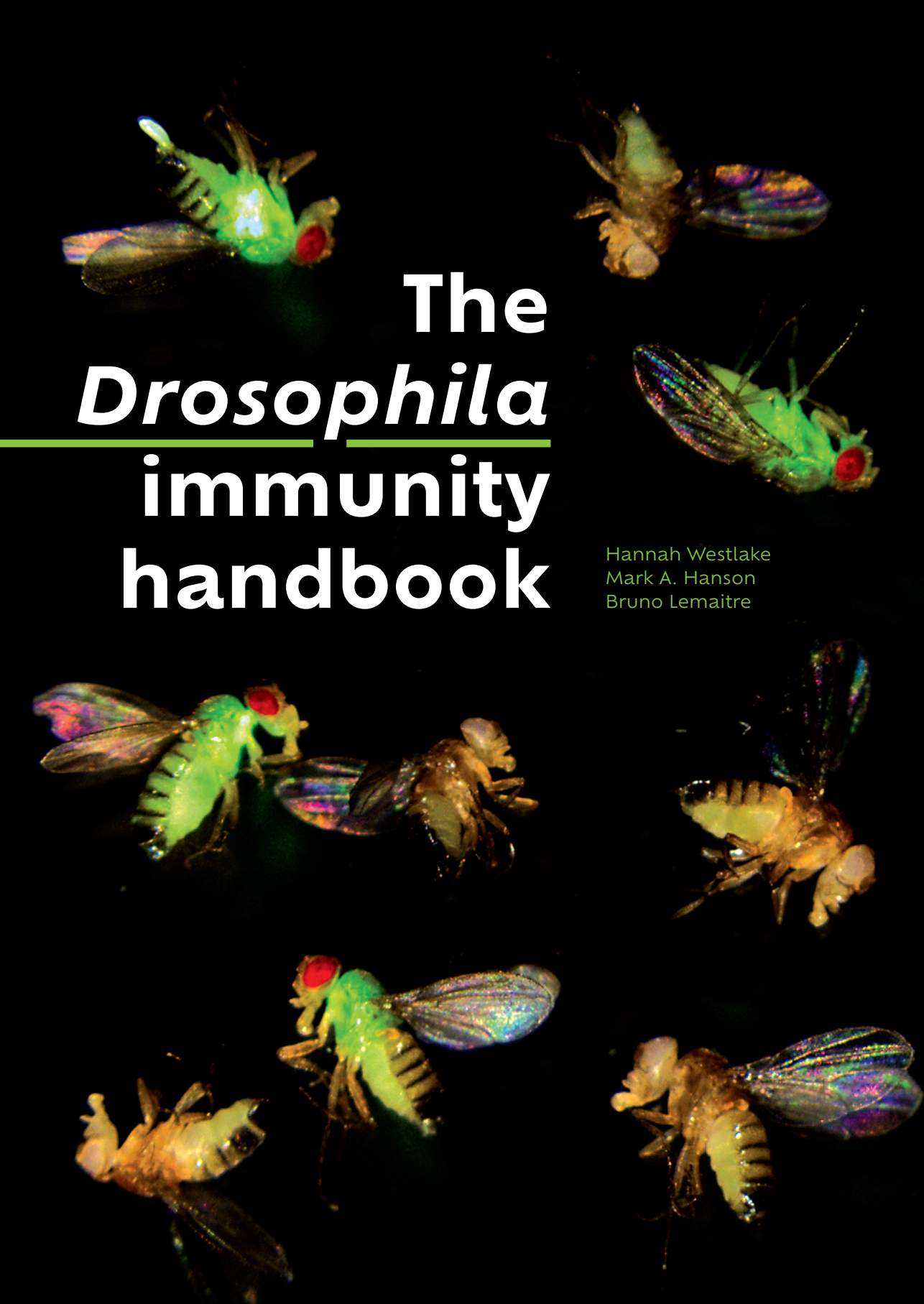

The *Drosophila* immunity handbook

Hannah Westlake
Mark A. Hanson
Bruno Lemaitre

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Animals possess efficient mechanisms for detecting and neutralizing infections. The application of *Drosophila* genetics to the study of these mechanisms has generated insights into insect immunity and uncovered general principles of animal host defense. These studies have shown that *Drosophila* has multiple defense "modules" that can be deployed in a coordinated response against distinct pathogens. These include physical barriers such as epithelia and chitin, the production of reactive oxygen species, antimicrobial factors, blood clotting, the melanization reaction, and complex cellular responses. These responses are accompanied by metabolic reprogramming to fuel the immune system and effectively combat pathogens. Recent studies have highlighted additional mechanisms that contribute to host defense, such as symbiont mediated immunity, disease tolerance mechanisms, and behavioral immunity. These studies reveal broader roles of the immune system beyond infection, notably in disease pathologies such as neurodegeneration or cancer. This remarkable animal model has given us a better understanding of the multiple roles of the immune system at the organismal level. Although it is difficult to summarize the sheer number of studies published on *Drosophila* immunity in recent years, here we aim to provide an overview of recent research trends, challenges, and discoveries in immunity through the lens of *Drosophila*. We hope that this overview will introduce scientists to the sophisticated fly immune system, draw interest to exciting recent findings in the field, and push new horizons of research by contextualizing existing research and highlighting exciting avenues to explore.

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Cover illustration: Fruit flies with mutated antimicrobial peptides (red eyes) let bacteria (green fluorescence) grow out of control, while wild-type flies (with normal antimicrobial peptides) suppress the infection.
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This overview is dedicated to Katja Bruckner and Ulrich Theopold, who made great contributions to *Drosophila* immune research. They will be missed.

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Abbreviations

AMP: Antimicrobial peptide
BLUD: Bacterial load upon death (see also PLUD)
CDN: Cyclic dinucleotide
circRNA: circular RNA
cGAS: Cyclic GMP–AMP (cGAMP) synthase
cGLR: cGAS-like receptor
CNS: Central nervous system
cRHIM: cryptic RIP Homotypic Interaction Motif
DAMP: Damage associated molecular pattern
DAP-type PGN: *meso*-Diaminopimelic amino-acid type peptidoglycan
DCV: Drosophila C virus
DHI: 5,6-dihydroxyindole
DIAP: *Drosophila* inhibitor of apoptosis
DOPA: 3,4-dihydroxyphenylalanine
DREDD: Death related ced-3/Nedd2-like caspase
dsRNA: Double-stranded RNA
EB: Enteroblast
EC: Enterocyte
ECM: Extracellular matrix
EE: Enteroendocrine cell
EGF-R: Epidermal growth factor receptor
ER: Endoplasmic reticulum
EV: Extracellular vesicle
FADD: Fas-associated death domain
FHV: Flock House Virus
GLR: cGAS-like receptor
GlcNAc: N-acetylglucosamine
GNBP: Gram-negative binding protein (misnomer – in *Drosophila,* these bind fungal glucans)
IBM: IAP-binding motif

IIV-6: Invertebrate iridescent virus 6
ISC: Intestinal stem cell
JAK-STAT: Janus kinase - signal transducers and activators of transcription
JNK: c-Jun N-terminal Kinase
lncRNA: long non-coding RNA
LPS: Lipopolysaccharide
Lys-type PGN: Lysine amino acid type peptidoglycan
MAMP: Microbe associated molecular pattern
MAPK: Mitogen activated protein kinase
miRNA: MicroRNA
ModSP: Modular serine protease
MurNAc: N-acetylmuramic acid
MVB: Multivesicular body
NF-κB: Nuclear factor-kappa-B
NO: Nitric oxide
NOS: Nitric oxide synthase
PAMP: Pathogen associated molecular pattern
PDGF/VEGF: platelet-derived growth factor/vascular endothelial growth factor
PGN: Peptidoglycan
PGRP: Peptidoglycan recognition protein
Pirk: Poor immune response upon knock-in (also called PIMS, Rudra)
PI 3 Kinase: Phosphatidylinositol *3-kinase*
PLUD: Pathogen load upon death (see also BLUD)
PO: Phenoloxidase
PPO: Prophenoloxidase
PRR: Pattern recognition receptor
PSC: Posterior signaling center
RDRP: RNA dependent RNA polymerase
RIP: Ribosome-inactivating protein
RISC: RNA-induced silencing complex
ROS: Reactive oxygen species
SCF complex: Skp, Cullin, F-box containing complex
siRNA: Small interfering RNA
SP: Serine protease
SPE: Spatzle processing enzyme
SPH: Serine protease homolog
SPPL: Set point pathogen load (see also SPBL)
SPBL: Set point bacterial load (see also SPPL)
Srgs: STING-regulated renes
ssRNA: single stranded RNA
STING: Stimulator of interferon genes
SWR: Systemic wound response
TCT: Tracheal cytotoxin (monomeric DAP-type peptidoglycan with an internal 1,6-anhydro bond)
TEP: Thioester-containing protein
TLR: Toll-like receptor
TNF-R: Tumor necrosis factor receptor

Toll-PO SP cascade: Toll-phenoloxidase serine protease cascade
UPR: Unfolded protein response
UTR: untranslated region
VLP: Virus-like particle
VSR: Viral suppressor of RNA silencing

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Goals of this review

The evolutionary history of the dipteran *Drosophila melanogaster* is shaped by various biotic interactions in its natural habitat, the decaying fruit. These interactions include beneficial and pathogenic microbes which trigger a diverse set of immune responses. Powerful genetic approaches and a wealth of genomic resources have given *Drosophila* one of the best characterized metazoan immune systems. *Drosophila* immune research has broad relevance due to conservation of innate immune mechanisms in mammals, and has major environmental, medical, and agricultural impacts by providing insight on how this ubiquitous insect group deals with its microbial environment. *Drosophila* also provides a powerful model to explore new concepts, enabling the study of immunity in an evolutionary framework at the organismal level.

Although *Drosophila* possess only innate and not adaptive immunity, recent studies have shown incredible complexity and specificity in *Drosophila* host defense. These studies encompass multilevel defense modules that can be selectively activated according to the nature of the infection. These discoveries have shifted our perception of innate immunity, revealing that diverse mechanisms, both immune and non-immune, contribute to host survival to infection. Beyond infection, innate immunity also has critical roles in whole-body homeostasis, for example by shaping the microbiota, and in non-infectious disease contexts such as cancer, neurodegeneration, and aging. The immune system is no longer viewed in isolation but as an integral factor in other physiological functions, working to promote fitness in a microbe-rich environment. The goal of this review (building on a previous review (Lemaitre and Hoffmann, 2007)) is to provide an overview of recent research trends, challenges, and discoveries in immunity through the lens of *Drosophila*, with updated interpretation and context from foundational findings in the field.

Author note

We have tried to be as comprehensive as possible in reviewing the field of *Drosophila* immunity. A notable exception is that we largely did not include articles exploring the pathogen side of immunity, including virulence factors of bacteria (e.g., *Pseudomonas aeruginosa, Pseudomonas entomophila, Serratia marcescens* or *Vibrio cholerae*), viruses, fungi or parasites. Although these topics are important to fully understand the immune system, as revealed by the strategies used by entomopathogens to suppress the immune system (D'Argenio et al., 2001; Davoodi and Foley, 2020; Dieppois et al., 2015; Dupas et al., 2003; Govind, 1999; Kurz et al., 2003; Lee et al., 2018; Limmer et al., 2011; Mortimer, 2013; Vallet-Gely et al., 2008; Vodovar et al., 2004; Younes et al., 2020), addressing them extensively would require another review. We apologize for articles that were missed or

important areas we could not cover in sufficient detail within the scope of this review. Readers are welcome to contact the authors to discuss possible omissions and errors. It is also important to note that some topics are covered in greater detail than others. This is partially due to the fact that *Drosophila* immune research has concentrated on what was considered important at different times (e.g., the extensive study of conserved signaling pathways in the late 90s to the early 2000s), while other topics received relatively little attention (e.g., cuticular and epithelial barriers, melanization) or are just beginning to emerge (e.g., behavioral immunity, sex differences).

This state-of-the-art description of the immune system takes advantage of an extensive reproducibility project, which offers new perspectives on literature published before 2010 (https://reprosci.epfl.ch/), see Westlake et al., 2024 for a summary). In light of this, we discuss articles that have been received with skepticism and topics that have produced conflicting results. This is in no way intended to judge the scientific value of these articles, but rather to highlight that science is an ongoing process.

Finally, we provide two extensive supplementary tables summarizing data that may be of use in future research (https://www.epfl.ch/labs/lemaitrelab/lemaitre-lab/resources).

Supplementary List 1: An updated list of *Drosophila* immune related genes

Supplementary List 2: A description of *Drosophila* host defense peptides

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1

General introduction: Concepts

Multiple factors contribute to evolutionary shaping of immune systems in living organisms. To situate the reader, we review some overarching concepts and terminology used in the study of *Drosophila* immunity before describing *Drosophila* immune modules in detail.

A. Factors that shape immune systems

An immune system must be adapted to the nature and diversity of microbes present in the host environment (Schmid-Hempel, 2021). Animals lose immune genes that are no longer beneficial: these are sometimes detectable in a pseudogenized form in the genome. A broad range of species from microbes to macroparasites are capable of infecting *Drosophila*, which accordingly possesses an array of immune programs to deal with diverse challenges (**Figure 1**). The developmental stage of the host at time of infection, and recurrence and severity of infection, are also key elements that shape immune reactions. *Drosophila* has defense mechanisms that are stage-specific, such as wasp egg encapsulation in the larva. Recurrent infections may favor evolution of long-lasting innate immune responses. The short lifespan and ephemeral ecology of *Drosophila melanogaster* has likely constrained the evolution of 'true' immune memory processes like the adaptive immunity of vertebrates. Instead, **priming** or **trained immunity** describe responses in *Drosophila* that enhance defense against repeated infection (Pradeu et al., 2024; Sheehan et al., 2020; Tang et al., 2023). These effects have been attributed to the persistence of immune effectors, increased levels of basal immunity, higher immune reactivity, and possible epigenetic changes in previously infected flies. **Transgenerational effects**, where protection is provided maternally to offspring, have been described in *Drosophila* for antiviral but not antibacterial defense (Mondotte et al., 2020; Radhika and Lazzaro, 2023; Vilcinskas, 2021). **Trade-offs** in energetic costs with other essential physiological functions, reproduction, and lifespan also shape immunity (Kraaijeveld et al., 2002). The level of **basal immunity** in the absence of infection is an important parameter influencing survival upon infection. Many immune modules are inducible, and are fully deployed only upon challenge, mitigating constitutive costs of immune defense (Harvell, 1990). Alternatively, some immune defenses are constitutively deployed and are not enhanced upon infection. The high cost of the immune system explains why immune modules with low utility are lost over time (Palmer and Jiggins, 2015; Ruzzante et al., 2022). Finally, the immune system is constrained by phylogeny. That is, *Drosophila* has specific features as an insect, for instance: an exoskeleton, larval growth by molting, metamorphosis, gas exchange through tracheae, and an open circulatory system. Although insect immune systems present a great deal of variation, fundamental mechanisms similar to

those found in *Drosophila* recur throughout the insect class. Thus, characterization of the *Drosophila* immune system has benefitted from studies in other insects, where some immune modules may be better studied, and *vice versa*.

