed, in contrast to flies, the larvae can be bought and used immediately, without having to invest in a whole set-up to grow flies, which unfortunately cannot be kept frozen and have to be regularly transferred manually to fresh vials. However, the understanding of the immune system of *Galleria* is much less advanced than that of *Drosophila* and this system suffers from the difficulty in interfering with the immune defenses of the moth. Thus, we believe that *Drosophila* is much better suited for the study of host-pathogen interactions because the degree of in-depth understanding of the mechanism of action of virulence factors with respect to the host's defenses that will be required in the long term can only be achieved using the sophisticated genetics available in *Drosophila* and *Caenorhabditis elegans*. In the following, we briefly describe what is know to date on the *Drosophila* immune system and will point the

reader to many excellent reviews that have been written on the subject. For the purpose of this article, we shall hardly deal with antiviral immunity, which is a subject of its own.

We first wish to draw the attention of the reader to the differences that exist between larvae and adult *Drosophila*, which constitute two distinct systems. The larva is undergoing constant growth, is characterized by the presence of thousands of hemocytes, and is under tight hormonal control, which influences the immune response [29]. It is technically much more challenging to work with larvae, that are not easily put to rest and that are very susceptible to injury because the hydrostatic pressure generated by larval movements is quite high, resulting often in fatal leakage after experimental manipulations such as injections. Also, the functional balance between the different arms of the immune response may be different, inasmuch as larvae grow within a microbe-rich environment, decaying fruits, and may be exposed to different pathogens than flying adults.

Resistance mechanisms: innate immunity in the fly

In this section, we deal with the classical view of innate immunity, the role of which is to attack and, if possible, to clear pathogens. Invertebrate immunity has been a subject of study for more than 130 years starting with the discovery of phagocytosis by Metchnikow in 1881 [30]. Following the demonstration by Metchnikow that phagocytosis was a host defense mechanism against bacterial infections in aphids in 1886, investigators first focused on the cellular immune response, until a humoral immune response was established in 1916 [31, 32]. However, the identification of the antimicrobial activity was delayed until the early 1980's, until Hans Boman purified the first AMPs from the *Hyalophora cecropia* moth [27]. Many studies that led to the current paradigm of innate immunity as we know it today were performed using the septic injury model in which a sharpened tungsten needle was first dipped into a microbial solution prior to pricking the insect (see box), thus bypassing the

tough physical barrier formed by the insect exoskeleton, the cuticle, which is constituted of proteins and chitin. An overview of the major arms of the *Drosophila* host defense triggered by a septic wound is provided in Fig. 1 [33].

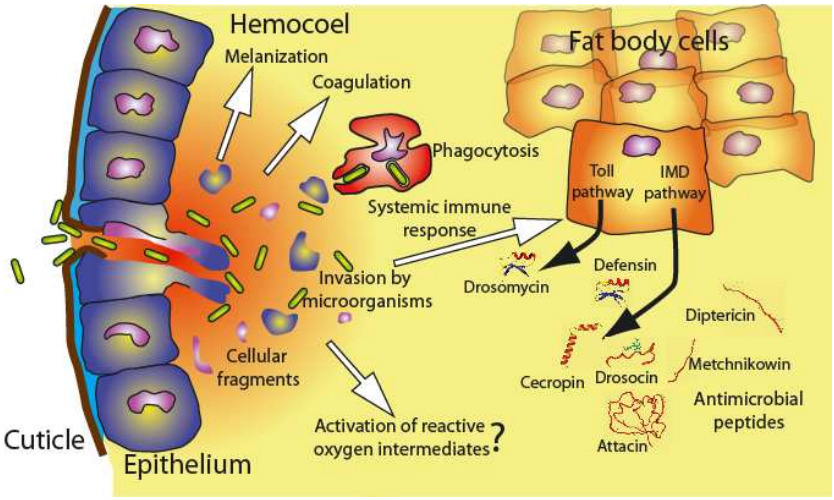


Figure1: **The *Drosophila* systemic immune reaction**.
A septic wound triggers the systemic immune reaction of the fly. The melanization and coagulation cascades are activated to trap pathogens and close the wound. Invading microbes are phagocytosed by hemocytes. In addition, the production of reactive oxygen intermediates might be triggered to fight microorganisms. A systemic infection, as well as wounding to some extend, induces the IMD and Toll pathway dependent production of antimicrobial peptides (AMPs) by the fat body and their secretion into the hemolymph.

Coagulation

Several reactions are triggered at the wound site. One of them is coagulation, which has been mostly studied in *Drosophila* larvae. It leads to the formation of a clot, which can be studied using proteomics [34]. The clot is formed by compounds found in the hemolymph (lipophorin, hexamerins and hexamerin receptor, fondue, tyrosinase-type phenoloxidase (PO)) and hemocyte-derived proteins such as tiggrin and hemolectin. Clot fibers are cross-linked by the evolutionarily conserved enzyme transglutaminase, which acts on Fondue [35]. Clot formation also appears to take place in the adult, although it seems to be smaller in a pullout assay; its importance in the adult host defense remains to be established. In contrast, the formation of a clot has been shown to be important for trapping invading bacteria in larvae. Larvae deficient for transglutaminase are much more susceptible to infection by an entomopathogenic nematode that uses a biological weapon, the bacterium *Photorhabdus* to

inactivate host defenses [35]. The factors that trigger coagulation remain elusive at present. In the horseshoe crab, at least two protease cascades converge to transform coagulogen into insoluble coagulin [36]. These protease cascades are triggered by microbial compounds such as bacterial lipopolysaccharide (LPS) or fungal ß-(1-3) glucans at exquisitely low concentrations and are therefore used clinically as bioassays for the detection of microbial contamination and infection. The proteases involved in the cascade appear to be very similar to the distinct proteolytic cascades that lead either to Toll pathway activation (see below) or the cleavage of proPO into an active enzyme.

Melanization

A secondary reaction that appears at the wounding site and which participates in hardening the initially soft clot is the activation of PO, which is a key enzyme required in the complex set of chemical reactions that process dopamin precursors and ultimately lead to the deposition of melanin on the clot. Melanization has been reported to generate reactive oxygen species (ROS), which may participate in killing invading microorganisms [37]. The activation of PO at the injury site may be elicited in larvae by host cell derived factors released through wounding, such as apoptotic cells and phosphatidylserine [38]. There is also a systemic activation of PO, which may be initiated by the detection of the bacterial compound peptidoglycan (PGN) through the Pattern Recognition Receptor (PRR) PGRP-LE (Peptidoglycan Recognition Protein-LE) as the overexpression of this PRR leads to PO activation [39]. PO cleavage is also triggered by Gram-positive bacteria and fungi. PO activation requires a functional Toll pathway, which may be required for a sustained production of active PO [40]. We have shown that the Gram-Negative Binding Protein 3 (GNBP3), which actually belongs to the ß-(1,3)-glucan binding protein family of PRRs, is required directly for PO activation independently of its role in Toll pathway activation [41].

We have also extended this finding to the two PRRs that mediate Toll pathway activation by some Gram-positive bacteria, namely GNBP1 and PGRP-SA [41]. The proteolytic cascades that ultimately cleave proPO are negatively regulated by the serine protease inhibitor Serpin 27A, which also regulates Toll pathway activation during development [40, 42]. There is some debate as whether PO plays an essential role in host defense against microbial infections [43, 44]. It appears, as will be most likely the case for many branches of host defense, that it depends on the microbe under study [43, 45]. A pathogen is often somewhat more susceptible to one type of host defense. Depending on its virulence factor armamentarium, it might be less well-equipped to confront specific types of host defense.

The cellular immune response

Whereas the adult appears to have only one class of hemocytes, the plasmatocytes, larvae have three distinct classes of hemocytes, namely plasmatocytes (90% of hemocytes), crystal cells, and lamellocytes [46]. The latter category is essentially not found in uninfected larvae. Lamellocytes differentiate upon the deposition of an egg laid down by parasitic wasps and more generally when particles too large to be phagocytosed are introduced into the hemocoel. Lamellocytes then form a tight capsule on top of a layer of plasmatocytes. Crystal cells actively rupture following an immune challenge and thus release PO crystals that dissolve into the hemolymph.

Hematopoiesis occurs in two waves, one that takes place during embryogenesis and one that occurs prior to metamorphosis in a dedicated structure, the lymph gland [Meister, 2003 #1363]. Of note, hemocytes can either be sessile or circulating, with some proliferation taking place also in the sessile hemocyte compartment [47]. Adult plasmatocytes have not been found to divide actively and actually comprise hemocytes of both embryonic and larval

origins [48]. Larval hemocytes are much more numerous (>5000) than adult hemocytes (1000-2000) [46].

As regards host defense against microbes, a main function of the cellular immune response is phagocytosis. A second function may be to participate in triggering a full systemic immune response in the fat body, possibly as a result of the emission of a secondary signal by hemocytes. Indeed, the Toll pathway ligand Spätzle (see below) is produced by plasmatocytes and is required for Toll pathway activation and thus acts as a cytokine [49]. A requirement for hemocytes to trigger systemically the Immune Deficiency (IMD) pathway (see below and Fig. 1) following an oral challenge with *Erwinia carotovora* has also been reported [50, 51]. Nevertheless, the IMD pathway can be induced efficiently in larvae devoid of hemocytes in a septic injury model [52]. It should be pointed out, however, that this secondary function of hemocytes may exist only in larvae. In adults, all evidence published to date does not support a role for hemocytes in triggering the systemic immune response, neither in oral or septic injury infection models [51, 53, 54].