B. Multiple ways to resist infection

Initial studies of the *Drosophila* immune system focused on effector mechanisms that directly combat pathogens, such as phagocytosis, antimicrobial peptides, or encapsulation. Today, we understand that *Drosophila* limit or combat infection in a greater variety of ways (Pradeu et al., 2024). First, flies exhibit **behavioral immunity**, a suite of behaviors that limit pathogen entry or contribute to sickness states promoting recovery (Davis and Schlenke, 2022; De Roode and Lefèvre, 2012; Montanari and Royet, 2021). Once an infection is established, a new suite of mechanisms in various physiological compartments limit pathogen growth inside the organism. **Cell-intrinsic immunity** refers to intracellular mechanisms such as antiviral defense. **Local immunity** takes place in epithelia in contact with the external environment, such as the gut or trachea. **Systemic immunity** takes place in the hemolymph (insect blood) and is usually divided into two branches: **cellular immunity** involving blood cells, and **humoral immunity** involving secreted substances produced by the hemocytes (*Drosophila* blood cells) and fat body, the fly analog of the liver. Finally, **reproductive immunity** deals with mechanisms that limit infection upon mating in male and female genital organs.

Immune programs are further divided into **resistance** mechanisms that directly target or limit growth of pathogens, and **disease tolerance** (or **resilience**) mechanisms that promote host survival without targeting pathogens (Howick and Lazzaro, 2017; Medzhitov et al., 2012). Although the complex interplay between survival and pathogen growth makes separating resistance and tolerance difficult (Hidalgo et al., 2022; Kutzer and Armitage, 2016a; Paulo et al., 2023), a common method is to plot survival against microbial load (Medzhitov et al., 2012; Schneider and Ayres, 2008). As survival outcomes are essentially determined by a race between pathogen-induced damage to the host competing against the host's ability to mitigate that damage, either by repairing itself or eliminating the pathogen, this approach reasonably assumes that microbial load is more greatly affected by disrupting resistance mechanisms than tolerance mechanisms. Disease tolerance mechanisms are quite diverse; any factors impacting fitness can indirectly affect host survival. For instance, a brain mutation making flies hyperactive may affect survival by wasting energy that then cannot fuel the immune system. In this review, we will focus on tolerance mechanisms that are closely related to the infection process. These include tissue repair, stress responses, detoxification, and mechanisms that protect the host against deleterious consequences of the immune system. **Nutritional immunity** is another mechanism that contributes to defense by preventing pathogens from benefitting from host nutrients such as iron. Finally, protection against pathogens might not be derived from *Drosophila* itself but from its microbiota, a process called **symbiont-mediated immunity** (Brownlie and Johnson, 2009). Microbial symbionts may promote survival directly by interfering with pathogen growth via niche competition or toxin production, or indirectly by stimulating the host immune system and increasing basal immunity. In this review, we primarily address how immune pathways and genes impact host defense, but the outcome of infection is influenced by multiple internal (stage, age, microbiota, sex, genetic backgrounds) and external factors that influence both patho-

gen growth and host defense in complex ways such as temperature (Cavigliasso et al., 2021; Fedorka et al., 2016; Kutch et al., 2014; Lazzaro et al., 2008; MacMillan et al., 2016; Salehipour-shirazi et al., 2017; Štětina et al., 2019), diet (Brown et al., 2009; Kutzer and Armitage, 2016b), hydration state (Zheng et al., 2018), CO_2 and oxygen concentration (Bandarra et al., 2014; Barretto et al., 2020; Helenius et al., 2009), social environment (Leech et al., 2017, 2017), mating status (see Consequences of mating, page 122), time of day and seasonality (Behrman et al., 2018; Lee and Edery, 2008; Stone et al., 2012) and past exposure to stresses and infections. Thus, many parameters can influence experimental results, including methodological choices such as the mode and site of infection, as exemplified by the differential impact on survival of inoculating flies in the thorax or the abdomen (Chambers et al., 2014).

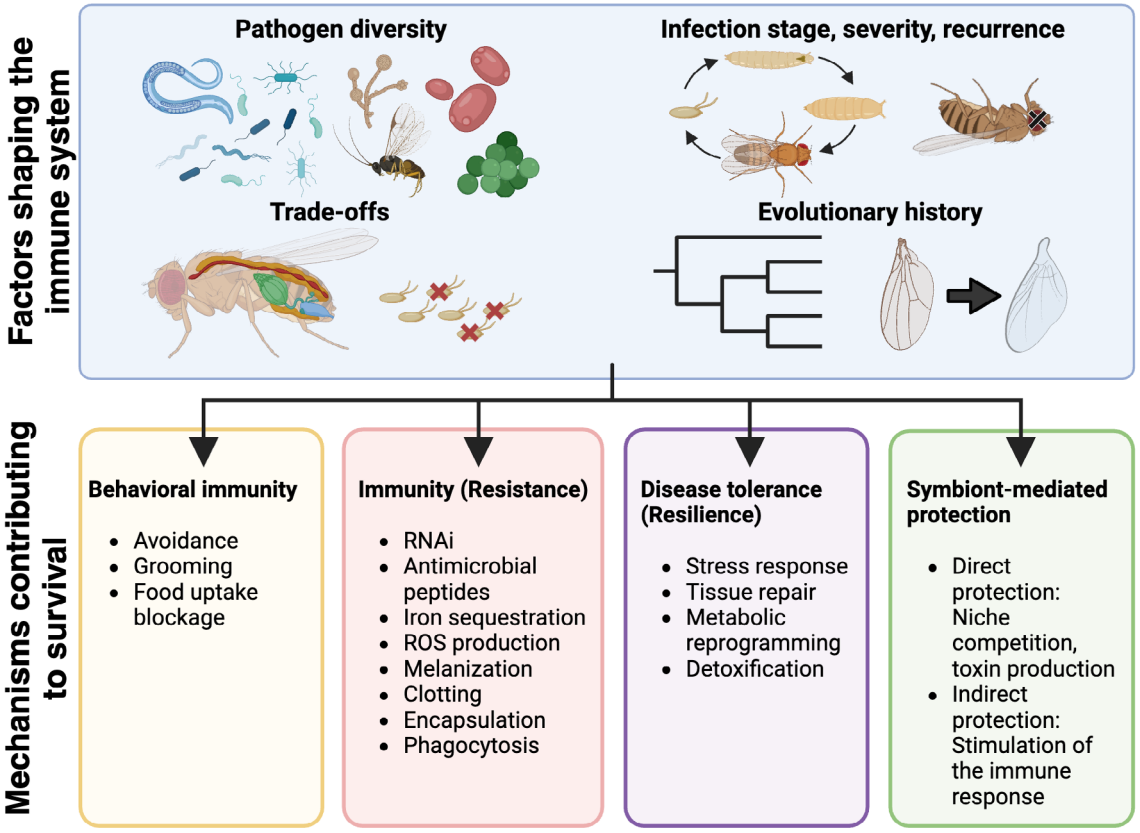


Figure 1 Different ways to resist infection
Multiple factors shape the evolution of the immune system to respond to pathogens and microbiota found in the host environment. These factors include the nature and number of pathogens in the environment, the life stage at which pathogens infect the host, trade-offs with other physiological needs such as reproduction, and evolutionary constraints. These evolutionary pressures have led to the selection of multiple mechanisms that contribute to survival upon infection. Figure created with BioRender.com, CC-BY-NC-ND.

Box 1 Modeling infection

Analysis of the *Drosophila* immune response has largely relied on using appropriate gene read-outs to monitor immune pathway activation in response to different stimuli, alongside experiments to see how pathway components and immune-responsive genes affect fly survival and pathogen growth.

Since large numbers of flies can easily be infected, *Drosophila* is especially suited to quantitative modelling of the immune system (Louie et al., 2016). One of the main determinants of survival outcome is the rapidity of the immune response, as evidenced by studies showing that survival depends on how quickly antimicrobial peptides are upregulated or lamellocytes are generated (Duneau et al., 2017a; Leitão et al., 2020). Despite identical controlled conditions, survival outcome can vary from individual to individual. This stochasticity was explained by characterizing survival as an outcome resulting from a tight arms race between pathogen growth and control by the immune system (**Figure Box 1**) (Duneau et al., 2017a). This model introduced parameters that determine infection outcome: the rate of microbe growth, the time taken to immunological control, the tipping point at which microbe load goes beyond what can possibly be controlled, and the titers at which microbe load settles following suppression (**set point pathogen load**) or kills the host (**pathogen load upon death**) (Lafont et al., 2021). Future efforts could expand these parameters to account for additional layers, such as microbe-dependent factors like shifts between protected and susceptible states (Ellner et al., 2021), or facets of the host response including tolerance to microbial or autotoxic immune damage.

Due to its ease of observation and simple binomial outcome, fly geneticists tend to define pathogenicity based on lethality. This criterion is rather restrictive compared to human disease studies: one of the most virulent bacteria in humans, *Shigella*, might not be considered pathogenic using this criterion, as it kills 'only' 3-10 % of those infected. A lack of readily assayed intermediate disease states has hampered efforts to advance definitions of pathogenicity in flies. However, recent studies have noted additional outcomes after infection visible to the naked eye which may allow scoring of intermediate disease states independent of mortality. These include: bloating of the abdomen after infection by Drosophila C virus, *Acetobacter* or *Pectobacterium* bacteria (Chtarbanova et al., 2014; Hanson et al., 2023; Zugasti et al., 2020), an erect wing response after immune stimulation (Hanson et al., 2021), or neurological symptoms associated with pathogen virulence factors (Huang et al., 2023; Smith et al., 2023). Reproductive fitness is also often ignored in *Drosophila* immune studies, despite research showing that there are parasites and pathogens that sterilize their hosts without affecting immediate survival (Bruner-Montero et al., 2023; Jaenike et al., 2010). Vigilance for such phenotypes will reveal their specificity or universality, and better demarcate disease progression. Presently, the underlying host factors that regulate these intermediate responses are poorly resolved.

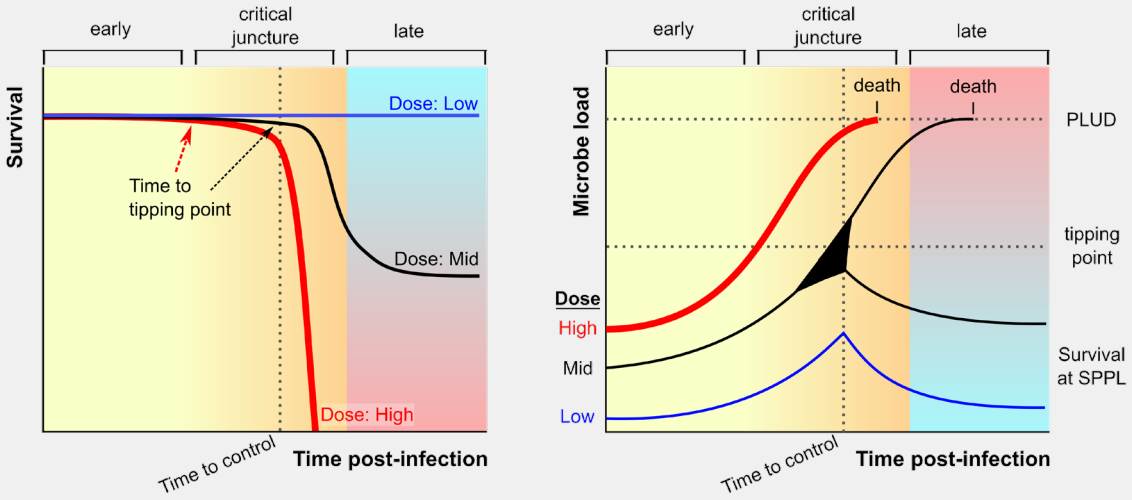


Figure Box 1 Modelling within-host pathogen growth dynamics
The survival outcome of a given infection (**left**) depends on initial pathogen dose, which determines subsequent growth dynamics within the host (**right**). For pathogens with moderate virulence, too high a dose overwhelms the host before the host can mount a successful immune response (**left**, **red line**), while a low dose creates an opposite dynamic where the pathogen is always controlled (**left**, **blue line**). These infection kinetics are described by Duneau et al. using formal parameters: *time to control*, *tipping point*, *Set Point Pathogen Load (SPPL)*, and *Pathogen Load Upon Death (PLUD)* (Duneau et al., 2017a). Infection kinetics play out over three main stages: (**i**) an *early* phase where recognition and immune signaling begins and pathogen growth is not controlled, (**ii**) a *critical juncture* that determines host outcome, where immune effectors start to control pathogen growth, and (**iii**) a final *late* phase where the results of the individual dynamics at the critical juncture become visible. Cryptic variation in individual host and bacterial states drives the stochasticity of the ultimate binary outcome: survival or death. Fundamentally, the host must mount a minimum threshold microbicidal activity to constrain pathogen growth. The *time to control* is a measure of how long it takes host immune activation to reach this threshold of microbicidal activity. Importantly, this threshold must be achieved before the pathogen reaches the *tipping point* microbial load, after which, microbial growth and total load will always exceed the potential for constraint by the host immune response. When *time to control* is very close to the time it takes a microbe to reach the *tipping point* microbial load, cryptic variation in host or bacterial condition can affect the ultimate outcome, even amongst identical infections. The black triangle at this juncture represents this uncertainty around potential trajectories. Ultimately, if microbe load exceeds the tipping point, microbial growth proceeds unchecked, and the host eventually succumbs to microbial burden at a relatively fixed *PLUD* determined by the limit of host tolerance to that pathogen. If microbial growth is successfully constrained, then the microbe load is controlled and reduces towards an *SPPL*. The *SPPL* is more stochastic than the *PLUD* and is affected somewhat by initial dose, suggesting that the microbe load at *time to control* determines the eventual *SPPL* for a given infected individual. Microbe load remains at *SPPL* for weeks, and in many cases pathogenic microbes are never fully cleared (Hidalgo et al., 2022). Microbes maintained at the *SPPL* may also stochastically break out into recurrent systemic infections, renewing the race between the host immune response and microbial growth even at late time points (Ramirez-Corona et al., 2022). Like adjusting pathogen dose, infection kinetics are also affected by how close the environmental temperature is to the ideal microbial growth temperature, or availability of nutrients, with similar predictable shifts that favor pathogen growth speed and increased host mortality. Figure adapted from (Duneau et al., 2017a).