Phagocytosis relies on a set of phagocytic receptors [[55, 56]. Phagocytosis may also be enhanced by putative opsonins such as the complement-like thioester-containing proteins (TEPs) and secreted protein isoforms of the Ig superfamily member Down Syndrome cell adhesion molecule (DSCAM) [57, 58]. The *DSCAM* gene can potentially form more than 38,000 splice isoforms, half of them coding for secreted forms of DSCAM. The other half may encode phagocytic receptors. It should be noted that evidence for a role of DSCAM and TEPS in phagocytosis has so far been obtained only in cell culture models and it remains to be established that the corresponding mutants are indeed more susceptible to some microbial infections. The same remark applies to several other putative receptors such as *Drosophila* scavenger protein CI (dSR-CI) [59]. Besides the Ig superfamily member DSCAM, putative *Drosophila* phagocytic receptors belong either to the scavenger family (Peste, dSR-CI,

Croquemort) or to a family of proteins containing several N-terminal specific EGF repeats known as Eater or Nimrod repeats (Eater, Nimrods, Draper) [23, 59-63]. Some of these receptors (Croquemort, Draper) are also required for the uptake of apoptotic cells. Some of the receptor downstream machinery has been identified in the case of apoptotic receptor genes and appears to be also required for uptake of microbes, although a developmental-stage specific role may exist, as documented for *ced-6* [60, 64, 65].

The microbial structures recognized by these receptors remain ill-defined at present. One exception is Draper, which binds to lipoteichoic acids (LTA) of *S. aureus* and *Bacillus subtilis* [60]. It is required for the LTA-dependent ingestion of *S. aureus*. However, it is also somewhat promiscuous as it is also required for the uptake of *Escherichia coli*, which does not synthesize LTA. Interestingly, Eater, Draper, Nimrod C1, and Peste have all been shown to be required for the uptake of *S. aureus* by cultured *Drosophila* cells, and the effects to be additive at least for the first three cited receptors [60]. It will be interesting to determine whether *Nimrod C1* and *Peste* mutants display an enhanced sensitivity to an immune challenge with *S. aureus* as already shown for *Eater* and *Draper* mutants. Thus, phagocytic receptors appear to be rather promiscuous and partially redundant. They may play an important role in the fight against natural pathogens of *Drosophila* as some of them appear to be under positive selection in *Drosophila* species, an indication of host-pathogen coevolutionary arms race [66].

The systemic immune response

This is a major defense system that is able to control most "casual" infections. It relies on the production by the fat body, a functional equivalent of the mammalian liver, of potent AMPs that are secreted into the body cavity where they fight microbial infections. Two major NF-κB pathways control the expression of AMPs as well as hundreds of other genes, the IMD

and the Toll pathway [67]. The former is essentially induced by Gram-negative bacteria and some Gram-positive bacilli and is required in the host defense against these pathogens as IMD pathway mutants display an enhanced sensitivity to this type of infection. In contrast, the Toll pathway is preferentially induced by some Gram-positive bacterial strains and by fungi. Of note, the Toll pathway has been initially identified for its role in embryonic development where it controls the establishment of dorso-ventral polarity. Many components of the Toll pathway have been identified through large-scale genetic screens investigating development. While the core intracellular pathway is essentially required both for development and immunity, the extracellular cascades that lead to Toll activation are distinct for each function.

The detection of infections

Two complementary modes of detection have been deciphered so far in adult flies. The first one relies on PPRs, that is receptors that have been selected during evolution for their ability to recognize microbial components that microbes cannot easily modify to escape detection because they are central to their biology [68].

In contrast to mammals and like birds, lipopolysaccharide (LPS), the major component of the Gram-negative bacterial cell wall, does not elicit a systemic immune response [69]. It was actually found that diaminopimelic-type peptidoglycan (DAP-PGN) is a strong elicitor of the IMD pathway [70]. DAP-PGN is bound directly by PGRP-LC, which is the IMD pathway transmembrane receptor [71]. DAP-PGN is also bound by PGRP-LE [72], a protein that can work apparently either as an intracellular receptor, or as a secreted receptor in a truncated isoform [73]. For instance, while PGRP-LC mutants are susceptible to some pathogenic Gram-negative bacterial infections, they do not succumb to a non-pathogenic challenge with *E. coli*, unless *PGRP-LE* is also mutated [74]. Thus, while PGRP-LC appears to be the main receptor of the pathway, it may be assisted by PGRP-LE in some circumstances.

Cristallography has revealed the major residues that allow PGRP-LC and PGRP-LE to discriminate between two types of PGN, DAP- and Lysine-type (this refers to constituents of the peptidic chains that crosslink glycan chains in PGN) [71, 72].

The receptor of the other pathway, Toll, is not itself a PRR, even though it is the founding member of the Toll-like Receptor family of PRRs. It contains the two domains that are characteristic of the family: an intracellular Toll-Interleukin Receptor domain that serves as a docking site for the MyD88 platform, and a leucine-rich extracellular domain, which binds a ligand, Spätzle. Prospätzle needs to be cleaved by proteases into an active ligand. A protease cascade that is activated by soluble PRRs has been delineated ([75] and references therein). The ß-(1,3) glucan binding domain of Gram Negative Binding Protein 3 (GNBP3) binds long chains of ß-(1-3) glucans found in the fungal cell wall [76]. In contrast, Toll pathway activation by Gram-positive bacteria relies on several PRRs, essentially PGRP-SA and to a lesser extent, depending on the bacterium, PGRP-SD. GNBP1 is also required in this process. It is not yet clear whether it acts fully as a PRR or might be required for PGN cleavage into shorter chains. One striking observation is that GNBP1, but not PGRP-SA, is dispensable for Toll pathway activation by short PGN fragments [77]. On the other hand, the concomitant overexpression of both GNBP1 and PGRP-SA is sufficient to induce the Toll pathway in the absence of any immune challenge [78].

In summary, the PRR detection system is highly conserved in insects and does not appear to be under positive selection, in keeping with its ability to detect essential components of the microbial cell wall [79]. However, it appears that some fungal pathogens may have developed the ability to avoid detection by GNBP3 [80]. They nevertheless trigger the Toll pathway by a distinct system that may have evolved under pathogen pressure. Namely, entomopathogenic fungi cross the cuticle by secreting proteases and chitinases that locally digest the cuticle (see Box). The fungus then crawls in through the microscopic hole

and enters the body cavity. One major fungal protease involved in crossing the cuticle is PR1, which is able to cleave the proform of a host protease, Persephone, thereby activating it [80]. This event triggers a distinct proteolytic cascade that ultimately targets Spätzle. Thus, Persephone appears to function as a bait for the detection of some foreign proteolytic activities, including bacterial proteases [81]. In other words, *Drosophila* is able to sense directly the enzymatic activity of virulence factors and thus to trigger the systemic immune response whenever a pathogen has developed strategies to neutralize the PRR-based sensing device. The expectation is that such a system is inherently dynamic and may be a focal point of interactions between host and pathogens. Indeed, Persephone appears to be under positive selection in natural *Drosophila* populations [82].

Signaling pathways

Toll and IMD are evolutionary conserved NF-κB pathways. The former is reminiscent of IL-1/MyD88-dependent TLR4 signaling whereas the latter is evocative of TNF and MyD88-independent TLR4 signaling [67]. This includes both phosphorylation cascades, *e.g.*, TAK1 and IKK kinases and K63-linked ubiquitin chains that are required for kinase activation in the IMD pathway. For both pathways, the subcellular localization of a transcriptional activator of the Rel family is controlled by intracellular signalling. Toll pathway activation leads to the phosphorylation and subsequent degradation of the cytoplasmic I-κB inhibitor, thus releasing DIF and/or Dorsal for nuclear uptake. Whereas Dorsal is required specifically for the developmental function of the Toll pathway and DIF for the immune function in adult, it appears that Dorsal and DIF are redundant in the larval stage, thus further underlining the stage-specific nature of the immune response. As regards the IMD pathway, Relish encodes both a transcription factor in its N-terminal domain and ankyrin repeats characteristic of I-κBs in its C-terminal domain. Following PGRP-LC activation, it is

thought that the DREDD caspase, which is loosely related to caspase 8 of vertebrates, cleaves Relish and thus releases the N-terminal domain from its cytoplasmic anchor, while IKKβ activity phosphorylates it, an event which is essential to transform Relish into an active transcription factor able to recruit the RNA polymerase complex [83]. The IMD pathway is negatively regulated at multiple levels, for instance by the degradation of PGN or negative regulation of AMP expression at the transcriptional level by the *caudal* repressor [84-86]. Much less is known as regards the negative regulation of the Toll pathway. Interestingly, the analysis of the genome of 12 *Drosophila* species suggests that the signaling pathways may be the target of virulence factors that would likely aim to hinder the systemic immune response [79]. As for drugs, it is formally possible to determine at which level a virulence factor acts by epistatic analysis. It is indeed often possible to design constitutively active forms of intracellular components, or to overexpress an intracellular component, to induce the signaling pathway independently of an immune challenge. If this signal-independent activation of the pathway is blocked by the drug or virulence factor, then the conclusion is that it acts at a level lower in the pathway than the component under investigation.

Effectors

The main effectors of the systemic immune response appear to be AMPs, which are regrouped in seven families [87]. They are able to kill microbes at specific concentrations *in vitro*. Some peptides such as Cecropin, Drosocin, and Drosomycin have been purified from flies and their concentrations have been found to be much higher than the minimum inhibitory concentrations determined *in vitro* against several types of microorganisms. Of note, some peptide families such as Diptericins and Attacins have not actually been purified from flies but have been first characterized in other insects, thus allowing their identification in flies by molecular biology techniques. Some peptides such as Drosomycin (100 µM), Drosocins

(40µM), Cecropins (20 µM), and Metchnikowin (10µM) are produced in very high concentrations. It is estimated that 24 hours after an immune challenge, the overall AMP concentration reaches the 300 mM mark. Some of these peptides are very stable (*e.g.*, Drosomycin, Drosocin) and can still be detected three weeks after the immune challenge, whereas others such as Cecropins are sensitive to proteases and are rapidly degraded [88]. Each AMP is effective against a range of microorganisms. Roughly, Attacins, Cecropins, Diptericins, and Drosocins are mostly active on Gram-negative bacteria. In contrast, even though they are present in very low concentrations (1 µM), Defensins are the AMPs that are most active against Gram-positive bacteria. Finally, Metchnikowin and Drosomycin are essentially antifungal, Drosomycin being only active against filamentous fungi. Because multiple AMPs with overlapping specificities are produced simultaneously, the genetic inactivation of single AMPs has not been reported. Likely, such an experiment would not yield conclusive results because of redundancy. One has to consider instead mutants which affect the transcriptional regulation of AMPs, namely mutants of the Toll or IMD pathways. It appears that most antibacterial peptides are mainly under the control of the IMD pathway: they are no longer induced in mutants and are expressed in the absence of an immune challenge when the IMD pathway is ectopically activated, *e.g.*, by overexpressing IMD or PGRP-LC. In contrast, the Toll pathway regulates the expression of Drosomycin. In mixed bacterial infections (Gram-positive and -negative bacteria), some AMP genes such as those of Cecropins, Attacins, and Defensins appear to be under the dual control of both pathways, which may account for synergies between the Toll and IMD pathways ([8, 89] and references therein). Metchnikowin appears to be regulated by either pathway. No AMPs are expressed in IMD-Toll pathway double-mutants [90]. This genetic background provides a convenient platform in which to test *in vivo* the activities of specific AMPs against a given microorganism. One only needs to express a specific AMP transgene under a constitutive

promoter. This approach confirmed expectations about the specific roles of some major AMPs [91]. Of note, some bacteria such as *Serratia marcescens* have been found to be resistant to all tested AMPs at high concentrations (200µM), thus explaining its high pathogenicity. This property is not directly linked to the secretion of bacterial proteases and is rather linked to its cell wall as an intact LPS is required for withstanding the action of the IMD pathway. Surprisingly, *S. marcescens* is sensitive to the action of Diptericin in the context of the digestive tract [54].

Microarray analysis has revealed that besides AMPs, each pathway also induces the expression of about 150-200 other genes, the function of many of which remains unknown [92-94]. Some members of the signaling pathway are upregulated, in what may constitute a replenishment reaction, whereas other are negative regulators of the pathway that provide a negative feedback loop. It is important to highlight that the effectors of the Toll pathway that act on classes of Drosomycin- and Metchnikowin-resistant microorganisms remain to be identified. Indeed, we have failed to find an *in vivo* activity of Metchnikowin against yeasts such as *Candida albicans* or *C. glabrata* (Joelle Asmar, DF, unpublished observations). Also, Defensins are mostly under the control of the IMD pathway. Yet, IMD pathway mutants are resistant to Gram-positive bacterial infections [95]. There seems to be no major AMP effector of the Toll pathway active against these Gram-positive bacteria. Thus, much remains to be discovered to understand fully the actual mechanism of action of the antimicrobial response.

Local immune responses

The development of reporter transgenes in which the gene encoding a naturally fluorescent protein (GFP, RFP...) is placed under the control of the promoter of an AMP gene has allowed the easy identification of tissues in which AMPs are expressed [96-98]. Here, we

shall focus on the intestine as oral infection models are increasingly being used in the community to understand host defense as well as to investigate host-pathogen relationships.

Intestinal defenses against microbial infections

The digestive tract is a tube formed of three major sections: the foregut, the midgut, and the hindgut. A diverticulum, the crop, is used as an extensible food storage organ. Its duct merges with the intestinal tract just before the beginning of the midgut. The paired Malpighian tubules, functional equivalents of kidneys, fuse with the digestive tract at the level of the border between mid- and hindgut. Both the foregut and the hindgut are covered with cuticle and are therefore as protected as any other organ of the fly. In contrast, the midgut is both the stomach and the section of the digestive tract in which food absorption takes place. The proventriculus or cardium, a valve-like structure positioned at the border between the foregut and the midgut, synthesizes a chitinoproteinaceous membrane known as the peritrophic matrix. This membrane lines the midgut and hindgut epithelium, thus defining two distinct compartments, an inner lumen that contains ingested food and microbes and the ectoperitrophic space delimited by the intestinal epithelium and the peritrophic matrix. Ingested microbes are thus not in direct contact with the epithelium. The function of the peritrophic matrix is fulfilled in vertebrates to some extent by the mucus secreted by intestinal goblet cells. The peritrophic matrix is semi-permeable and allows the passage of digestive enzymes and defense molecules secreted by the intestinal epithelium while at the same time permitting the intake of nutrients from the lumen by the epithelium. This matrix thus constitutes an effective barrier against most bacteria.

This physical barrier is complemented by two largely inducible chemical defenses, an oxidative shock and the production of AMPs by the epithelium (Fig. 2) [54, 99-102]. As compared to other tissues, there is an additional level of complexity that has to be taken into

account in the digestive tract, namely the presence of a commensal microbiota, the composition of which influences the lifespan of flies [85, 103]. While the *Drosophila* microbiota is not developed in young flies, it progressively fills up the lumen of the gut in older flies, causing a stress on the intestinal epithelium [104]. As compared to mammals, the complexity of the microbiota is much lower as it is composed by only a dozen bacterial species [85]. Ideally, the immune response should be triggered only when pathogenic bacteria are present in the lumen, and not by the microbiota.

The dual oxidase (DUOX) enzyme mediates the reactive oxygen species (ROS) response [99]. This transmembrane enzyme contains both a NADPH-oxidase domain and a myeloperoxidase domain, which allows it to synthesize oxidative radicals such as $HClO^{\cdot}$ that are potent microbicides. This enzyme is regulated at two distinct levels. First, it is activated by calcium ions that are released from the endoplasmic reticulum through IP_3 [105]. This results from the activation of an unidentified G protein coupled receptor (GPCR) by a high microbial burden. The activated GPCR in turn triggers a Gαq-phospholipaseCβ (PLCβ) axis that results in the synthesis of the intracellular messengers IP_3 and diacylglycerol. The activation of DUOX takes place within a few seconds. There is a low basal activity of DUOX under normal conditions that is sufficient to prevent the growth of dietary yeasts such as *Saccharomyces cerevisiae*. Of note, the release of low levels of calcium activates calcineurinB, which in turns positively regulates the MKP3 phosphatase, an inhibitor of the p38 MAPK pathway [105]. This pathway plays a key role in the second level of DUOX regulation, that is, its transcription [106]. When pathogenic bacteria are present in large amounts, the p38 MAPK pathway is activated and overrides MKP3-mediated inhibition. The p38 MAPK is activated by a dual influence. The first is that of the IMD pathway, which bifurcates downstream of the TAK1 kinase to also activate the p38 pathway, in addition to its role of activating the IKK complex for subsequent phosphorylation of Relish. The second

input depends on the unknown GPCR that works upstream of PLCβ and leads to MEKK1 phosphorylation. Whereas the IMD pathway is presumably activated by PGN, the GPCR is activated by an as yet unidentified microbial compound that is distinct from PGN. The DUOX response is so potent that it kills flies lacking the immune responsive catalase, which protects the host from its own armentarium [100].

In response to a strong bacterial challenge, some AMPs such as Diptericin, Attacin, and Cecropin are produced in the gut epithelium (Fig. 2), most noticeably at the entrance of the midgut, that is in the proventriculus. Because bacteria are not in direct contact with the ingested microbes, it appears likely that PGN fragments are released in the gut lumen, either as the result of bacterial growth and proliferation, or, alternatively, as the result of digestion and lysis by AMPs. Multiple negative regulatory feedback loops set the threshold over which the IMD pathway gets activated in the digestive tract [84, 86, 107]. One of them is the Caudal transcription factor, which specifically represses in the distal part of the midgut the expression of AMPs but not of other IMD pathway-regulated genes [85]. In its absence, the IMD pathway is upregulated, which results in a modified composition of the commensal microbiota. A major bacterial species is quantitatively strongly reduced whereas a minor species, which is more resistant to the action of the IMD pathway is positively selected. This bacterial species is however detrimental to the fly when selected for as it causes a decreased lifespan of *Caudal* mutant flies.