2

Ecological context of *Drosophila* and its microbiota

Drosophila melanogaster has a world-wide distribution and is closely associated with human activity (Throckmorton, 1975). This fly is generally found associated with decaying fruits (**Figure 2**). This ephemeral and seasonal ecosystem, which contributes to the fast life cycle of the fly, is shared with two other genetic model organisms: the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae* (Félix and Braendle, 2010). Decaying fruits house a complex ecosystem shaped by dynamic changes in fungal and bacterial composition during fruit fermentation. In this microbe-rich environment, *Drosophila* interacts with a broad range of symbionts, opportunists, and pathogens. This diversity of cohabitants has likely shaped the sophisticated *Drosophila* immune system, which faces different selective pressures compared to insects feeding on non-rotting foods, such as bees, silkworms, aphids, or weevils (Gerardo et al., 2010; Hammer et al., 2023; Heddi and Zaidman-Rémy, 2018). Furthermore, a short life cycle allows the *Drosophila* genome to evolve markedly over short (human) timescales. Although this is beneficial for genetic studies, *Drosophila* strains have been kept by geneticists for several decades on food treated with fungicide and a microbiota less diverse than that found in natural habitats (Chandler et al., 2011; Chen et al., 2022; Pais et al., 2018). This reduced pathogen pressure on lab-reared *Drosophila* may facilitate fixation of mutations in key

Figure 2 The rotting fruit, an ephemeral ecosystem
Drosophila larvae and adults develop in decaying organic matter, such as the Opuntia fruit shown here. The fruit was damaged by bird pecking, and colonized by bacteria, yeasts, and flies laying eggs in the fruit. *Drosophila* can serve as vectors for microbes, transferring them between food substrates. B.L. Personal photo, taken in Tunisia.

immune genes, such as *imd*, *NimC1* and *PPO3,* mutations of which have been serendipitously discovered in laboratory stocks. Pre-existing natural polymorphisms are also common in immune genes, and may become fixed in stocks derived from wild-caught individuals given relaxed infectious pressure in the lab (Arunkumar et al., 2023; Hanson et al., 2019a; Smith et al., 2023). Other aspects of fly care (e.g., diet, temperature, flipping frequency) also impact infection outcome in important ways, with different severity according to fly genetic background. Collectively, detailed analysis of local microbiota conditions and fly care practices should not only improve repeatability of immune studies, but also clarify why marked differences in infection outcome are seen across laboratories.

A. Pathogens of *Drosophila*

Recent studies using DNA sequencing and sampling of wild fly populations have developed a better picture of the variety of viruses, bacteria, fungi, microsporidians, protozoans (notably trypanosomes), and macroparasites (nematodes, mites, parasitoid wasps) associated with flies (**Figure 3**) (Carton et al., 1986; Chandler et al., 2011; Webster et al., 2015). Although some important observations have been made in natural conditions, our understanding of how pathogens affect fly survival or fitness in the wild is limited. Surveys of wild populations reveal that virus or parasitoid wasp infections can be widespread (up to 85% of larvae in a population can be infested by parasitoid wasps) (Carton et al., 1986; Subasi et al., 2023; Wallace and Obbard, 2023; Webster et al., 2015). This is also likely the case for bacterial and fungal pathogens, although we lack data. One study suggests that bacteria can be major factors affecting regional and seasonal fly immunity population genetics (Behrman et al., 2018).

While viruses and parasitoids are highly co-evolved pathogens, most bacterial and fungal pathogens of *Drosophila* are considered opportunistic, as *Drosophila* is just one among many possible host species found in the environment. Due to host-pathogen arms races, co-evolved pathogens whose survival depends on a specific host are expected to display more mechanisms that suppress the host immune system (see **Box 7**, Wasps target the *Drosophila* immune system, page 103). Consistent with this, many suppressors of *Drosophila* immunity have been observed in viruses and parasitoid wasps. Some pathogens manipulate *Drosophila* behavior to disseminate themselves, such as the fungal entomopathogen *Entomophthora muscae* (Bonning and Saleh, 2021; Elya et al., 2023, 2018; Yang et al., 2021). *Drosophila* is also a vector of plant pathogens such as *Pectobacterium carotovorum Ecc15*, a bacterium that induces soft rot of various plants including potatoes (Kloepper et al., 1981). While all *P. carotovorum* strains induce plant rot by producing pectinolytic enzymes, only certain strains possess virulence factors that allow colonization of the *Drosophila* gut, indicating that specific mechanisms have evolved to allow even opportunistic bacteria to hitchhike on flies (Basset et al., 2000; Basset et al., 2003; Muniz et al., 2007; Vieira et al., 2020).

B. Routes of infection

Use of natural pathogens and natural infection routes that mimic challenges faced by *Drosophila* in the wild is ultimately the gold standard in studying the immune system

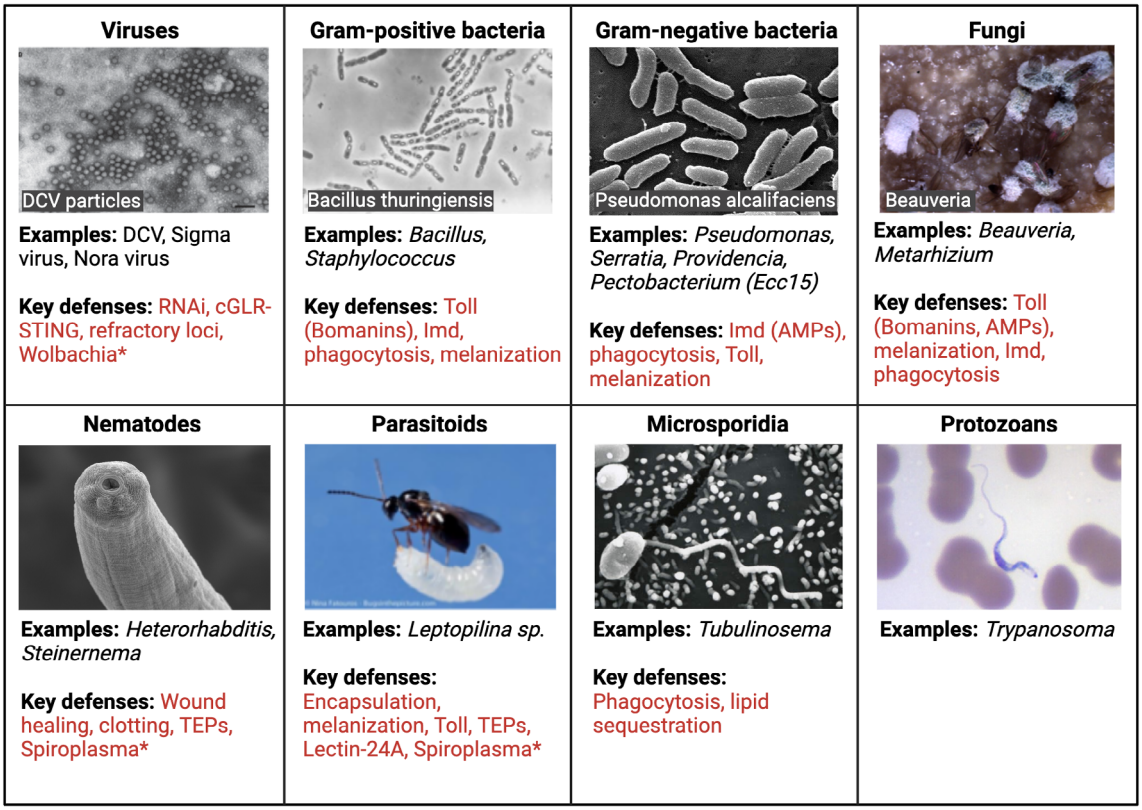


Figure 3 Enemies of Drosophila and corresponding host defenses
Drosophila can be infected by pathogens belonging to many different classes (Caravello et al., 2022; Franchet et al., 2019; Ryckebusch et al., 2024; Teixeira et al., 2008; Xie et al., 2013). The main mechanism(s) of defense used to combat each class of pathogen are indicated, although defenses may be pathogen-specific. Asterisks (*) indicate symbiont-mediated immunity.
Photo credits: *Drosophila* C Viruses (Dostert et al., 2003); *Baccillus thuringiensis* (Le *Bacillus thuringiensis* (Bt) en question, Biofil N°128 2020 p. 23-25); *Pseudomonas alcalifaciens* (CDC/ Pete Wardell, Public domain, via Wikimedia Commons); *Beauveria bassiana* (B. Lemaitre CC); *Steinernema* entomopathogenic nematodes (Mirayana M. Barros, Dennis Chang, Dihong Lu, and Adler R. Dillman via Wikimedia Commons). *Leptopilina* parasitoid wasp; *Microsporidia* (Wikimedia commons, unlicensed CDC); Trypanosome (Ed Uthman from Houston, TX, USA, CC BY 2.0).

(Neyen et al., 2014; Troha and Buchon, 2019). This also allows study of pathogen-specific entry routes. Although oral or topical infection routes are considered the most 'natural', systemic infections are likely more common than appreciated in the wild. A recent survey of wild-caught flies found that ~31% show abdominal or genital scars, likely due to mites or mating injuries (**Figure 4A, B**) (Subasi et al., 2023). Larvae likely experience many wounds from wasp infestation, nematode attack, or incidental scrapes from the food substrate. While injection or septic injury as employed in the lab is undoubtedly artificial, this approach mimics such infection routes (**Figure 4C**). An inevitable drawback of using natural pathogens is that they may be co-evolved to suppress aspects of the immune response we want to study. As a result, study of the *Drosophila* immune system

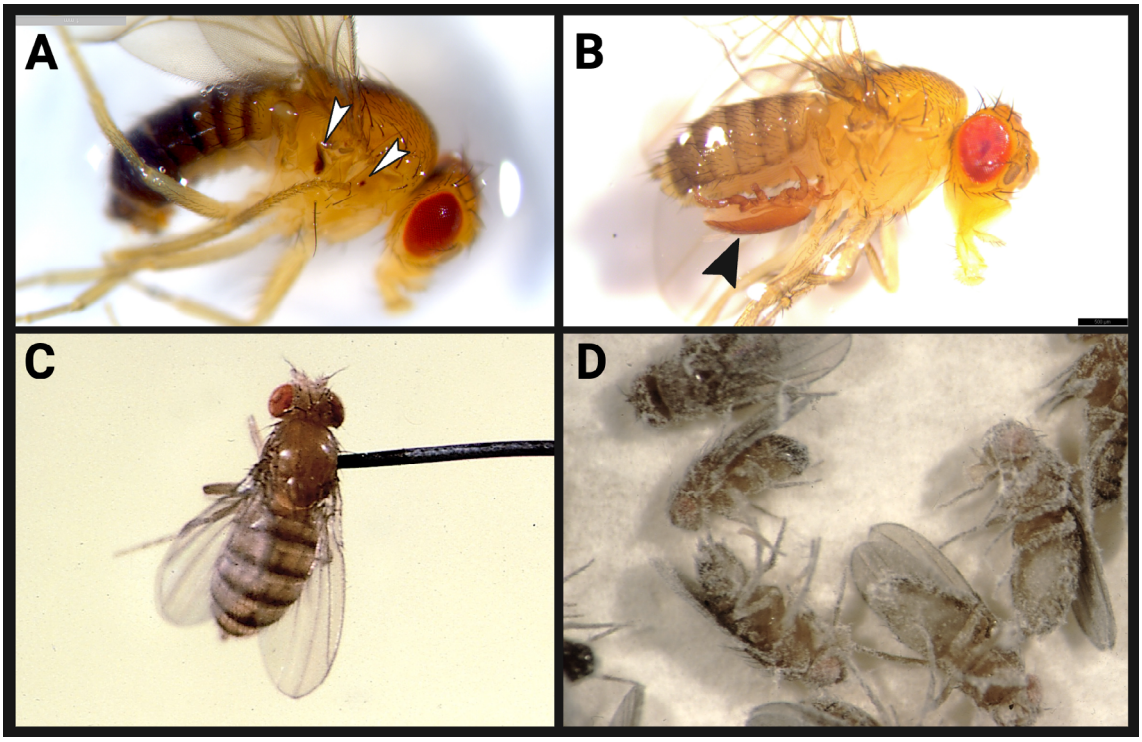


Figure 4 Septic injury and natural infection in wild and lab-reared flies
A Wounds on a *D. melanogaster* male collected from the wild. Arrowheads indicate melanized spots on the thorax. The cause of this damage is not known but may be due to a mite. Photo courtesy of Bengisu S. and Sophie A.O. Armitage. See also (Subasi et al., 2023). **B** Female *D. melanogaster* with a *Macrocheles* sp. mite attached to the abdomen (arrowhead). Photo courtesy of Bengisu S. and Sophie A.O. Armitage. See also (Subasi et al., 2023). **C** An adult fly undergoing septic infection in the lab. The fly is pricked with a 0.2mm needle dipped in a concentrated bacterial pellet to introduce systemic infection (Photo credit, B. Lemaitre CC). **D** Flies naturally infected with fungal spores. Anaesthetized flies were rolled on a lawn of sporulating *Beauveria bassiana* (Photo credit, B. Lemaitre CC).

has greatly benefitted from use of non-native pathogens, which allows better control of the multiple parameters influencing infection. This approach enables exploration of the underlying properties of the immune response without confounding factors such as suppressors or behavioral manipulation by natural pathogens.

C. The gut microbiota

The gut of laboratory-maintained fly stocks hosts low bacterial diversity (1–30 species) compared to wild-caught flies (Broderick and Lemaitre, 2012; Chandler et al., 2011; Chen et al., 2022; Wong et al., 2011). The most common species are members of just three major families: Lactobacillaceae, a family of lactic acid-producing bacteria (e.g., *Lactiplantibacillus, Leuconostoc*); Acetobacteraceae, a family of acetic acid-producing bacteria (e.g., *Acetobacter, Gluconobacter*); and occasionally Enterobacteriaceae.