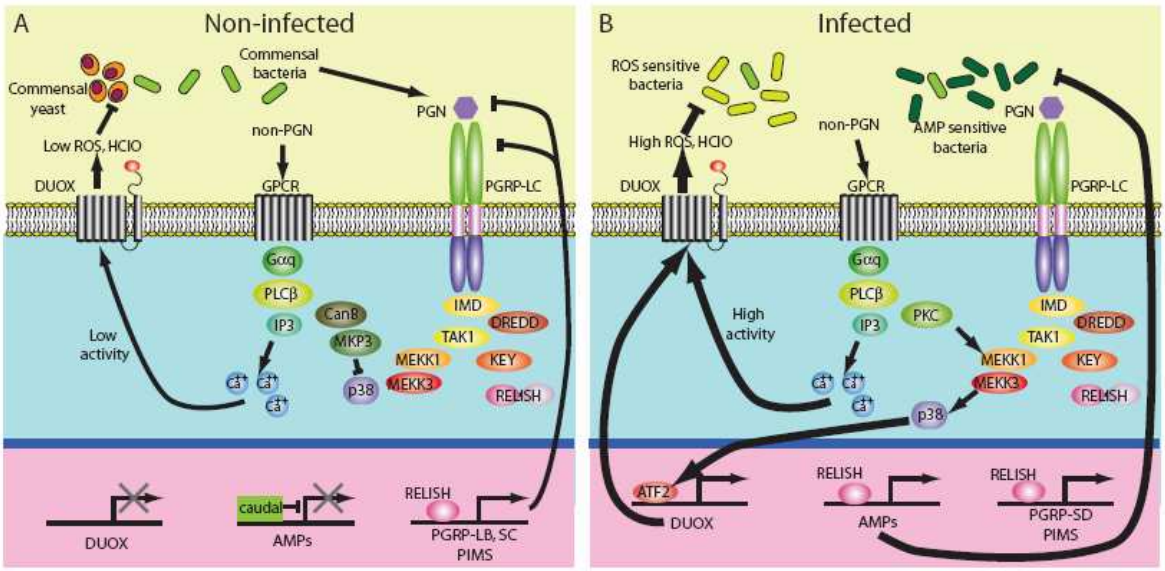


Figure 2: **Epithelial immune response in the *Drosophila* midgut**. **A**: In non-infected gut carrying a low microbial burden, the transcription of AMP genes is inhibited by the transcriptional regulator Caudal. The commensal microbiota just weakly trigger a reaction leading to the production of a low concentration of ROS that keeps nutritional yeasts from growing. **B**: Under infectious conditions however, the microbial burden is high, therefore triggering a strong activation of the IMD pathway by peptidoglycan (PGN) fragments and of the unknown G-protein-coupled receptor (GPCR) independent of PGN. The IMD-controlled Relish translocation into the nucleus is strong enough to overcome Caudal-dependent inhibition of the AMP genes. The IMD pathway, in addition, leads to the activation of the p38 pathway via TAK1, which in turn induces the ATF2-dependent transcription of the *Duox* gene. The activation of the unknown GPCR receptor by non-PGN compounds induces IP_3-dependent release of Ca^{2+} ions, which boost DUOX activity. Thin arrows: weak activation/inhibition; thick arrows: strong activation/inhibition

The other major component of host defense: endurance to infections

We have discussed so far the first facet of host defense, namely resistance. These are the mechanisms that the host employs to attack the pathogen, resulting in the clearance or neutralization of the pathogen. This is the commonly well-studied aspect of immunity. There is however another less widely know aspect of immunity that was first discovered in plants. The initial observation by agronomists at the end of the 19th century was that some cultivars yielded still relatively important crop grains than others despite being affected by fungal diseases ([108] and references therein). This property was called tolerance and has been recently extended to animals [109-111]. Because the word "tolerance" has a precise meaning in vertebrate immunity, we suggest to rather use the word endurance, which is less heavily connoted. What is endurance? A short definition is that it encompasses all processes used by the host to withstand and repair damages inflicted either directly by the pathogen or by the

host's own immune response. Thus, endurances lies at the border between infection biology and the study of stress resistance and homeostasis. One might think of it as a reallocation of resources to palliate the negative effects of stress and damages.

Endurance mechanisms are not expected to target the pathogen, thus should not have major effects in terms of microbial titer. As a result, manipulating endurance of the host should not lead in the long term to the development of resistance of the pathogen as exemplified by the common occurrence of antibiotic-resistant pathogens. A telling example is the strategy used to remedy the negative effects of a microbial infection, that is the easy and cheap treatment to cure cholera patients of the lethal effects of the infection. It suffices to have the patient drink a simple solution that contains just salt and sugar to reestablish ionic balance at the level of the digestive epithelium.

Some examples of endurance mechanisms have been proposed in *Drosophila*. Here, we shall illustrate this concept by focusing on recent insights gained using oral infection models in *Drosophila* (Fig. 3) [2, 18, 103, 112, 113]. The basic finding is that some pathogens such as *Pseudomonas entomophila* and *S. marcescens* damage enterocytes, possibly by the secretion of virulence factors such as toxins, phospholipases, proteases, etc. Yet, despite the induction of extensive cell death, for instance in the *S. marcescens* model, the integrity of the digestive tract is preserved. The key observation was that infection (or the induction of apoptosis or the JNK stress response pathway) triggers the compensatory proliferation of intestinal stem cells (ISCs), which normally have a rather slow rate of proliferation. The existence of this response of ISCs was elegantly demonstrated by clonal analysis in which fluorescently-marked clones were generated only in proliferating cells. Thus, a classical technique of developmental genetics of *Drosophila* was here useful to study host defense to intestinal infections. One of the pathways that controls the compensatory proliferation of ISCs has been identified. It is the JAK-STAT pathway. The Unpaired family of ligands are

expressed by enterocytes in response to stress and will act on the Domeless receptor present presumably on ISCs, thus inducing the nuclear translocation of STAT in the nucleus. The JAK-STAT pathway is required to act both on the proliferation of ISCs and their subsequent differentiation into functional enterocytes. JAK-STAT pathway mutants usually succumb more rapidly to intestinal infections, likely because gut damage can no longer be repaired and this would lead to the loss of the integrity of the epithelium. It is possible using the Gal4/UAS technology to specifically target genes in either ISCs/enteroblasts or in enterocytes by using the relevant Gal4 drivers thus allowing a precise dissection of the pathway.

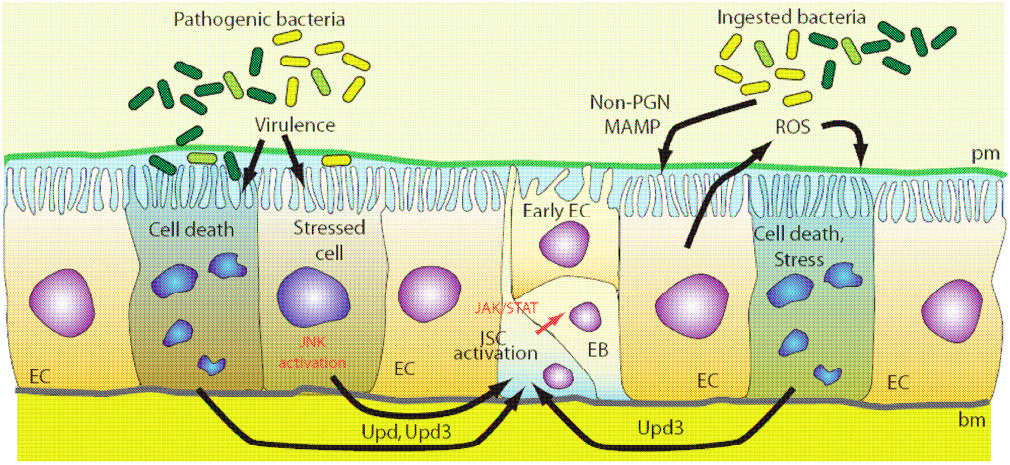


Figure 3: **Regulation of gut homeostasis**. Ingested bacteria harm the epithelial cells directly by secreting virulence factors and indirectly by triggering an oxidative burst. The reactive oxygen species (ROS) that are produced damage the microbes as well as the epithelial cells. Damaged epithelial cells produce the cytokines Upd and Upd3 that trigger JAK-STAT activation in neighboring intestinal stem cells (ISCs), which leads to compensatory proliferation. Stressed cells activated the JNK pathway, which synergizes with the JAK-STAT pathway in activating ISC proliferation and epithelium renewal. pm: peritrophic matrix, bm: basal membrane, EC. Enterocyte, EB: enteroblast, PGN: peptidoglycan, MAMP: microbe-associated molecular pattern.

The plant phytopathogen *Erwinia carotovora carotovora* (*Ecc*) provides an example of the requirement for endurance in limiting the negative effects of the host immune response [103]. *Ecc* is not a fly pathogen. The phytopathogenic bacteria may be disseminated by *Drosophila* between plants because of their ability to colonize the midgut of the fly. Upon ingestion by adult flies, *Ecc* triggers a strong ROS response that is detrimental to the gut epithelium. Indeed, when the compensatory proliferation of ISCs is blocked genetically by targeting the JAK-STAT pathway, mutant flies display alterations of the gut epithelium and

succumb to a challenge with this bacterium. The proliferation of ISCs induced by *Ecc* was blocked when the ROS response was abrogated by inactivating DUOX, thus demonstrating that the host's own immune response was the primary source of intestinal damage.

It is likely that endurance mechanisms cover a wide range of physiological processes, such as sleep for instance. Infected flies tend to sleep more, like humans, and this phenomenon has been linked to the IMD pathway [114, 115]. *Drosophila* appears to be a model of choice to identify genes involved in these processes because of the power of its genetics, especially the possibility to perform extensive mutagenesis screens. We have recently performed a genome-wide screen involving more than 20,000 survival experiments to identify genes involved in the host defense against ingested *S. marcescens* [2]. While we have found genes involved in host resistance and also genes required for the homeostasis of the intestinal epithelium, we have isolated also many genes with an as yet unidentified role in host defense and predict that many will actually be shown to be involved in other, possibly unidentified, endurance mechanisms.

Host-pathogen interactions

The fly is a powerful system to study host-pathogen interactions and has been used widely to model infections by diverse microorganisms. These microbes can be grouped into several classes. A first distinction to be made is that between pathogens and non-pathogens. As for human patients, this distinction depends both on the microbe's genomic makeup with its set of virulence factors, but also importantly on the host, which may present immunodeficiencies or endurance defects as exemplified by *Ecc* ingested by JAK-STAT pathway mutant *Drosophila* flies. A second distinction is to determine whether the microbe is a human pathogen that the fly is highly unlikely to encounter in its natural environment, even though *Drosophila* is a human commensal, or an opportunistic pathogen able to infect a broad

range of organisms. This is the case of pathogens such as *P. aeruginosa* or *S. marcescens* that infect plants, invertebrates and vertebrates, humans included. The study of host-pathogen relationships is likely to be most relevant in this instance given the basic biology shared by metazoans and the use of the same pathogenic microbial strain for infection. Indeed, several studies have demonstrated that common virulence factors are required to infect nematode worms, flies, and mammals [116, 117]. It is also nevertheless possible that sets of virulence factors are well-suited for certain hosts and not others, depending on their array of defenses. Another category of pathogens are entomopathogens that have evolved specific traits to overcome specifically insect or, in some cases, fly host defenses. Indeed, some fungal and viral pathogens manipulate even the behavior of their hosts [118]. Thus, the process of host-pathogen interactions is likely to be highly dynamic as the result of an evergoing arms race between the host evolving new defenses and the pathogen developing novel virulence strategies. Several natural bacterial and fungal entomopathogens have been described. The ideal case would be an obligate parasite that imperatively needs to infect *Drosophila* to fulfill its host cycle as the evolutionary pressure would be highest in this system. This may be the case of the intracellular fungal parasites *Microsporidia*, such as *Tubulinosema ratisbonensis* [119]. One severe limitation to the study of host-pathogen relationships using entomopathogens is the scarcity of models in which the pathogen can be genetically manipulated.

Other parameters that are to be taken into account is whether the microbe is intracellular or extracellular, or both. For instance, it has been shown that autophagy is an important cellular defense against the facultative human intracellular parasite *Listeria monocytogenes* [120]. Intracellular parasites may be studied also in cell culture models such as S2 cells. Indeed, several studies have reported high-throughput genome-wide RNAi

screens in which genes required for pathogen survival have been identified. These screens can be largely automated and RNAi libraries that cover the whole genome are available [20].

The studies performed so far using *Drosophila* as a model often reflect the scientific background of the investigators. Microbiologist will use the fly as a living test tube to screen for microbial mutants with altered virulence. In contrast, Drosophilists are more likely to use microbes as tools to study *Drosophila* innate immunity. However, the study of host-pathogen relationships, that is the thorough investigation of the specific interactions between both genomes, still remains in its infancy, possibly because it represents a formidable challenge that will ultimately require the close collaboration between microbiologists and immunologists.

The use of *Drosophila* to model infections by human pathogens needs to be carefully considered. For instance, human pathogens are adapted to grow at the relatively high temperature of 37°C, a barrier temperature that flies cannot endure for prolonged periods; the infections are performed at most at 29°C. Indeed, the temperature barrier is thought to be the key limitation that prevents many fungi from being human opportunistic pathogens [121]. Therefore, flies may be sensitive to some microbes that may actually be non-pathogenic at high temperatures. Thus, when assessing the potential pathogenicity of uncharacterized microbial strains to be later used in mammalian models, it might be necessary to test experimentally whether temperature is a limiting condition. This cannot be performed in *Drosophila*. However, the Greater Wax Moth *Galleria mellonella* may be used to this end, because it endures temperatures up to 37°C. Thus, one may in principle easily test whether a microbe that is virulent in the 18-29°C range (temperatures at which *Drosophila* are usually kept) is still virulent at 37°C.

For *L. monocytogenes*, temperature is a key factor as it fails to be virulent at low temperatures. Indeed, it has been shown that the expression of the key virulence factor

regulator PrfA is under the control of a thermosensitive riboswitch, that is a RNA secondary structure that gets destructured at 37°C [122]. At low temperature (30°C), the secondary structure masks the Shine-Dalgarno sequence required to initiate the translation of PrfA and consequently, the PrfA-dependent virulence program is not launched. Intriguingly, PrfA appears to be required for virulence in *Drosophila* cells, even though they are cultured at the restrictive temperature [123]. Thus, *Listeria* may have evolved a strategy for expressing this virulence factor *in vivo*, despite the apparent high temperature requirements. *Listeria* does kill wild-type flies at temperatures below 30°C and is thus virulent.

As mentioned above, one limitation of entomopathogens is often the scarcity of genetic resources already available to study the host-pathogen equation from the pathogen's perspective. The opportunistic pathogen *P. aeruginosa* bypasses these limitations as it has been intensively studied since it is a major human pathogen that affects immunosuppressed and burn wound patients. *P. aeruginosa* is also the major colonizer of the respiratory tract of cystic fibrosis (CF) patients. Severe blood stream infections can as well be caused by *P. aeruginosa*-contaminated catheters or other surgical instruments [124, 125]. *P. aeruginosa*'s ability to form biofilms renders it rather resistant to clinical cleaning procedures. In addition, multiple natural or acquired resistances to antibiotics often complicate therapy [125]. Thus, much is known about its basic biology and virulence (more than 40,000 publications are found in Pubmed). An ordered library of transposon insertions covers about 80% of its genome [3], which renders possible large-scale screens for virulence factors without having to screen mutant collections at random, that is having to screen much larger numbers of mutants. In the following section, we describe the various approaches that have been applied to study host-pathogen interactions using a substantial part of the palette of *Drosophila* and *P. aeruginosa* tools.

- ***P. aeruginosa***

In *Pseudomonas-Drosophila* host-pathogen interaction studies the *P. aeruginosa* strains PAK (clinical strain), PA01 (reference strain) and PA14 (clinical strain) are commonly used [112, 126-133] in two basic types of infections: the septic injury and the oral infection models. The *Drosophila* cellular and humoral immune responses have been shown to be crucial for fighting *P. aeruginosa* infections [129, 132]. Systemic infection induces both the IMD and the Toll pathway [132]. Highly virulent isolates of *P. aeruginosa* such as PA01 and PA14, have been suggested to counteract the humoral immune response by downregulation of NF-κB signaling [134, 135]. The suppression of NF-κB pathway activity in mammals seems to be dependent on N-3-oxo-dodecanoyl-homoserine lactone, a *P. aeruginosa* quorum-sensing signal molecule [135]. Quorum-sensing has been shown to be very important for *P. aeruginosa* virulence. Quorum-sensing systems enable the bacteria to synchronize their gene expression programs when bacterial density reaches a threshold. The expression of many virulence factors, e.g. pyocyanin, rhamnolipids, and proteases, has been shown to be quorum sensing dependent (reviewed in [136, 137]). The principle of quorum sensing is the following: the bacteria synthesize and secrete signal molecules (N-acylhomoserine lactones or quinolones) that they are also able to sense. In the case of a low bacterial density, these molecules stay under a threshold concentration in the medium. When the cell density increases, the concentration of these signaling molecules reaches the threshold and triggers specific gene expression profiles by binding to specific receptors, that are also transcription factors. Three quorum-sensing systems are known in *P. aeruginosa*: the two N-acylhomoserine lactone dependent systems, Las and Rhl. LasI and RhlI synthesize the signaling molecules, which subsequently bind to and activate the transcriptional regulators LasR and RhlR. The third system is the quinolone-dependent Pqs system. The quinolone molecules that are used as signal have been termed **P**seudomonas **q**uinolone **s**ignal (PQS)

(reviewed in [136, 137]). In addition to the quorum sensing systems, the type III secretion system, that allows to inject effector molecules directly into the cytoplasm of host cells, is of particular importance for *P. aeruginosa* virulence [138, 139]. Its effector proteins are: ExoS, ExoT, ExoU and ExoY. ExoS and ExoT are capable of inhibiting phagocytosis by disrupting actin cytoskeletal rearrangements, focal adhesion and important signal transduction cascades [140]. ExoU and ExoY are cytotoxins with phospholipase and adenylate cyclase activities respectively [141, 142]. *P. aeruginosa* strains express different combinations of these proteins, that have profound and often devastating effects on epithelial barrier function and wound healing [125]. Some *P. aeruginosa* strains are also cyanogenic. It has been shown that cyanogenic human isolates are more virulent in a *Drosophila* septic injury infection model than non-cyanogenic isolates [143].