Yeasts such as *Hanseniaspora, Pichia, Starmerella* or *Saccharomyces* are also found (Broderick and Lemaitre, 2012; Chandler et al., 2011; Mure et al., 2023; Wong et al., 2011). Recent studies have further emphasized the importance of microbes, viruses and endosymbionts circulating in laboratory *Drosophila* stocks, as these cryptic factors can influence experimental results (Habayeb et al., 2009; Hanson et al., 2023; Hanson and Lemaitre, 2023; L'heritier, 1958; Teixeira et al., 2008). Interestingly, flies tend to favor microbially diverse environments, and are attracted to bacteria and yeast compositions able to provide specific metabolites, such as derivatives of ethanol and acetate catabolism (Fischer et al., 2017).

Unlike mammals and social insects, (Engel and Moran, 2013; Martinson et al., 2017), *D. melanogaster* does not harbor a core microbiota distinct from the environment. Instead, microbes are ingested and colonize the host gut, and gut microbes are in turn excreted and colonize the external environment. Excreted bacteria can then modify the external ecological niche to favor growth and recolonization of bacteria beneficial to the fly, including strains that more persistently colonize the intestinal tract (Gould et al., 2018; Pais et al., 2018; Storelli et al., 2018). These persisters may be better able to resist the action of host immune effectors (Arias-Rojas et al., 2023), or may colonize host-constructed physical niches (Pais et al., 2018; Dodge et al., 2023). Pulse-chase studies show that the cardia, a segment of the adult foregut, can selectively bind and stabilize colonization of bacteria with strain-level specificity. There is no similar niche in larvae, which feed continuously and therefore have a more constant and abundant microbiota, while adults ingest food in intermittent sips (Storelli et al., 2011). In the laboratory, emerging adults have almost no bacteria in the gut, and frequent flipping of flies on sterile medium maintains a low microbiota load in adults (Blum et al., 2013; Wong et al., 2011).

The microbiota influences host traits in many ways, providing a food source, stimulating digestion, and driving anabolic pathways that promote larval growth and oogenesis (Elgart et al., 2016; Lesperance and Broderick, 2021; Ridley et al., 2012; Shin et al., 2011; Storelli et al., 2011). The microbiota further complement fly metabolism by providing vitamins (notably B group vitamins), cholesterol (from yeast), and amino acids that ameliorate or complement the diet (Consuegra et al., 2020; Sannino et al., 2018). It is therefore not surprising that microbiota manipulations can have multiple effects on the host. While germ-free flies are easily cultivated in typical laboratory conditions, microbial associations greatly promote growth in nutrient-poor conditions, emphasizing the importance of live bacteria to fly development (Mure et al., 2023; Shin et al., 2011; Storelli et al., 2011). Unfortunately, it can be difficult to distinguish whether these benefits come from direct action of bacteria, or indirect processing of the food substratum by bacteria into metabolites that nourish flies. Interactions between the microbiota and diet or aging further emphasize the difficulty in ascribing direct or indirect effects. For this reason, the role of the gut microbiota is best defined in terms of its interaction with the nutritional environment (Keebaugh et al., 2019).

Direct antagonistic interactions between microbiota and entomopathogens have been described in several insect species (Blum et al., 2013; Fast et al., 2018; Glittenberg et al., 2011; Gould et al., 2018; Lee et al., 2018; Sibley et al., 2008). However, to date little is known about how *Drosophila* microbiota impact host survival to pathogenic microbes. The microbiota may impact host survival by stimulating basal immunity, by engaging in direct niche competition, or by influencing host metabolism. In larvae, microbiota protect the host upon ingestion of *Candida albicans* (Glittenberg et al., 2011). A recent series

of *in vitro* and *in vivo* experiments revealed that *Lactiplantibacillus plantarum* improve fly survival by inhibiting the growth of three invasive Gram-negative bacteria through acidification of both internal and external environments, including culture media, fly food, and the gut itself, while *A. tropicalis* suppresses this effect by quenching acids (Barron et al., 2024). Microbiota-mediated priming of antiviral responses has also been observed in *Drosophila* (Sansone et al., 2015). Recent work has shown a protective role of *Drosophila* cuticular microbiota against *Beauveria* topical infection, preventing fungus establishment that is reciprocally combatted by the pathogen through secretion of a fungal Defensin (Hong et al., 2023a, 2023b; Hong et al., 2022). Future studies disentangling the complex interplay of host-microbiota-pathogen interactions will be needed to clarify how the microbiota affects the *Drosophila* response to infection.

D. *Drosophila* endosymbionts

Drosophila melanogaster can harbor two facultative endosymbiotic bacteria, *Wolbachia* and *Spiroplasma*, that reside inside the host and are vertically transmitted. These bacteria are insect specialists with a reduced genome size and are fully integrated into the biology of their host. Although they are not detected by the immune system, these symbionts can have profound impacts on host physiology and protection against pathogens.

i) Wolbachia

Wolbachia is the most widespread and widely studied facultative endosymbiont, estimated to infect 50% of all terrestrial arthropod species, including a significant fraction of wild and lab *Drosophila* strains (Clark et al., 2005; Porter and Sullivan, 2023; Weinert et al., 2015; Werren et al., 2008) (**Figure 5A**). *Wolbachia* resides in the cytoplasm of cells at high loads and colonizes germ cells to facilitate transmission (Fast et al., 2011). While most *Wolbachia* strains do not have detectable effects on *D. melanogaster* fitness, some with high proliferation rates are pathogenic (Chrostek et al., 2013; Fry et al., 2004; Min and Benzer, 1997). How are potentially pathogenic facultative endosymbionts maintained in natural populations? In many species, *Wolbachia* manipulates reproduction through cytoplasmic incompatibility to increase spread in the population, but this mechanism is likely less significant in the *Wolbachia-Drosophila* interaction (Bourtzis et al., 1996; Yamada et al., 2007). Instead, *Wolbachia* has been shown to increase *Drosophila* survival to certain viral infections (Bruner-Montero and Jiggins, 2023; Hedges et al., 2008; Teixeira et al., 2008) (**Figure 5B**). This important discovery led to the use of symbiont-mediated protection to reduce the impact of human arboviruses transmitted by mosquitoes (Sinkins, 2013). It also reveals how an immune phenotype - here increased resistance to a virus - can be mediated by a symbiont rather than the host. The mechanisms behind the protective effect of *Wolbachia* against host viral infections are not fully understood, but likely do not depend on stimulation of the host immune system or bacterial toxins. *Wolbachia* protection is modulated by temperature and is dependent on symbiont titer, which is regulated by a group of eight genes called *octomom* (Chrostek et al., 2021; Chrostek and Teixeira, 2015). As viruses and *Wolbachia* co-exist in the cytosol, *Wolbachia* might alter or compete for a cytosolic factor required for virus success, such as cholesterol (Caragata et al., 2013; Pimentel et al., 2021; Wong et al., 2015). Protection

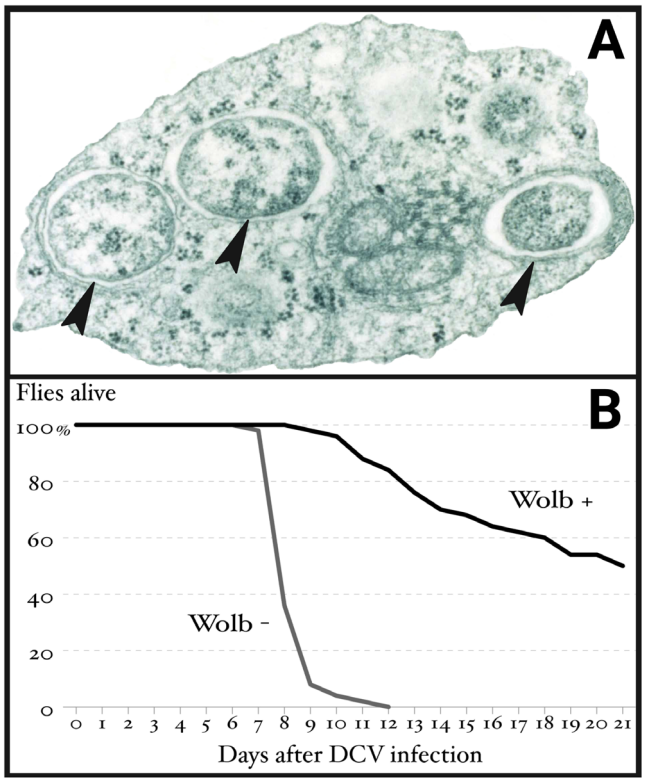


Figure 5 Wolbachia, an intracellular endosymbiont
A *Wolbachia* in an insect cell (arrowheads). From ("Genome Sequence of the Intracellular Bacterium Wolbachia," 2004) CC BY. **B** The presence of *Wolbachia* protects *Drosophila* males against DCV viral infection. Wild type males with (Wolb+) or without (Wolb-) *Wolbachia*. Flies were treated with tetracycline to eliminate *Wolbachia.* Adapted from (Teixeira et al., 2008) CC BY.

against viruses might not be the only benefit provided by *Wolbachia*; a recent preprint proposes that *Wolbachia* may supplement flies with pyrimidines, helping to buffer the effects of nutrient-poor conditions on fly development (Lindsey et al., 2023). Thus, while its contributions to defense are robustly confirmed, the benefits of *Wolbachia* to the host could extend beyond what is presently known.

ii) Spiroplasma

In contrast to cosmopolitan *Wolbachia*, *Spiroplasma poulsonii* only infects 0-5% of wild *Drosophila* and is not maintained in lab stocks, but recent advances now allow its cultivation *in vitro* (Haselkorn, 2010; Masson et al., 2018). *Spiroplasma* is an extracellular bacterium with no cell wall that resides in the hemolymph of larvae and adults (**Figure 6**). It colonizes the female germline at the adult stage by co-opting the yolk uptake machinery (Herren et al., 2013), a mechanism also used by other insect pathogens to ensure vertical transmission (Brasset et al., 2006; Fukatsu, 2021; Guo et al., 2018; He et al., 2019; Huo et al., 2019, 2014; Wei et al., 2017). The titer of this symbiont is tightly controlled by lipid and

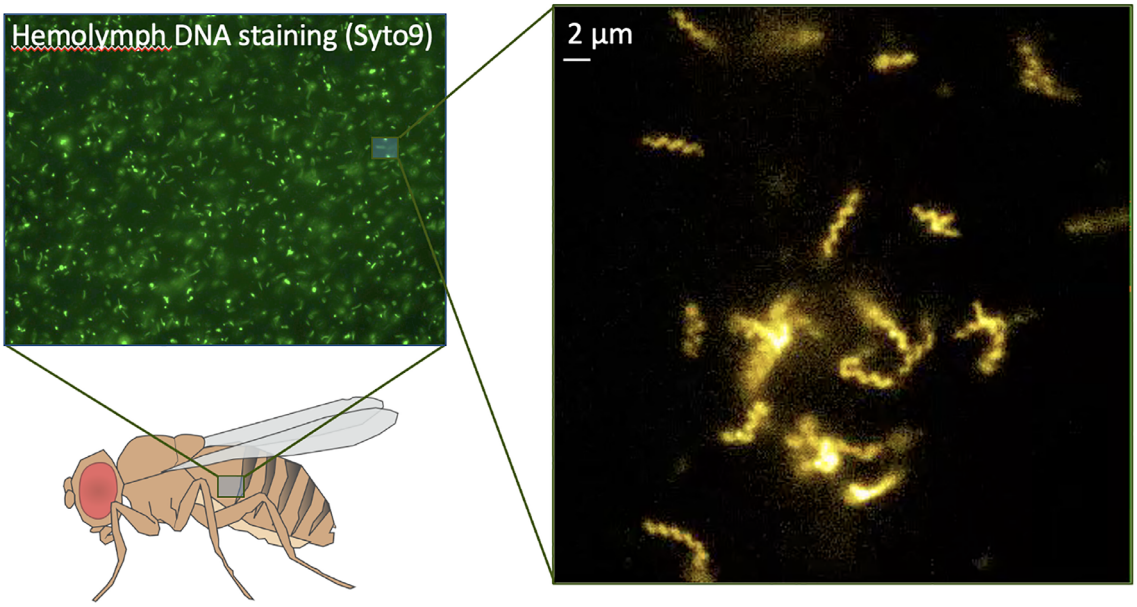


Figure 6 Spiroplasma, an extracellular endosymbiont
Spiroplasma poulsonii is a facultative endosymbiont of *Drosophila*. *Spiroplasma* reside in the hemolymph as shown by DNA staining (**left**), but are vertically transmitted by colonizing the oocytes by using the Yolk uptake machinery. This symbiont has a helical shape (**right**) and no cell wall. Credits, *Drosophila*: Mark Hanson clip art (CC-BY 4.0), *Spiroplasma*: Florent Masson, Alex Persat et B. Lemaitre (see also Masson et al., 2021).

iron availability (Herren et al., 2014; Marra et al., 2021b). *Spiroplasma poulsonii* grows slowly and does not impact the fitness of young flies, but kills old flies.

Strikingly, *S. poulsonii* produces a toxin called SPAID that targets the male-specific dosage compensation (MSL) complex (Harumoto and Lemaitre, 2018; Veneti et al., 2005). This kills male embryos, and is thought to favor bacterial transmission by increasing the proportion of infected females, perhaps through reduced larval competition (Martins et al., 2010; Ventura et al., 2012). Importantly, *Spiroplasma* increases survival of drosophilid flies targeted by parasitoid wasps and nematodes, providing another striking example of endosymbiont-mediated defense (Ballinger and Perlman, 2017; Jaenike et al., 2010; Xie et al., 2010). *Spiroplasma* may compete for host lipids to impede wasp development (Paredes et al., 2016). However, a key mechanism of *Spiroplasma*-mediated defense is the production of ribosomal toxins (RIPs) that cleave parasite ribosomal RNA at the sarcin-ricin loop (Ballinger and Perlman, 2017; Hamilton et al., 2016). Production of RIPs preferentially targets nematodes or developing wasps present in the hemolymph, a compartment shared with *Spiroplasma*. The specificity of different RIPs to different parasites prevents collateral damage to the host (Ballinger et al., 2019), though RIPs can have detrimental effects on old flies at very high titers, contributing to the reduced lifespan of *Spiroplasma*-infected females (Garcia-Arraez et al., 2019).

3
The antiviral response

Recent surveys have revealed an incredible diversity of RNA and DNA viruses, some of which are vertically transmitted, that infect flies in natural populations (Wallace and Obbard, 2023; Webster et al., 2015). It is therefore not surprising that the antiviral response of *Drosophila* is complex, and in some cases, virus specific. To date, RNA interference (RNAi) is the best characterized antiviral mechanism in insects. Viruses activate distinct transcriptional responses in *Drosophila* (Kemp et al., 2013). They also impact host survival differently depending on the route of infection (Mondotte and Saleh, 2018). We note that the trend of calling every pathway or process that promotes survival to viral infection 'antiviral' has caused confusion. Although most of the immune or repair signaling pathways (Toll, Imd, JAK-STAT) have been implicated in some way in antiviral defense, whether these pathways directly sense or eliminate viruses or instead primarily contribute to host disease tolerance is poorly characterized. Evidence of a generic transcriptional response to viruses in flies like the interferon response observed in mammals remains elusive, but promising results have recently indicated a role for the cGAS-STING-Relish pathway in broad antiviral defense.

A. Restriction factors

Genome Wide Association Studies (GWAS) using panels from highly polymorphic wild-type flies have revealed that host resistance to viruses is greatly impacted by a small number of major-effect loci (Cogni et al., 2016). Cogni and colleagues found that three quantitative trait loci (QTLs) were responsible for 90% of heritable resistance to Drosophila C Virus infection, while five QTLs explained 42.2% of the resistance to Sigma virus. These studies show that pathogenicity can be determined by just a few important host loci. These loci explaining heritability can vary across populations (Smith et al., 2023) or evolutionary timescales.

Two major refractory loci, *ref(2)P* for Sigma virus and *pastrel* for DCV, have been well characterized, although the mechanisms by which they block virus propagation are still not determined. *Drosophila* Sigma viruses infect natural populations of *D. melanogaster* at frequencies of 0–15% (Carpenter et al., 2007). Sigma-infected flies are paralyzed or killed when exposed to high concentrations of carbon dioxide, which provides a simple assay for detecting Sigma virus infection. A complex mutation in *ref(2)P* (homologous to mammalian *p62*) reduces the replication rate of Sigma virus (Brun and Plus, 1980; Contamine et al., 1989). Ref(2)P is involved in the autophagic clearance of cytoplasmic protein bodies (Bartlett et al., 2011) and has also been linked to Toll pathway activity (Avila et al., 2002). CHKov1 and Ge1 have also been identified as Sigma virus restriction factors, of which Ge1 is a component of P bodies involved in RNA metabolism

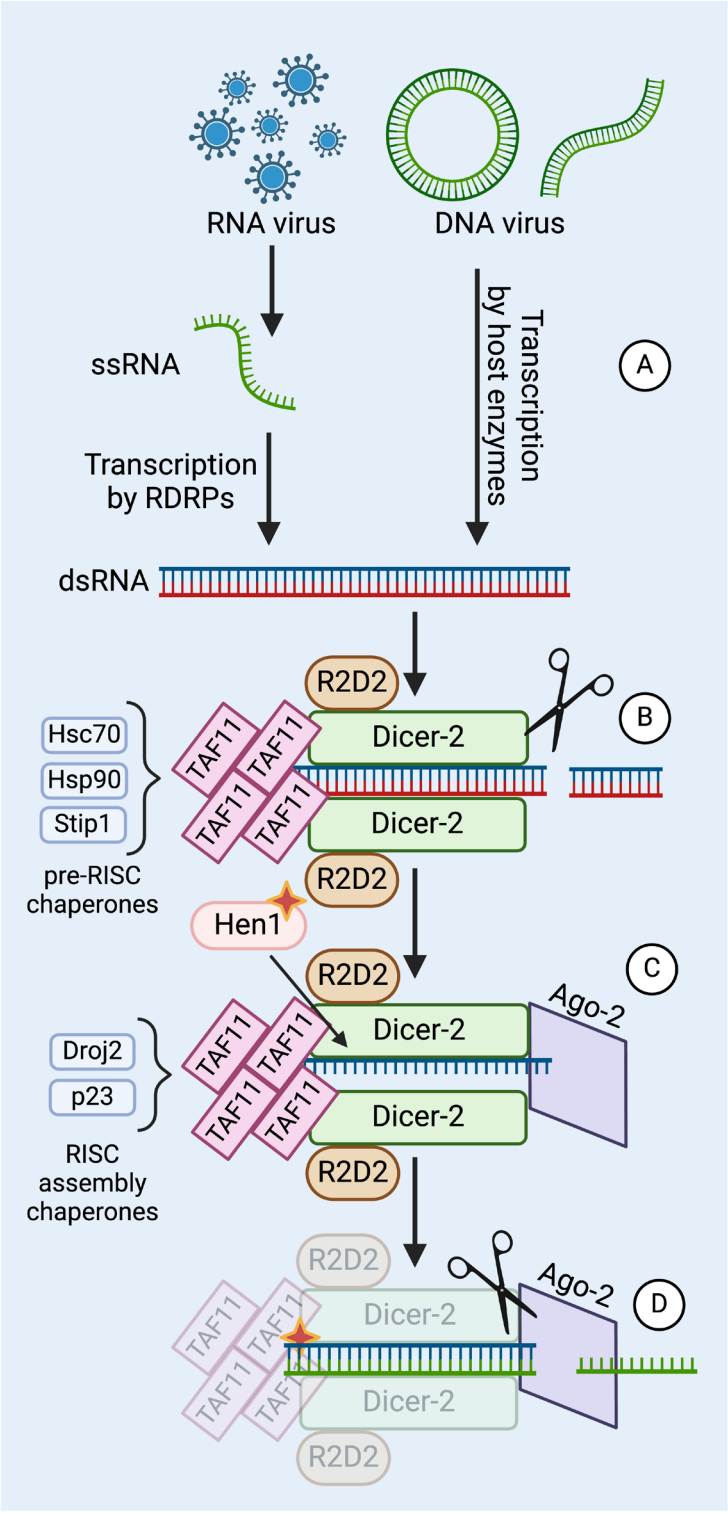
(Cao et al., 2016; Magwire et al., 2011). Similarly, a polymorphic site in the *pastrel* gene strongly affects resistance to Drosophila C virus (Magwire et al., 2012). The function of *pastrel* is unknown, but the fact that increased expression of this gene enhances protection to DCV, and that it is regulated by the cGAS-STING pathway, suggests that it could be an effector (Cao et al., 2017; Hédelin et al., 2024).

B. RNAi

Small interfering RNA in the RNA interference response (siRNAi) is a central element of *Drosophila* antiviral defense against both RNA and DNA viruses, as it is in nematodes and plants (reviewed in (Bonning and Saleh, 2021; Mussabekova et al., 2017)). In this system, the helicase RNase Dicer-2 senses long dsRNA in the cytoplasm and cleaves it into 21-nucleotide siRNA duplexes (**Figure 7**). One siRNA strand is then matured and incorporated into the RISC (RNA-induced silencing complex) composed of Dicer-2, Ago-2, and R2D2. The endonuclease Ago-2 binds the siRNA and guides the RISC to cytoplasmic RNA with a complementary sequence, inducing cleavage and degradation of the targeted RNA (Deddouche et al., 2008; Galiana-Arnoux et al., 2006; Lee et al., 2004; Liang et al., 2015; Liu et al., 2003). The siRNAi response also controls DNA viruses such as Invertebrate Iridescent Virus 6 (IIV-6) (Bronkhorst et al., 2012; Jayachandran et al., 2012; Sabin et al., 2013), which produces dsRNA by convergent transcription using host-DNA-dependent RNA polymerase II (De Faria et al., 2022).

The siRNAi response has features of both innate and adaptive immunity, as it is activated by a molecular pattern (dsRNA) and has a high degree of specificity to its target. The antiviral role of the siRNAi response is clearly demonstrated by (**i**) enhanced virus proliferation and increased susceptibility in flies deficient for RNAi components such as Dicer-2, R2D2 or Ago-2 (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Zambon et al., 2006), and (**ii**) the fact that many *Drosophila* viruses encode suppressors of RNAi (Bonning and Saleh, 2021; Bronkhorst et al., 2014; Mussabekova et al., 2017; Van Mierlo et al., 2014). Furthermore, RNAi genes are among the fastest evolving genes, likely as a consequence of a virus-host arms-race (Obbard et al., 2006). The RNAi pathway largely functions cell autonomously (Roignant et al., 2003), but exogenous dsRNA can be taken up by cells by endocytosis (Saleh et al., 2006), possibly involving the scavenger receptor SR-C1 (Ulvila et al., 2006).

Figure 7 The siRNAi pathway
The siRNAi pathway is a cell-autonomous response in *Drosophila* that targets viral RNA for cleavage and degradation in a series of steps: **A** RNA viruses produce dsRNA through viral RNA-dependent RNA polymerases (RDRPs), while DNA viruses often make use of host nuclear enzymes. This dsRNA may be released by cell lysis and taken up by neighboring cells to propagate the siRNAi response. **B** dsRNA is bound by the RISC (RNA-induced silencing complex) loading complex comprised of TAF11, R2D2 and Dicer-2. Dicer-2 cleaves the dsRNA to 21-nt siRNA with a 2-nt overhang. **C** Ago-2 is recruited to activate the RISC. The passenger strand of dsRNA (red) is ejected and degraded while the guide RNA (blue) is retained and stabilized by methylation (star) by the methyltransferase Hen1. **D** The RISC binds viral RNA complementary to the guide sequence (green), which is cleaved by Ago-2 and degraded. Several chaperone proteins (Hsc70, Hsp90, Stip1 (also known as Hop, Hsp70/Hsp90 Organizing Protein Homolog), Droj2, p23) participate in the siRNA process at different steps to enhance efficiency of the response. Adapted from (Bonning and Saleh, 2021; Mussabekova et al., 2017). Figure created with BioRender.com, CC-BY-NC-ND.


RNA virus
DNA virus
ssRNA
Transcription by host enzymes
A
Transcription by RDRPs
dsRNA
R2D2
Dicer-2
TAF11
B
Hsc70
Hsp90
Stip1
pre-RISC chaperones
Hen1
C
Ago-2
Droj2
p23
RISC assembly chaperones
D

Some evidence suggests that endogenous retrotransposons can act to form episomal chimeric DNA called vDNA containing both RNA virus and retrotransposon sequences (Goic et al., 2013; Karlikow et al., 2016; Poirier et al., 2018). These vDNAs can then produce new siRNA, amplifying and extending the duration of the RNAi-mediated antiviral response. The presence of episomal vDNA may provide long-lasting protection and could even be integrated into the genome to form endogenous viral elements with antiviral potential. Oral ingestion of DCV can provide long term protection in adult flies (Mondotte et al., 2018), and antiviral protection can be transmitted over more than five generations (Mondotte et al., 2020). These fascinating findings that provide evidence of priming and transgenerational effects in the antiviral response await confirmation by other laboratories. In another example of cross-talk between endogenous transposable elements and virus protection, a polymorphic transposable element is associated with increased resistance to Drosophila A virus (Brosh et al., 2022). Finally, one report suggested that Dicer-2 can, in addition to its RNAi function, regulate a transcriptional response that includes *Vago*, a gene associated with antiviral defense (Deddouche et al., 2008).

C. cGAS-STING

In mammals, cyclic GMP–AMP (cGAMP) synthase (cGAS) produces the cyclic dinucleotide 2′3′-cGAMP in response to cytosolic DNA to activate an antiviral interferon response through IRF3 and NF-κB (Decout et al., 2021). Recent studies have highlighted a similar role of the cGAS-like receptor STING-Relish pathway in the *Drosophila* antiviral response (**Figure 8**). Two of three cGAS-like proteins encoded in the genome, cGLR1 and cGLR2, have been identified upstream of STING. cGLR1 is activated by double-stranded RNA to produce the cyclic dinucleotide 3′2′-cGAMP, whereas cGLR2 produces a combination of 2′3′-cGAMP and 3′2′-cGAMP in response to an as-yet-unidentified stimulus. 2’3’-c-di-GMP is a potent agonist of STING signaling in *Drosophila*, including across species where other cyclic dinucleotides show variable efficacy in triggering host immunity (Cai et al., 2023; Holleufer et al., 2021; Slavik et al., 2021). STING then activates the Imd pathway transcription factor Relish through IKKβ to regulate the expression of a set of ‘STING-Regulated Genes’ (Srgs), which are potentially antiviral (Goto et al., 2018, p. 201; Hua et al., 2018; Segrist et al., 2021). Expression of *STING* itself is also down-regulated in *Relish* mutant flies, suggesting a positive feedback loop (Goto et al., 2018). Only the downstream part of the Imd pathway is involved in this response, indicating that Relish can be regulated by a ‘non-classical pathway’ to produce distinct transcriptional output (Schneider and Imler, 2021) (see The Humoral-Imd pathway, page 47 and **Box 4**, Alternative modes of Imd pathway activation, page 54).