P. aeruginosa *septic injury models in the* Drosophila *host*

In many studies of *Drosophila-Pseudomonas* host-pathogen interactions a model of systemic infection by inoculating the bacteria directly into the body cavity of the fly has been used. *P. aeruginosa* proliferates rapidly in the hemocoel and kills its host quickly (in less than 48h) [126]. In addition to the effects of quorum-sensing and the type III secretion systems, many virulence factors have been described to be important for septic injury induced systemic infection in *Drosophila*. *P. aeruginosa* PA01 strains mutant for twitching motility show impaired virulence in wild-type flies[126, 131]. The genes implicated in twitching motility are found in seven loci dispersed along the PA01 genome. Most mutations hitting three of these loci led to impaired virulence in *Drosophila*, whereas mutations in the other loci had an effect on twitching motility but not on virulence. Therefore it is hypothesized that those three loci, that amongst others encode the gene cluster *pilGHIJKL chpABCDE*, could encode a signal transduction system that controls adaptation for surface growth and twitching motility as well

as expression of factors, that are required for full virulence in *Drosophila*. Interestingly, a *chpA* mutant partially regained virulence in flies deficient for both the IMD pathway and the phenoloxidase cascade, suggesting that ChpA is important for overcoming the flies' immune response [131].

Mutations in *P. aeruginosa* that affect resistance to oxidative stress, affect also bacterial virulence [144, 145]. A mutation in OxyR a H_2O_2-responsive activator renders bacteria highly susceptible to H_2O_2. *oxyR* mutants display impaired virulence in *Drosophila* and mice [145]. IscR, which encodes an iron-sulfur cluster assembly regulator homologue, was also shown to be required for *P. aeruginosa* peroxide resistance. *iscR* mutant bacteria have reduced Catalase A (KatA) activity, the major enzyme needed for H_2O_2 detoxification [144]. Flies infected with *iscR* mutant PA14 succumb significantly slower to the infection than flies infected with wild-type PA14. The peroxide susceptibility as well as the impaired virulence were restored in *iscR* mutants that expressed an additional copy of *katA*, suggesting that both phenotypes are dependent on the lack of KatA expression. These findings suggest that full virulence of *P. aeruginosa* is dependent on its ability to overcome host-generated oxidative stress.

4000 PA14 transposon insertion mutants have been screened to identify *P. aeruginosa* multihost virulence factors in a *Drosophila* infection model [146]. After the main screen and several rounds of retests 15 genes were identified that are important for full virulence in *Drosophila*. Next, the mutants displaying an attenuated virulence in *Drosophila* were tested in a murine peritonitis model. 13 such mutants displayed also a significantly reduced virulence, thus establishing *Drosophila* as a useful model for the high-throughput identification of new multihost virulence factors. Out of the 15 transposon insertion mutants, *hudR* was characterized further. *hudR* codes for a MarR/SlyA family transcription factor that represses the transcription of *hudA*. The elevated HudA levels in *hudR* mutants are sufficient for

virulence attenuation since HudA overexpression in wild type bacteria produces reduced virulence. HudA-like genes are exclusively found in *P. aeruginosa* strains and in some ascomycete fungi, but their physiological and biochemical functions remain to be established [146].

Several studies [133, 147, 148] used *Drosophila* infection models to determine the pathogenic potential of environmental and clinical isolates of *P. aeruginosa*. Vives-Flórez *et al.* [148] compared the virulence of five environmental and five clinical *P. aeruginosa* strains and found no differences in the degree of virulence displayed by these strains when using either a *Drosophila* or a lettuce model. They conclude that it is therefore a mistake to consider environmental *P. aeruginosa* strains as less dangerous than clinical isolates. The introduction of high concentrations of *P. aeruginosa* strains in bioremediation projects, to detoxify e.g. contaminated soil, might thus represent a risk for the wild life inhabiting the bioremediation area as well as for people entering the treated zone. It should however be established that the environmental strains are able to withstand 37°C.

Comparison of different isolates from burn wounds and CF patients has been performed as well [133, 147]. The isolates from burn wounds showed rather strong virulence in both septic injury and oral infection models [133], whereas the virulence of the CF isolates was more diverse. Most isolates displayed similar levels of virulence in both models, but some exhibited high virulence in one and low virulence in the other infection model, which underlines that the two models represent distinct infectious processes. The authors conclude that the populations infecting CF patients are highly inhomogeneous and speculate that more virulent strains might be responsible for primary infection, cross-infection and pathogenic processes, while the less virulent strains might rather play a role in chronic colonization [133]. Indeed, Las quorum sensing-deficient strains are often recovered from infected patients.

The genetic tools available in the *Drosophila* model give the possibility for the experimenter to ectopically express bacterial virulence factors directly in fly tissues (using the UAS-Gal4 system). Avet-Rochex *et al.* [129] overexpressed the *P. aeruginosa* type III secretion effector ExoS under the control of an eye-specific driver. The expression of the toxin induced a rough eye phenotype that could be partially or completely reverted by the overexpression of either the Rac1 or Rho1 GTPase respectively. This demonstrates that ExoS acts as a negative regulator of Rho GTPases. To determine the tissue in which bacterial ExoS exerts its function during infection, the authors overexpressed ExoS either ubiquitously, in the fat body or in hemocytes. The activation of antimicrobial-peptide genes was not effected during infection of flies, that expressed ExoS ubiquitously, suggesting that Rho GTPases are not implicated in NF-κB signaling. In keeping with this, flies in which ExoS was expressed in the fat body showed wild-type survival to *P. aeruginosa* infection. In contrast, flies expressing ExoS in hemocytes succumbed faster to the infection than wild type flies (Fig. 4). This suggests that Rho GTPases contribute to cellular immunity. Phagocytosis of gram-negative as well as gram-positive bacteria was impaired in flies with ExoS-expression in hemocytes, pointing to a general role of Rho GTPases in phagocytosis, that is independent of specific pathogen recognition and engulfment. The inhibition of phagocytosis probably results from actin polymerization defects due to ExoS activity [129]. In addition, *exoS* mutant *P. aeruginosa* that display impaired virulence in wild-type flies, showed normal virulence in ExoS-expressing flies (Fig. 4). This study demonstrates nicely that *Drosophila* is a very useful model to study the mechanisms of action of *P. aeruginosa* virulence factors. In another study Avet-Rochex *et al.* [149]were able to show that Rac2, a Rho GTPase that is important for the phagocytosis of several bacteria, is the main target of ExoS.

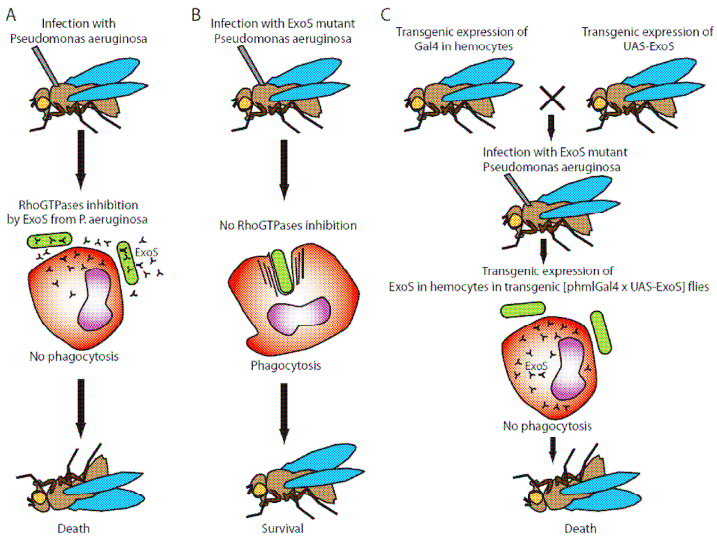


Figure 4: ExoS-dependent inhibition of phagocytosis.
A: *P. aeruginosa* secretes ExoS to block the phagocytic machinery. ExoS inhibits the Rac2 Rho GTPase that is needed to phagocytose the bacteria. Wild-type *P. aeruginosa* are therefore able to establish fatal infections. **B**: *exoS* deficient *P. aeruginosa* are not capable of preventing their phagocytosis, and therefore show impaired virulence. **C**: The virulence of *exoS* mutant bacteria is completely restored in flies, that express transgenic *exoS* in their hemocytes. In this case the hemocytes are unable to phagocytose *exoS* deficient bacteria, which allows them to establish a lethal infection.

A similar approach can be used to decipher antimicrobial properties of mammalian host resistance genes that *Drosophila* is lacking. Besides its ability to degrade organophosphates, paraoxonase 1 is a lactonase that degrades the N-acylhomoserine lactone (3-oxo-dodecanoyl-homoserine lactone) used by the LasI/LasR system. It has previously been shown that quorum sensing deficient bacterial mutants are less virulent in a septic injury model. The overexpression of transgenic human Paraoxonase-1 (hPON1) in *Drosophila* (the *Drosophila* genome does not encode any paraoxonases) conferred resistance to PA01 infection [150]. hPON1 overexpressing flies infected with wild-type PA01 showed survival rates similar to those exhibited by wild-type flies infected with bacteria double mutant for the two N-acylhomoserine lactone producers LasI and RhlI. The virulence of *lasI*/*rhlI* mutant bacteria in wild-type flies, but not in hPON1-expressing flies, could be restored by feeding the flies with 3-oxo-dodecanoylhomoserine lactone. In addition, quorum-sensing responsive genes were significantly down-regulated in PA14 bacteria infecting hPON1-expressing flies as compared to PA14 bacteria infecting wild-type flies. In summary, these data suggest that hPON1 degrades the signal molecule that is needed to activate the Las quorum-sensing system and the subsequent expression of virulence factors during infection, and thereby reduces the virulence of invading bacteria in humans [150].