Several observations support a role of cGLR-STING-Relish in the *Drosophila* antiviral response. First, the Imd pathway has previously been implicated in viral resistance (Avadhanula et al., 2009; Costa et al., 2009), and silencing of IKKβ or Relish increases DCV replication (Goto et al., 2018). Second, several insect viruses inhibit the antiviral response by hijacking a suppressor of the Imd pathway named Diedel (Lamiable et al., 2016b), or by producing enzymes called poxins that degrade 2′3′-cGAMP (Silva et al., 2020). The existence of poxins may explain the emergence of the alternate cyclic-nucleotide messenger 3’2’-cGAMP produced by cGLR1 and cGLR2. Third, injection of 2′3′-cGAMP reduces viral titer and susceptibility in a STING-dependent manner (Cai et al., 2020). The recent identification of this pathway raises important questions on the

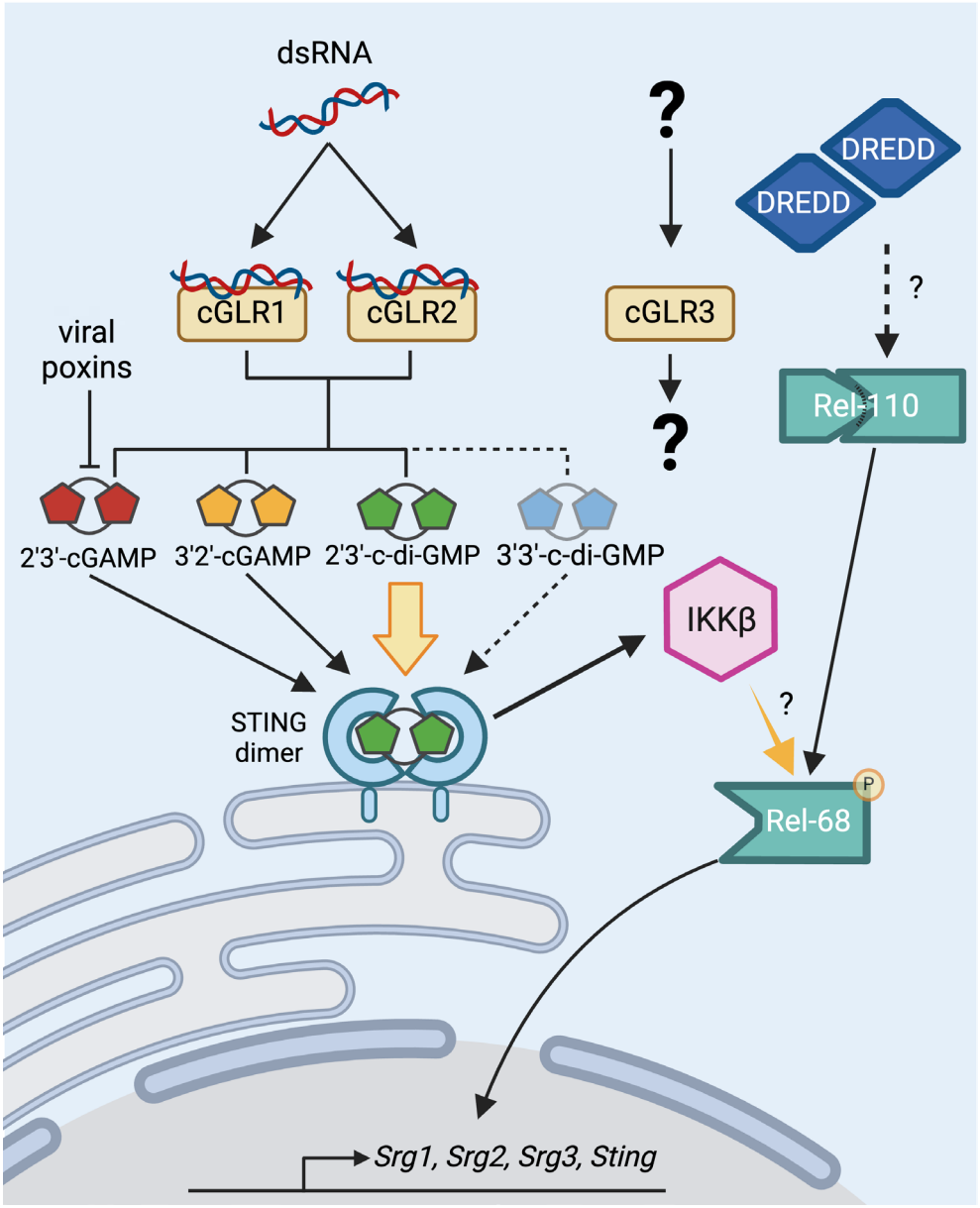


Figure 8 The cGLR-STING pathway
The *Drosophila* cGLR-STING pathway activates a distinct transcriptional response via noncanonical activation of the Imd pathway transcription factor Relish. dsRNA from viral or endogenous sources binds the cGAS-like receptors cGLR1 and cGLR2, which produce several cyclic dinucleotides (CDNs) with varying ability to activate STING. CDNs bind and activate a dimeric STING receptor embedded in the endoplasmic reticulum, leading to activation of the IKKβ kinase and the transcription factor Relish. This activation likely involves Relish phosphorylation by IKKβ and cleavage by DREDD as in Imd signaling. How this mode of Relish activation results in a distinct transcriptional response from Imd signaling is not yet well understood. Transcription of *STING-related genes* (*Srgs*) and *STING* itself act as readouts of this pathway. Adapted from (Cai et al., 2023, 2022; Slavik et al., 2021). Figure created with BioRender.com, CC-BY-NC-ND.

nature of the ligands involved in virus detection beyond dsRNA, and more importantly identification of the antiviral effectors downstream of this pathway.

STING, which is strongly enriched in the gut, might also be directly activated by di-nucleotides produced by bacteria. Despite compelling evidence supporting a role of a STING-Relish pathway in antiviral immunity, *Relish* mutants display only a modest susceptibility to systemic infection by various viruses (Ryckebusch et al., 2024). The high expression of *STING* in the gut (Leader et al., 2018) might point to a more significant role for this pathway in mucosal immunity. Moreover, STING has been implicated in lipid metabolism and autophagy, and may have broader functions beyond virus surveillance (Akhmetova et al., 2021). Nazo, a putative antiviral factor regulated by the STING pathway (Goto et al., 2018), encodes the *Drosophila* homolog of human *c19orf12* gene implicated in neurodegeneration. In *Drosophila*, Nazo has recently been shown to have a role in triglyceride lipid homeostasis (Sreejith et al., 2024). Thus, it cannot be fully excluded that STING affects defense against viruses indirectly through a role in lipid metabolism.

D. Other responses to viruses

In addition to RNAi and cGAS-STING-Relish, the JAK-STAT, Toll and p38 pathways have been linked to antiviral defense in certain contexts, although the effects of genetic background were not always considered in these early studies (Dostert et al., 2005; Ferreira et al., 2014; West and Silverman, 2018; Zambon et al., 2005). It is unclear if these pathways indeed orchestrate an antiviral response *sensu stricto* (e.g., production of antiviral effectors), or instead promote repair or tolerance to cell debris and tissue damage induced by viruses. In addition, processes such as autophagy (Shelly et al., 2009) and apoptosis (Liu et al., 2013; Nainu et al., 2017; Settles and Friesen, 2008), and molecules including heat-shock proteins (Merkling et al., 2015), Pherokines 2/3 (Sabatier et al., 2003), virus-induced RNA 1 (Vir-1) (Dostert et al., 2005), Vago (Deddouche et al., 2008) and antimicrobial peptides have been implicated in host defense to certain viruses (Feng et al., 2020; Hanson and Lemaitre, 2020). Hemocytes and the cellular response may also contribute (Lamiable et al., 2016a). Studies also suggested that Toll-7 functions as a pattern recognition receptor for viruses, triggering autophagy (Moy et al., 2014; Nakamoto et al., 2012). However, a follow-up study did not find a role for Toll-7 in antiviral autophagy (Lamiable et al., 2016a). Future studies should analyze how these processes and putative effectors are activated by viruses and how they affect and contribute to survival.

As viruses are expected to cause lysis of infected cells, tropism to different organs may cause diverse and specific pathologies. Sigma virus infects widely, but especially targets the cephalic and thoracic ganglia and induces paralysis after exposure to CO_2 (Longdon et al., 2012; Tsai et al., 2008), while systemic DCV infection causes intestinal obstruction by invading the smooth muscles surrounding the crop (Chtarbanova et al., 2014). Flock house virus (FHV) is a cardiotropic virus, and genes such as dSUR that control viremia in the heart are protective (Eleftherianos et al., 2011). Moreover, cryptic infections such as those caused by Nora virus can affect phenotypes such as longevity in certain genetic backgrounds, which may act as a confounding factor in immune studies (Habayeb, 2006; Hanson and Lemaitre, 2023).

4

The systemic antimicrobial response

Immunity in *Drosophila* has mostly been investigated by introducing microbes directly into the body cavity using either a needle dipped in concentrated bacteria/fungi or injection of a dilute bacterial solution (Neyen et al., 2014; Troha and Buchon, 2019). This mode of infection triggers a potent immune reaction referred to as the systemic response (Buchon et al., 2014; Ferrandon et al., 2007) (**Figure 9**). This response, which will be the focus of the next six chapters, consists of (**i**) the production of immune effectors by the fat body and hemocytes through the Toll and Imd pathways (Sections **4** and **5**), (**ii**) the melanization reaction (Sections **6** and **7**), (**iii**) wound healing and disease tolerance[1] mechanisms regulated by the JAK-STAT, MAPK and JNK pathways (Sections **7** and **8**), and (**iv**) phagocytosis by hemocytes (i.e. insect blood cells) (Section **9**). A subset of these modules is activated depending on the characteristics of the infecting microbe, as exemplified by early studies showing that the Toll pathway is primarily activated by Gram-positive bacteria and fungi, while the Imd pathway responds to Gram-negative bacteria (Lemaitre et al., 1997, 1996; Rutschmann et al., 2002) (see **Box 5**). These reactions are supported by metabolic reprogramming to fuel protein production for the immune response (Sections **5C**, **9E**) and physiological changes that mitigate tissue damage caused by both host immune effectors and pathogens. Both resistance and disease tolerance are intricately linked and cooperate to promote host survival (Galenza and Foley, 2019). The systemic response is by far the best studied immune reaction in *Drosophila*, and its study has strongly shaped our view of the insect immune system. We should however be aware that many of these processes may be unique to systemic immunity, and that processes in other tissues such as epithelia involving the same molecules may not function the same way.

A. The humoral Toll pathway

In *Drosophila*, the Toll pathway is activated by microbial cell wall components (fungal glucans and peptidoglycan), microbial proteases (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007; Royet and Dziarski, 2007), and other mechanisms that are less well-characterized. Microbial recognition occurs either through direct detection of microbes by secreted pattern recognition receptors in the hemolymph or by sensing perturbations in this compartment. This leads to activation of complex cascades of

1 Tolerance has multiple meanings in immunology, but we can distinguish disease tolerance or resilience as the capacity to endure infection, and immune/self-tolerance as the ability of the immune system to avoid damaging self-tissues (see Multiple ways to resist infection, page 18).

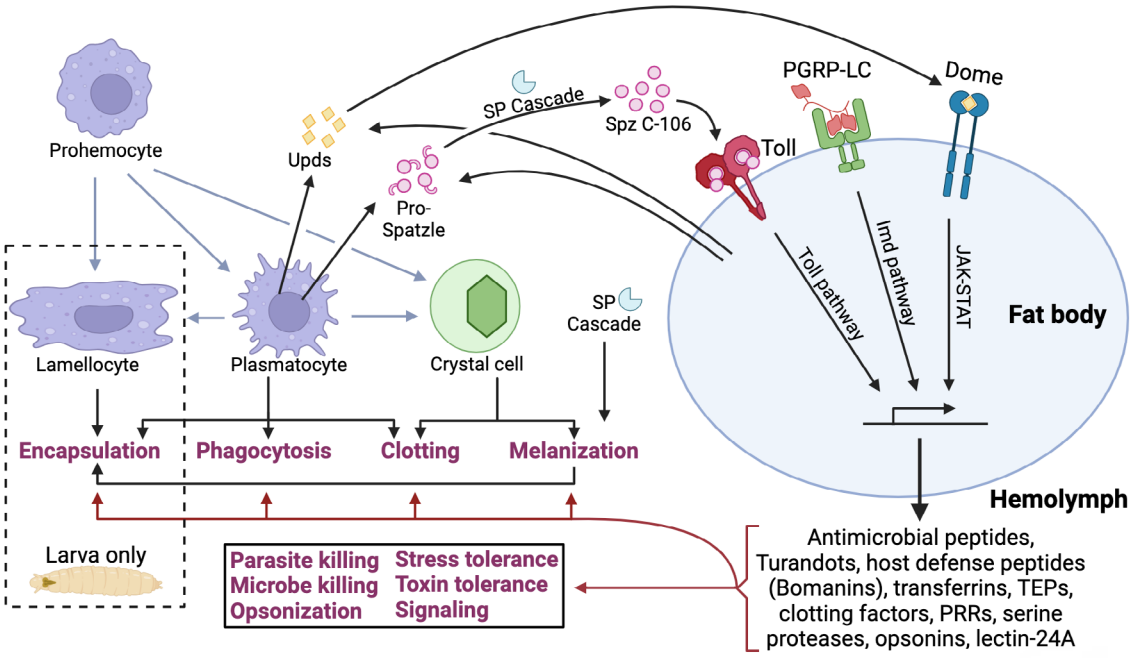


Figure 9 The systemic immune response
Schematic overview of *Drosophila* systemic immune modules. Detection of microbial pathogens elicits an array of interconnected and synergistic defense modules in immune-responsive tissues, including the fat body which is an analogue of mammalian liver (Arrese and Soulages, 2010), and in hemocytes, the *Drosophila* blood cells. Lamellocytes contribute to wasp encapsulation and are only found in larvae (Lanot et al., 2000). SP, serine protease. Created with BioRender.com, CC-BY-NC-ND.

serine proteases (SPs) that bifurcate to regulate both cleavage of the neurotrophin-like Toll ligand Spatzle (Spz), which initiates the intracellular Toll pathway *sensu stricto*, and the melanization reaction involving phenoloxidases (POs). This extracellular cascade is referred to here as the Toll-PO SP cascade, as it regulates both phenoloxidase cleavage and ligand-mediated activation of the Toll receptor. The Toll pathway regulates expression of hundreds of genes (De Gregorio et al., 2002b). These include genes that encode small effector peptides such as the Bomanins and Drosomycin, but also many proteins involved in melanization (e.g., serine proteases, serpins), clotting (Fondue), and nutritional immunity (transferrin). *Toll*-deficient flies are viable but display marked susceptibility to infections by Gram-positive bacteria and fungi as well as other pathogens (Lemaitre et al., 1996; Rutschmann et al., 2002; Ryckebusch et al., 2024). The Toll pathway also has important roles in hematopoiesis and cellular responses (Louradour et al., 2017; Qiu et al., 1998). In larvae, Toll pathway activation in the fat body is sufficient to activate lamellocyte differentiation in peripheral compartments (see Systemic Immunity: Cellular response, page 91), revealing a broad impact on the immune response (Schmid et al., 2014).

i) Recognition and Toll-PO SP signaling

The Toll pathway can be activated by a broad range of exogenous and endogenous stimuli including fungi, Gram-positive and Gram-negative bacteria, microbial proteases, and reactive oxygen species (ROS) (**Figure 10**). Two secreted pattern recognition receptors (PRRs), GNBP3 and PGRP-SA, sense fungal glucan and bacterial peptidoglycan (PGN) respectively, and can initiate this cascade (Gobert et al., 2003; Gottar et al., 2006; Leulier et al., 2003; Michel et al., 2001; Mishima et al., 2009; Pili-Floury et al., 2004). Interestingly, PGRP-SA and GNBP3 are phylogenetically derived from muramidase and glucanase enzymes, respectively, and likely evolved as PRRs by losing catalytic activity while retaining binding affinity for microbial cell wall components (Hughes, 2012).

Genetic studies in *Drosophila* and biochemical analysis in other insects have shown that binding of GNBP3 to fungal β-glucan leads to activation of an apical serine protease, ModSP, that initiates the Toll-PO SP cascade (Buchon et al., 2009c; Takahashi et al., 2015; Wang and Jiang, 2007). A recent study also found a role for the highly inducible GNBP-like 3 protein in preventing suppression of the Toll pathway by the entomopathogenic fungus *Metarhizium robertsii,* which uses the effector protein Tge1 to block the *Drosophila* β-glucan receptor GNBP3 (Lu et al., 2024). ModSP is also activated by PGRP-SA, which can sense lysine-type peptidoglycans from Gram-positive bacteria but also DAP-type peptidoglycan from Gram-negative bacteria and bacilli (Filipe et al., 2005; Leulier et al., 2003). Although PGRP-SA seems to preferentially bind lysine-type peptidoglycan over DAP-type peptidoglycan, more recent studies suggest that peptidoglycan quantity and accessibility play an important role in responsiveness of the Toll pathway primarily to Gram-positive bacteria rather than Gram-negative bacteria (Atilano et al., 2011; Leulier et al., 2003; Vaz et al., 2019). Gram-negative bacteria possess a thin layer of peptidoglycan hidden under a layer of lipopolysaccharide (LPS), making it inaccessible to secreted PRRs. In contrast, Gram-positive species expose an external thick layer of peptidoglycan which is accessible to secreted PRRs such as PGRP-SA when not covered by modifications such as teichoic acid (**Box 2**). Thus PGRP-SA may only be able bind to peptidoglycan of Gram-positive bacteria when it is accessible, for instance at the septum during bacterial division (Atilano et al., 2011). As described in other insects (Kim et al., 2008; Tabuchi et al., 2010; Wang et al., 2022), binding of at least two PGRP-SA molecules to polymeric peptidoglycan recruits GNBP1, which functions as an adaptor to increase local ModSP concentration enough to undergo autoactivation (Buchon et al., 2009c; Filipe et al., 2005; Gobert et al., 2003; Park et al., 2007; Pili-Floury et al., 2004; Westlake et al., 2024). The precise localization of ModSP and the remaining Toll-PO cascade SPs during activation has not yet been established, and may occur at the surface of microbes, on lipid vesicles, or freely in the hemolymph.

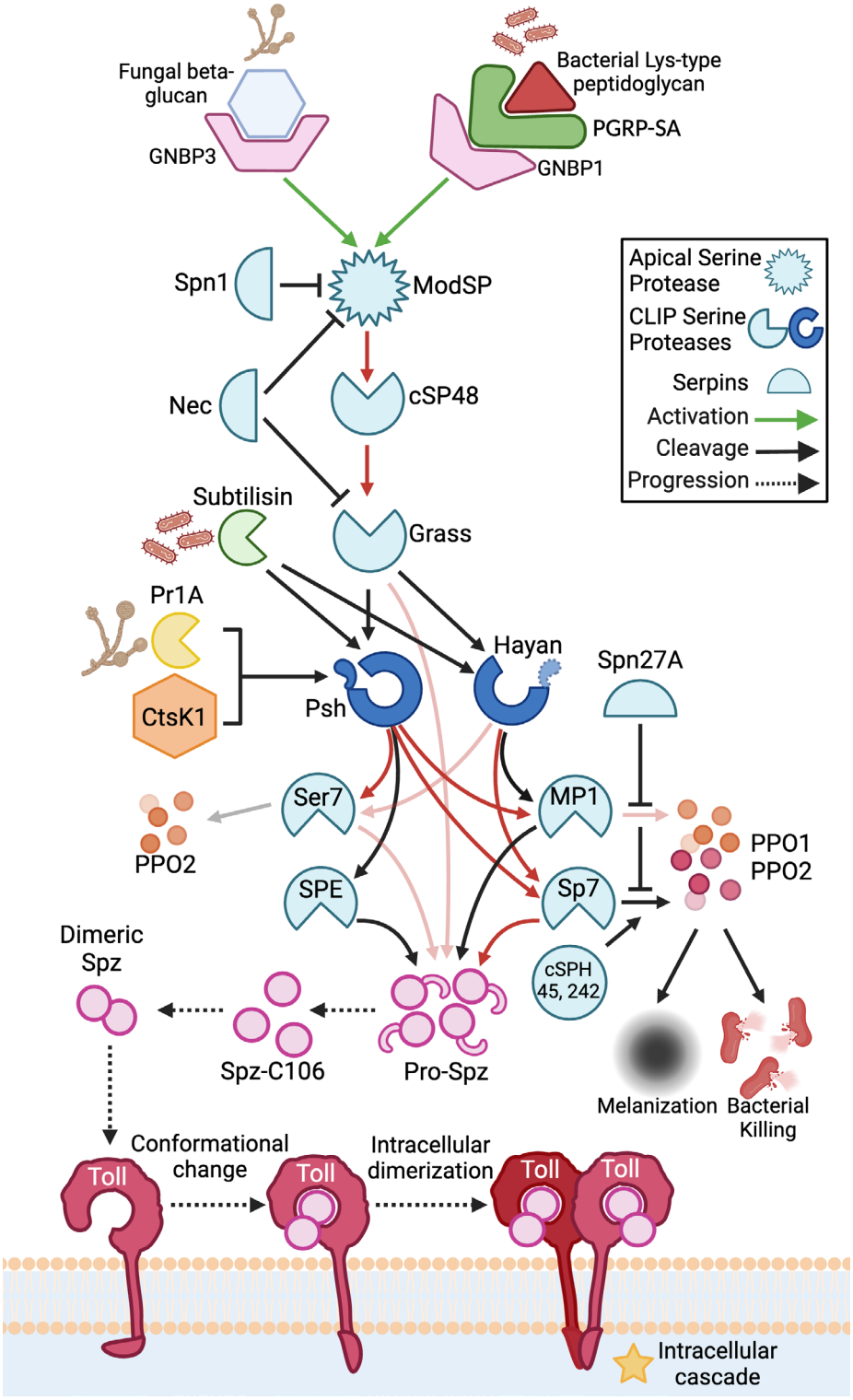
ModSP cleavage triggers sequential activation of several serine proteases that shape the signal activating the Toll pathway and melanization response (Buchon et al., 2009c; Chamy et al., 2008; Dudzic et al., 2019) (**Figure 10**). Many of these SPs are CLIP domain[2] serine proteases, a large gene family of proteases found in insects and mollusks

2 The CLIP domain is a protein domain found in the N-terminal part of some serine proteases involved in sequential proteolytic cascades, such as the one regulating Toll pathway during early embryogenesis or immunity. Among the 147 SPs and 57 SPHs (Serine Protease Homologs with no catalytic activity) identified in *Drosophila melanogaster*, 28 SPs and 14 SPHs contain a regulatory CLIP domain (Jang et al., 2008; Veillard et al., 2016).

that are secreted as zymogens and activated upon cleavage by an upstream serine protease (Jang et al., 2008; Piao et al., 2005; Veillard et al., 2016). An extensive biochemical analysis recently showed that ModSP cleaves CLIP domain Serine Protease 48 (cSP48) to activate Grass, which then cleaves Persephone (Psh) and Hayan (Shan et al., 2023). These are two partially redundant Toll pathway regulators that cleave both (**i**) the Spatzle Processing Enzyme (SPE) to activate the Toll ligand Spatzle and the intracellular Toll cascade resulting in gene expression, and (**ii**) Sp7 and Ser7 to activate melanization through PPO1/PPO2 (Chamy et al., 2008; Dudzic et al., 2019; Jang et al., 2006; Kambris et al., 2006; Ligoxygakis et al., 2002b; Shan et al., 2023). Skanda (CG15046), a serine protease homolog with no catalytic activity encoded in the Psh-Hayan gene cluster, also appears to function with Hayan and Persephone in the activation of Spatzle (B. Lemaitre, unpublished). A loss-of-function mutation in *SPE* only partially suppresses Toll pathway activation by *M. luteus* in larvae, suggesting the existence of another Spatzle-processing serine protease (Yamamoto-Hino and Goto, 2016). Consistent with this, at least MP1 and Sp7 are also capable of cleaving Spatzle *in vitro* (Shan et al., 2023). Furthermore, N-glycosylation of the Spatzle precursor modulates Toll pathway activation in response to infection (Yamamoto-Hino et al., 2015) and N-glycosylation is involved in immune activation in other contexts (see Encapsulation, page 100 and **12A**, Autoimmunity, page 127).

In addition to sensing microbial cell wall components through the pattern recognition receptors PGRP-SA and GNBP3, the Toll pathway can be directly activated by microbial proteases at the level of Persephone (Chamy et al., 2008; Gottar et al., 2006; Ming et al., 2014). Persephone (and likely Hayan) has a 'bait region' that can be cleaved by microbial proteases such as subtilisin of *Baccillus subtilis*, or the cuticle-degrading protease PR1 from entomopathogenic fungi *B. bassiana* and *M. anisopliae* (Issa et al., 2018; Nakano et al., 2023). PR1 does not directly activate Persephone, which contains an unusual histidine residue that requires specific cleavage by the endogenous cathepsin CtsK1 (also called 26-39p) after initial processing of the Persephone bait by PR1. In con-

Figure 10 The Toll-PO SP cascade
Schematic representation of the serine protease cascade leading to Toll and phenoloxidase activation. Arrows in red indicate cleavage events that have been demonstrated biochemically but have not yet been shown genetically (Shan et al., 2023). Faded arrows indicate minor associations. PGRP-SA acts as a pattern recognition receptor for peptidoglycan and requires GNBP1 as a cofactor, while GNBP3 binds fungal glucans. Both of these receptors activate the serine protease cascade through the apical serine protease ModSP, which auto-activates upon clustering. Fungal PR1A can activate Toll by cleaving the bait region of Persephone, which is then further matured by the endogenous cathepsin CtsK1 (29-36p, (Issa et al., 2018)). Similar activation of Hayan has not yet been demonstrated, but the bait region is conserved in one Hayan isoform (Dudzic et al., 2019). *Bacillus* subtilisin can directly cleave and activate Persephone, and likely also Hayan. Terminal serine proteases maturate Spatzle leading to Toll activation (largely SPE, but recent results indicate that other proteases also participate (Shan et al., 2023)), and PPO1/2 leading to melanization. The non-catalytic serine protease homologs cSPH45 and cSPH242 act as cofactors in PPO1/2 activation by Sp7 (Jin et al., 2023). Following cleavage, mature Spatzle (Spz-C106) forms dimers which bind to and produce an intracellular conformational change of the Toll receptor. This allows dimerization of the Toll receptor in a 2(2 Spz : 1 Toll) complex, which initiates intracellular signaling. Many steps involved in the Toll-PO SP cascade are not fully established. Figure created with BioRender.com, CC-BY-NC-ND.


Fungal beta-glucan
GNBP3
Bacterial Lys-type peptidoglycan
PGRP-SA
GNBP1
Spn1
ModSP
Nec
cSP48
Subtilisin
Grass
Pr1A
CtsK1
Psh
Hayan
Spn27A
Ser7
MP1
PPO2
PPO1
PPO2
SPE
Sp7
cSPH 45, 242
Dimeric Spz
Spz-C106
Pro-Spz
Melanization
Bacterial Killing
Conformational change
Intracellular dimerization
Toll
Toll
Toll
Toll
Intracellular cascade
Apical Serine Protease
CLIP Serine Proteases
Serpins
Activation
Cleavage
Progression

trast, processing of Persephone by the upstream SP Grass or by subtilisin is independent of CtsK1. As proteases can act as virulence factors that pathogens employ to infect insects, the activation of the Toll and melanization pathways by microbial proteases is similar to effector-triggered immunity (ETI) which activates plant innate immunity in response to virulence factors (Liegeois and Ferrandon, 2022; Pradeu et al., 2024).

The Toll-PO SP cascade is tightly regulated by serine protease inhibitors (serpins), which block serine proteases via a suicide mechanism[3] (Reichhart, 2005). Serpins that negatively regulate the Toll-PO SP cascade include Necrotic (Nec), Spn1 (Fullaondo et al., 2011), Spn27A (De Gregorio et al., 2002a; Ligoxygakis et al., 2002c), Spn28D (Scherfer et al., 2008) and likely Spn5 (Ahmad et al., 2009). Mutations in these lead to constitutive activation of Toll (Spn1), phenoloxidases (Spn27A, Spn42a) or both (Nec, Spn5). Necrotic was initially thought to be a direct inhibitor of Persephone, as mutation of *persephone* suppresses the constitutive activation of Toll observed in *necrotic* mutants (Ligoxygakis et al., 2002b). However, a recent biochemical analysis reveals that Necrotic inhibits both ModSP and Grass upstream of Persephone (Shan et al., 2023). Despite progress, we are still far from understanding the full complexity of the Toll-PO SP cascade and its regulation: dozens of genes encoding SPs and serpins, some of which are upregulated upon infection, have not yet been functionally characterized. Furthermore, some protein associations that have been demonstrated biochemically have not yet been validated genetically (see **Supplementary list 1**).

Activation of the Toll pathway by endogenous stimuli is less well characterized, although reactive oxygen species (ROS) have been shown to induce Toll activity to a certain extent. Increases in ROS induce maturation of Persephone and cleavage of Spatzle by MP1, another CLIP serine protease (Nakano et al., 2023). Toll activation by ROS is also observed in other contexts: in response to injury (Chakrabarti and Visweswariah, 2020), apoptosis and stimulation of lamellocyte production upon wasp parasitization in larvae (Louradour et al., 2017) (see Encapsulation, page 100). Thus, the Toll pathway broadly surveys the hemolymph compartment by sensing not only microbes but also disrupted homeostasis.

3 Unlike most small protease inhibitors (e.g., Kunitz-type inhibitors) that disrupt target proteases by a competitive (lock-and-key) mechanism, each serpin irreversibly disrupts the structure of a single target protease and is consumed in the process. Serpins contain a Reactive Center Loop (RCL) domain that is cleaved by the targeted SP, leading to a covalent ester bond between the SP and the serpin that distorts the active site (catalytic triad) of the SP and inhibits catalysis (Huntington, 2011; Reichhart, 2005).