An interesting experimental approach was followed by Heo *et al.* [151]. The authors used *Drosophila* as a model organism to study the efficacy of antibacterial phage-therapy. Flies fed for 12h on a medium containing either of two lytic bacteriophages (MOK1 or MPK6) were significantly less susceptible to subsequent septic injury with PA01. The pretreated flies displayed also a smaller bacterial load than non-treated flies. The efficacy of the two bacteriophages as therapeutic phages was also tested in a murine peritonitis model and yielded similar results. Mice injected with either bacteriophage showed significantly less mortality and lower bacterial burden than control mice. This study shows that *Drosophila* can be a valid model to investigate innovative treatments of bacterial infection.

An evolutionary approach has been used to study the dynamics of host defense across generations of flies consistently exposed to *P. aeruginosa* [128]. Males and females were infected with *P. aeruginosa* and subsequently only the survivors were allowed to seed the next generation. The offspring was treated the same and again only surviving flies were used to give progeny. This selective pressure was kept for ten generations. In parallel flies of the same original stock were kept also for ten generations in the absence of any selective pressure. Interestingly, the survival rate to *P. aeruginosa* infection went up from 15% in the original population to 70% in the selected population within six generations and stayed constant from then on. The adaptation of the fly population to *P. aeruginosa* was costly since selected flies, especially females, displayed reduced longevity and fecundity. Therefore, there is a trade-off between adaptation and fitness. This is underlined by the fact that the population resistance to *P. aeruginosa* infection decreased rapidly after the selective pressure exerted by constant exposition to *P. aeruginosa* was removed. After 10 generations the progeny of both selected and non-selected populations was infected with *P. aeruginosa* and microarray analysis was performed. Comparison of the microarray data revealed differential transcription levels between the two fly populations for around 400 genes, amongst which several known

immunity-related genes were found, including some thought to play a role in the cellular immune defense. This study demonstrates that an evolutionary approach can be used to identify genes implicated in host defense against infections.

P. aeruginosa *oral infection models in the* Drosophila *host*

Besides the septic injury model, oral infection models have been widely used to infect flies with *P. aeruginosa*, even though it is not always clear why investigators privilege one model of infection over the other. The cause of death in oral infection models has rarely been investigated. The protocols used to feed flies are diverse and differ on three points. One is starvation. If flies are starved before feeding on a bacterial solution, most of the food that is taken up is delivered to the crop, a storage organ. In contrast, when flies are fed on bacteria without previous starvation, the food is distributed directly throughout the entire intestine. A second parameter is the addition of bacterial broth or not to the sucrose medium that contains the bacteria and on which the flies feed. Bacteria proliferate to a certain extent on media containing bacterial broth, whereas in a sucrose only medium, bacteria are not likely to proliferate, and are possibly quiescent. A third point is the amount of bacteria given to the flies, which may have an impact on fly survival rates. Diverse combinations of these important technical differences in experimental setups lead to varying virulence of the bacteria and therefore different outcomes of the infection in terms of gut damage and survival of infected flies.

P. aeruginosa oral infection has been reported to cause c-Jun N-terminal kinase (JNK)-dependent apoptosis in enterocytes, the main cell type of the intestine [112]. Infection induced stress leads to JNK pathway activation, which triggers apoptosis. Apoptosis in turn promotes strong compensatory proliferation of the intestinal stem cells (ISCs). This proliferation and subsequent differentiation constitute an endurance mechanism as described

above (Fig. 3). JNK mutant flies succumb faster to PA14 oral infection. In animals that express a latent oncogenic form of Ras1 an infection by *P. aeruginosa* leads to severe overproliferation of epithelial cells. The intestine develops a multilayered epithelium with altered apicobasal polarity reminiscent of dysplasia. It therefore has been suggested that infection can boost predisposition effects leading to stem cell-mediated tumorigenesis [112].

The oral infection model is used in several laboratories to study bacterial virulence factors and modulators. QscR, a homologue of LasR and RhlR, is encoded in the *P. aeruginosa* genome. Interestingly, no additional homologues of LasI and RhlI have been found. This RhlR/LasR homologue, named QscR, has been reported to be a repressor of quorum sensing in *P. aeruginosa* [130]. It likely exerts its function by repressing *lasI*. Therefore in *qscR* mutants, LasI is overexpressed leading to an overstimulation of the Las quorum sensing system, which positively regulates the Rhl and Pqs systems. As a result quorum sensing in general is overactivated in *qscR* mutant bacteria. *qscR* mutant bacteria show a hypervirulence phenotype in a *Drosophila* oral infection model, thus underlining the importance of quorum sensing effectors as virulence factors.

In addition, PqsE a key component of the Pqs quorum sensing system has been shown to be important for virulence in *Drosophila* [152]. *pqsE* mutant bacteria show reduced virulence in an oral infection model. Quorum sensing regulation is tightly connected to the surrounding iron concentration [152]. Low iron activates the Pqs quorum sensing system, while high iron suppresses it. Concentrations of free iron within a host are low as there is a competition between host and pathogen for acquiring iron. Therefore low iron concentration might be an indication for a hostile environment requiring expression of virulence and fitness-related genes. In the case of virulence factor-dependent tissue damage, iron concentrations

become high and downregulate quorum sensing, which might be a mechanism favoring host survival and the establishment of chronic infections.

P. aeruginosa adapts to nutritional deficiencies via the stringent response. It produces the guanine nucleotides ppGpp and pppGpp through the GTP pyrophosphokinase RelA. *relA* mutant bacteria are hypovirulent in a *Drosophila* oral infection model [153]. Under low magnesium conditions RelA enhances the production of 3-oxo-dodecanoylhomoserine lactone and thereby triggers the Las quorum sensing system, which in turn activates the Rhl system. In contrast, RelA activation reduces the production of the PQS signal. The Las/Rhl upregulation leads to a heightened production of virulence factors. The low virulence of relA mutants suggests that the adjustment of cellular ppGpp and pppGpp levels might be an important regulatory mechanism under pathogenic conditions.

Some human infections are polymicrobial infections, in which several microorganisms infect the host at the same time. In the lungs of CF patients many bacterial strains can be found. The different microorganisms interact and influence each other, which makes the study of such infection even more difficult than host-pathogen interaction involving a single pathogen and a host. Sibley *et al.* [127] developed a *Drosophila* oral infection model to study such polymicrobial infections. They studied 40 oropharyngeal isolates in combination with PA01 by feeding the flies on a mixture of PA01 and a particular oropharyngeal strain. The microorganisms were diverted to the crop, which was apparently damaged. It has not been formally established whether crop damage is the cause of death of infected flies, a proposition that is difficult to demonstrate and that requires extensive characterization of the model. The authors found three distinct classes of microorganisms: class I consists of microorganisms that kill flies on their own and enhance PA01 killing when fed in combination. Class II are not killing the flies when fed alone, and do not influence PA01 killing when fed in combination. The most interesting class is class III, which consists of microorganisms that are not

pathogenic or even beneficial when fed alone to the flies, but that enhance fly killing when fed in combination with PA01. 38% of the oropharyngeal isolates where able to act synergistically with PA01 and 48% where pathogenic on their own, although less pathogenic than PA01. The enhanced killing of flies in mixed infections of PA01 and class I microorganisms is likely to be an additive effect, whereas the effects of class III microorganisms cannot be explained that easily. Expression profiles of PA01 virulence genes change in coinfection with class III microorganisms compared to PA01-only infection, suggesting that the presence of the other microorganism influences PAO1 gene expression. Interestingly, distinct members of class III had a different influence on the expression profile of PA01, an observation that suggests that the interactions between PA01 and those microorganisms are not based on a single mechanism. Coinfection with PA01 and a class III microorganism triggered the *Drosophila* humoral response strongly as witnessed by *Drosomycin* expression. Indeed, the induction of *Drosomycin* expression was stronger than could possibly be accounted for by an additive effect of the two microorganisms. How some microorganisms influence PA01 transcriptional profiles and the resulting host immune response remains unclear. The underlying mechanisms are likely to be rather complex. The *Drosophila* model for polymicrobial infections will allow to decipher these infections by giving the possibility to study the effect of bacterial genes as well as host genes in an *in vivo* model.

- ***Fungal models of infection***

Several infection models have been used to study the interactions between *Drosophila* and fungal pathogens. The septic injury model has been widely used. For instance, wild-type flies survive an injection of *C. albicans* or *C. glabrata*, but Toll pathway-deficient flies succumb rapidly to this infection [80, 154, 155]. However, septic injury bypasses natural routes of infection, which may involve specific virulence processes such as the colonization

of the digestive tract and interactions of the pathogen with epithelial receptors. An alternative infection model consists in spraying fungal spores directly onto the fly exoskeleton by rolling the insects over a fresh carpet of sporulating fungi (as for the entomopathogenic fungi *Beauveria bassiana* and *Metharizium anisopliae* or the human pathogen *Aspergillus fumigatus* [80, 156, 157]. Oral infection models in which *Drosophila* feeds on a lawn of fungi have been used for *A. fumigatus*, *C. albicans*, *Cryptococcus neoformans*, *Cryptococcus kuetzingii*, *Cryptococcus laurentii*, or *Saccharomyces cerevisiae* [157-159]. One should be cautious when using sucrose in an oral infection model using fungi, as yeast may ferment the sugar into ethanol, which is lethal to the flies when produced in too large quantities (Joelle Amar, Ghullam Hussain, Dominique Ferrandon, unpublished observations).

Opportunistic pathogens such as *Candida*, *Aspergillus* as well as *Cryptococcus species* cause disease in humans either through mucosal and skin infections or through potentially lethal invasive mycoses of almost all inner organs, especially in immunocompromised patients. *Drosophila* wild-type flies resist septic injury with these human pathogens. However, *Toll* deficient flies are highly sensitive and succumb to a systemic infection due to *Candida*, *Aspergillus*, as well as *Cryptococcus* [41, 80, 157-159]. Consequently, Toll pathway immunocompromised flies have been then extensively used as hosts to study human pathogens. For example, Chamilos and co-workers have shown that in agreement with findings in the mouse model of invasive candidiasis, *C. albicans* deficient for the regulatory genes CPH1 and EFG1, which are blocked in the yeast phase, was almost avirulent in *Toll* mutant flies [159]. Remarkably, the use of *Toll* mutant flies has allowed to uncouple hyphal proliferation of *C. albicans* with tissue proliferation. Indeed, *cdc35 C. albicans* deletion mutant that does not undergo the yeast-to-hyphae transition, show a strong reduced virulence toward the *Toll* mutants but are still able to invade the tissue of the flies [154].

These interesting findings highlight the fact that *Drosophila* deprived of a functional

Toll humoral response are still able to survive to some extent attenuated forms of fungal infection. What are the immune barriers that still impede fungal proliferation in Toll pathway mutant flies? Phagocytosis is unlikely to contribute to the remaining host defenses in the case of *C. albicans* infection. Indeed, *Toll* mutant flies with impaired phagocytosis die as rapidly as non-treated flies [41]. Surprisingly, it has also been reported that wild-type flies devoid of hemocytes are more prone to *C. albicans* infections. Agglutination as well as melanization may participate in the remaining immune defenses against *C. albicans* infection in a *Toll* mutant background.

In contrast to what has been shown with *Candida* spp. and *Aspergillus*, wild-type fruit flies died rapidly following infection with a variety of zygomycetes, including *Rhizopus*, *Mucor* and *Cunninghamella* species[160]. Chamilos and co-workers have focused on the role of iron as a key enhancer of virulence in zygomycetes fungi. Interestingly, iron metabolism is rather similar in flies and humans[161, 162]. Flies on high-iron diet developed an enhanced susceptibility to zygomycetes infection compared to those receiving normal food. In contrast, treatement of flies with deferasirox, an iron chelator that induces iron starvation to Zygomycetes, protected them from zygomycosis, a situation reminiscent of what is happening in human. These remarkable findings obtained in flies might give some clue to understand why iron-overloaded patient develop severe and sometimes lethal zygomycosis.

Due to its inherent advantages as a host model, *Drosophila* has also been extensively for testing antifungal agents. Lionakis and co-workers have demonstrated that adult flies fed with voriconazole, the most common drug used for the treatment of invasive aspergillosis in humans, shows better survival rates and lower tissue fungal burdens than those not exposed to voriconazole [157]. Voriconazole is a triazole effective against *Aspergillus* but not against *zygomycetes spp*.. The use of voriconazole in patients could therefore facilitate the emergence of zygomycosis infection especially due to *Rhizopus*. Lamaris and colleagues have indeed

shown that *R. oryzae* exposed to voriconazole presented increased virulence in both flies and mice [163].

Mixing fluconazole, another triazole antifungal drug, into food significantly protected *Toll* mutant flies from fluconazole-susceptible *C. albicans* strains, thus validating the efficiency of the drug in *Drosophila*. Interestingly, fluconazole had no effect on flies infected with fluconazole-resistant *C. krusei* strains.

In conclusion *D. melanogaster* model is a promising medium-throughput *in vivo* model for large scale studies of fungal virulence mechanisms and for testing drug efficacy against fungi.

- ***Manipulating genetically both host and pathogen in the fly: the case of viral infections***

The above examples have illustrated the scope of techniques that are available to study host-pathogen interactions. In the case of some viruses, it is possible to go even a step further. We shall focus here on the Flock House Virus (FHV), a member of the nodaviridae family of non-enveloped riboviruses. Its genome is made up of two single-stranded, positive-sense RNAs. RNA1 encodes proteinA, an RNA-dependent RNA polymerase while RNA2 encodes a capsid protein. RNA1 is also processed to generate the smaller RNA3, which codes for the RNAi inhibitor protein B2 (reviewed in [164]). The trick was to produce transgenic lines that allow each the expression of a specific genomic RNA [165]. While each line expresses only one genomic RNA, the offspring obtained by crossing the transgenic RNA1 line to the transgenic RNA2 line yielded flies that express the two RNAs. Those flies succumbed to viral infection as the full functional genome of FHV was reconstituted from the transcripts. By using a modified version of RNA1, which did not allow for the production of RNA3, viral proteins generated from the transgenes produced a virus that was lacking B2 and was only weakly replicating and thus was controlled by the *Drosophila* immune response. This led to

the hypothesis that siRNAs might be involved in the antiviral defence. This prediction was confirmed as it was found that Dicer-2 mutants (Dicer-2 is an essential enzyme required for the generation of siRNAs) are more susceptible to an array of viruses, but normally resistant to other microbial challenges. Interestingly, the viral titre of transgenic flies expressing the B2-deleted version of FHV was restored to normal levels, thus confirming that B2 does indeed target Dicer-2 and therefore allows a successful infection of flies by FHV[165]. Thus, it is possible in this transgenic system to manipulate genetically both host and pathogen in the fly, a very powerful and elegant approach to dissect their interactions.

Conclusions

This review has documented the vast array of techniques that have been implemented to study host-pathogen relationships in the *Drosophila* model. A few points are worth emphasizing. One of them is that the distinct infection models (septic injury, oral infection or natural entry of entomopathogenic fungi) should be studied in detail to understand their specific features. As exemplified by our work on *S. marcescens*, even a very potent entomopathogen in the septic injury model may become much less virulent in an oral infection model. Indeed, whereas the introduction of one bacterium in the hemocoel via a wound suffices to kill the fly in about a day, bacteria that gain access to the hemocoel through the gut appear to have downregulated their virulence program. Thus, a thorough understanding of microbial pathogenesis in one model requires a correct understanding of the basic biology of the infection in this model. Indeed, we have recently discovered that flies orally infected with *P. aeruginosa* PA14 actually succumb to a systemic infection in the hemocoel and not from gut damages (Limmer *et al*., in preparation). This means that mutant bacteria that display an impaired virulence in this model may do so either because i) they fail to endure intestinal defenses, or ii) they are unable to cross the intestinal barrier, or iii) they

downregulate their virulence program in the hemocoel, or iv) they fail to reactivate their virulence programs once in the hemocoel, or v) because they are unable to overcome specific host defenses of the host. Thus, the interpretation of a mutant phenotype may be much more complex than originally envisioned. This has a direct bearing when one attempts to translate knowledge gained in the *Drosophila* system to infection in other hosts. Only in this way will it become possible to correctly interpret findings in model systems.

A second point to emphasize is that of the specificity of interactions between host and pathogens that are derived from the genetic makeups of both host and pathogen. One ideal case is that of a virulence trait of a pathogen that is required to overcome a given host defense. A microbe mutant for this virulence factor will be less pathogenic to the wild-type host. In contrast, the host impaired for that specific host defense is likely to be more susceptible to attacks by the wild-type pathogen. The expected outcome of the interaction between the mutant microbe and the immunodeficient host is that the mutant microbe will regain substantially its pathogenicity only in the immunodeficient host. When this happens, there is then a clear indication of the specificity of the interaction and a meaningful insight is gained on the specific function of the virulence factor. There are relatively few studies that have established in *Drosophila* this correspondence between loss of virulence of the pathogen and immunodeficiency of the host (e.g., [54, 101, 129, 131]). Thus, the field of host-pathogen interactions in the *Drosophila* model is ripe for considerable progress in the years to come. A detailed understanding of virulence strategies of the pathogen may allow the development of innovative methods to alter the pathogenicity of microbes. For instance, it has been proposed to interfere with quorum sensing systems of the patients using the non-bactericidal drug azithromycin [166] to decrease the virulence of *P. aeruginosa*. This however had the unexpected consequence that patients treated with this drug tend to select for more virulent quorum-sensing competent bacteria that would otherwise be competed by *lasR* defective

strains that have a selective advantage as they are cheats that do not pay the metabolic price of synthesizing the N-acylhomoserine lactone. Promising alternatives may be to boost either the host resistance, for instance by finding drugs that stimulate the induction of host defenses or block the weapons the pathogen uses to inactivate specific host defenses. *Drosophila* appears to be especially well suited to study in-depth endurance mechanisms, which might form the basis for novel therapies that would optimize this aspect of host defense.

Finally, the *Drosophila* system is not adapted for high-throughput screening of drugs as recently developed for *C. elegans*. One way to do it would be to perform studies on embryos and young larvae that can be handled by automated sorters. However, this will require many challenging adjustments such as exactly synchronizing the larva so that they are all tested at the same developmental stage. Also, the host defense of larvae is still imperfectly understood at present. *Drosophila* may be better suited for medium-throughput screens that are aimed at understanding host-pathogen relationships in detail. It may also be put to good use to understand the mechanisms of action of other drugs isolated in screens performed on other infection models thanks to its elaborate genetics and advanced knowledge of its host defense.

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