Box 2 Structural composition of bacterial cell walls

The cell walls of bacteria are composed of many complex polymers that are specific to prokaryotes, and are used by eukaryotic immune systems to detect invaders. Peptidoglycan is an essential glucopeptidic polymer restricted to the cell wall of both Gram-negative and Gram-positive bacteria, consisting of long glycan chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic (MurNAc) acid residues that are cross-linked to each other by short peptide bridges (Garde et al., 2021; Mengin-Lecreulx and Lemaitre, 2005; Vollmer et al., 2008). Gram-negative bacteria have a thin layer of peptidoglycan trapped between an outer lipopolysaccharide-coated lipid bilayer and the primary cell membrane. Lipopolysaccharide (LPS) is highly immunogenic in mammals, but *Drosophila* appears to lack receptors for this molecule (Kaneko et al., 2004; Leulier et al., 2003). Previous results showing activation of the Imd pathway by LPS were subsequently shown to be linked to the presence of contaminating peptidoglycan (notably in the LPS provided by Sigma™). Gram-positive bacteria have a thick layer of peptidoglycan that is sometimes covered by teichoic acid. The presence of teichoic acid can impede recognition of bacteria by secreted PGRPs (Atilano et al., 2017, 2011; Attieh et al., 2019; Tabuchi et al., 2010). Peptidoglycan from most Gram-positive bacteria differs from Gram-negative peptidoglycan by the replacement of meso-diaminopimelic acid (DAP) with lysine at the third position in the stem peptide chain (**Figure Box 2**). Some groups of Gram-positive bacteria such as *Bacillus* species that include many insect pathogens and symbionts (*Bacillus thuringiensis, Lactobacillus*) produce DAP-type peptidoglycans, but these are often amidated and have reduced affinity for Imd pathway pattern recognition receptors. The terminal monomer of DAP-type peptidoglycan of Gram-negative bacteria is called tracheal cytotoxin (TCT), a molecule that strongly activates the Imd pathway. Research has demonstrated that the fly immune system senses polymeric and monomeric (notably TCT) peptidoglycans through a number of PGRP receptors to initiate immune signaling cascades (Aggarwal and Silverman, 2007; Kaneko et al., 2004; Leulier et al., 2003; Lim et al., 2006; Royet et al., 2005; Stenbak et al., 2004). To date, there is no formal evidence that the major immune elicitors that activate vertebrate immunity (such as LPS, flagellin, teichoic acid, lipoteichoic acid, or prokaryotic DNA/unmethylated CpG) can activate the *Drosophila* Toll or Imd pathways, although they may be involved in other immune reactions (e.g., phagocytosis, behavioral immunity).

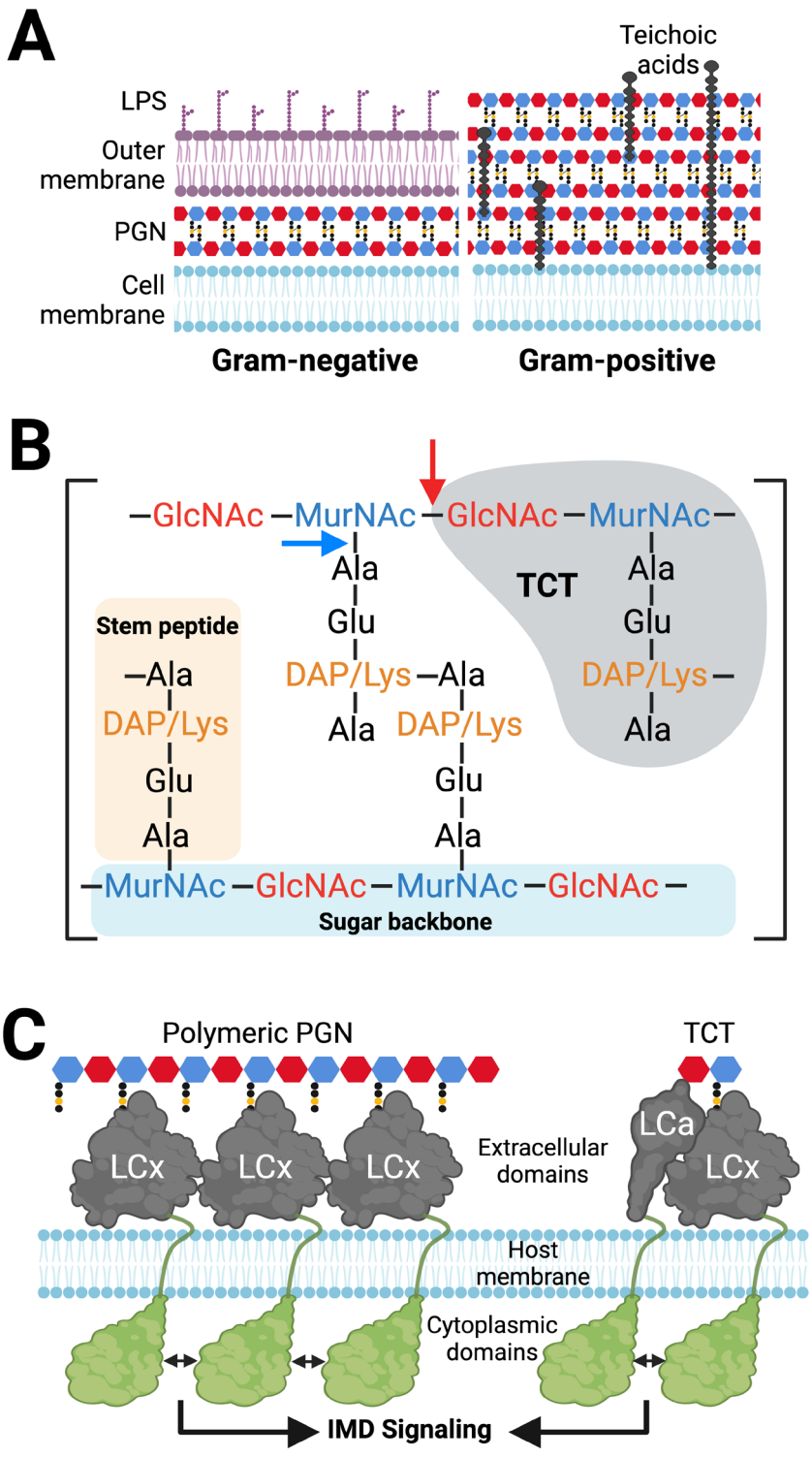

A
Teichoic acids
LPS
Outer membrane
PGN
Cell membrane
Gram-negative
Gram-positive
B
–GlcNAc–MurNAc–GlcNAc–MurNAc–
Ala
Glu
DAP/Lys
Ala
TCT
Stem peptide
–Ala
DAP/Lys
Glu
Ala
–MurNAc–GlcNAc–MurNAc–GlcNAc–
Sugar backbone
C
Polymeric PGN
TCT
LCx
LCx
LCx
LCa
LCx
Extracellular domains
Host membrane
Cytoplasmic domains
IMD Signaling

Figure Box 2 Structure, degradation and immune recognition of peptidoglycan

A Structure of Gram-positive and -negative cell walls. Gram-negative cell walls (**left**) comprise a single layer of peptidoglycan (**PGN**) trapped beneath a secondary membrane decorated with lipopolysaccharide (**LPS**). Gram-negative bacteria release small amounts of peptidoglycan when dividing that can be recognized by the pattern recognition receptors PGRP-LC (transmembrane), PGRP-LE (intracellular) and PGRP-SD (secreted) upstream of the Imd pathway (Iatsenko et al., 2016; Kaneko et al., 2004; Leone et al., 2008; Lim et al., 2006; Neyen et al., 2012). Gram-positive cell walls (**right**) comprise many layers of peptidoglycan but lack a secondary membrane. However, Gram-positive bacteria may be decorated with **teichoic acids**, which are poly-phosphoglycerol or -phosphoribitol polymers covalently linked by phosphodiester bonds to (**i**) the C6 of the MurNAc in peptidoglycan, or (**ii**) lipids in the plasma membrane. These modifications can interfere with peptidoglycan recognition by immune receptors.

B Schematic of the structure of peptidoglycan. Peptidoglycan, a major component of bacterial cell walls, comprises a **sugar backbone** of repeating disaccharide units of **GlcNAc** (N-acetylglucosamine) and **MurNAc** (N-acetylmuramic acid) joined by β-1,4-glycosidic linkages (**red arrow**). MurNAc bears covalently linked **stem peptides** (also called peptide bridges or tetrapeptides, as these are often composed of L-Ala-D-Glu-L-Lys/*meso*DAP-D-Ala, although these can be found also as di, tri or pentapeptides) that are cross-linked to the stem peptides of a second sugar backbone. Stem peptides may incorporate ***meso*DAP** (*meso*-diaminopimelic acid, typically in Gram-negative peptidoglycan) or L-**lysine** (typically in Gram-positive peptidoglycan) at the third position. In some bacteria such as *Bacillus* species, DAP is amidated or otherwise modified, which appears to reduce recognition by immune receptors (Vaz et al., 2019). Enzymes can cleave peptidoglycan in ways that either increase or reduce immunogenicity. SltY cleaves β-1,4-glycosidic linkages (**red arrow**) to produce peptidoglycan monomers called **TCT** (tracheal cytotoxin), which are highly diffusible and a strong elicitor of the Imd pathway (Kaneko et al., 2004; Neyen et al., 2012; Stenbak et al., 2004; Zaidman-Rémy et al., 2006). The immunogenicity of TCT is dependent on an internal 1,6-anhydro bond in MurNAc. In *E. coli* peptidoglycan, this bond occurs naturally only at the terminal end of each peptidoglycan chain, and therefore constitutes only ~5% of all GlcNAc-MurNAc bonds. Although muramidases such as lysozyme cleave the same bond as lytic transglycosylases such as SltY, muramidases fail to generate an 1,6-anhydro bond upon cleavage, and thus generate less immunogenic peptidoglycan monomers. Structural characterization of several PGRPs has revealed features associated with specificity to *meso*DAP-versus L-lysine-type peptidoglycan, and presence or absence of enzymatic amidase activity (Chang et al., 2006, 2005, 2004; Kim et al., 2003; Leone et al., 2008; Lim et al., 2006). Amidase PGRPs, which are typically N-acetylmuramoyl-L-alanine amidases, remove stem peptides from the sugar backbone (**blue arrow**) to reduce immunogenicity of peptidoglycan, and may be specific to *meso*DAP or L-lysine-type peptidoglycans (Gelius et al., 2003; Kim et al., 2003; Mellroth et al., 2003; Mellroth and Steiner, 2006; Orlans et al., 2021; Zaidman-Rémy et al., 2011, 2006). PGRP-LB reduces immunogenicity of both *meso*DAP-type polymeric peptidoglycan and monomeric TCT.

C Peptidoglycan binding to PGRPs. PGRP-LCx is thought to cluster as a result of binding polymeric *meso*DAP-type peptidoglycan, triggering association of PGRP-LCx cytoplasmic domains and initiating Imd signaling (**left**). PGRP-SA clusters on L-lysine-type peptidoglycan and activates Toll in a similar way. PGRP-LCx or -LCa alone do not have high affinity for TCT but form a heterodimeric complex that stabilizes interaction with TCT and initiates signaling (**right**). Adapted from Lim et al., 2006. Created with BioRender.com, CC-BY-NC-ND.

ii) Toll signaling

Drosophila Toll is activated by the neurotrophin-like protein Spatzle (Spz) (Lemaitre et al., 1996; Schneider et al., 1994; Tauszig et al., 2000; Valanne et al., 2022) (**Figure 11**). A dimer composed of two mature Spatzle proteins binds one Toll receptor, causing a conformational change that allows dimerization of intracellular Toll domains (DeLotto and DeLotto, 1998; Hashimoto et al., 1988; Hu et al., 2004; Lemaitre et al., 1996; Parthier et al., 2014; Weber et al., 2003). While Spatzle is the main ligand activating Toll upon septic injury in adults, the constitutively active Spatzle-like protein Spatzle-5 (Spz5) may also play a role in Toll pathway activation in some contexts (Nonaka et al., 2018). Upon dimerization, the intracellular TIR domain of Toll recruits the adaptor MyD88 (Tauszig-Delamasure et al., 2002). MyD88 localizes to the cell membrane by binding phosphatidylinositol 4,5-bisphosphate (PIP2)-rich membrane regions, and membrane localization is promoted by ubiquitination of MyD88 by Sherpa (Kanoh et al., 2015; Marek and Kagan, 2012). MyD88 recruits the adaptor Tube and the Pelle kinase through homotypic interactions of their death domains, leading to activation of Pelle (Galindo et al., 1995; Grosshans et al., 1999, 1994). Pelle phosphorylates Cactus, an IkB homolog, triggering its rapid proteasomal degradation (Belvin et al., 1995; Daigneault et al., 2013; Geisler et al., 1992; Nicolas et al., 1998). Degradation of Cactus releases the NF-κB transcription factors Dif and Dorsal, which then translocate into the nucleus and activate the Toll transcriptional program (Ip et al., 1993; Lemaitre et al., 1995a; Reichhart et al., 1993; Valanne et al., 2022). Dif (Dorsal-related immunity factor) and Dorsal are encoded by two clustered genes arising from a recent duplication that have overlapping but distinct functions that have not yet been fully clarified[4]. However, Dif plays a more important role in adult host defense, while only Dorsal is involved in embryonic dorsoventral patterning (Gross et al., 1996; Lemaitre et al., 1995a; Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000a). Note however that widely used Dif mutants may display a weaker phenotype than initially published (Le Bourg, 2011).

The intracellular part of the Toll pathway is very similar to the Toll-like receptor (TLR) NF-κB cascade that regulates innate immunity in mammals, emphasizing the conserved role of this pathway in innate immunity (Gay and Keith, 1991; Lemaitre et al., 1996; Schneider et al., 1991). There are however three notable differences between Toll signaling in *Drosophila* and TLR-NF-κB signaling in mammals: (**i**) TLRs are pattern recognition receptors that directly sense microbial molecules ('MAMPs') (Brennan and Gilmore, 2018; Leulier and Lemaitre, 2008) whereas in *Drosophila* the endogenous Spatzle proteins act as Toll receptor ligands; (**ii**) *Drosophila* Tube has no homolog in mammals, and is considered a degenerate copy of the Pelle/IRAK kinase that serves as a scaffold for Pelle (Sun et al., 2004); and (**iii**) the *Drosophila* IRAK homolog Pelle directly phosphorylates the IkB homolog Cactus, while in mammals IkB is phosphorylated by the IκB-kinase (IKK) (Daigneault et al., 2013).

The Toll pathway has many other functions beyond immunity in *Drosophila*, including regulation of early embryonic dorsoventral patterning, muscle attachment, and wound healing (Belvin and Anderson, 1996; Capilla et al., 2017; Green et al., 2016;

4 Both Dorsal and Dif express a B isoform that contains the Rel homology domain (RHD) but lacks the nuclear localization domain. These B isoforms are conserved in other species. The function of the B isoform is poorly defined, but Dorsal B seems to play a major role at neuromuscular junctions (Gross et al., 1999; Zhou et al., 2015).

Halfon and Keshishian, 1998). Although there are nine Toll genes in the *Drosophila* genome, only Toll-1 (Toll) is confidently implicated in *Drosophila* immunity (but see Akhouayri et al., 2011; Bettencourt et al., 2004; Lamiable et al., 2016a; Nakamoto et al., 2012; Narbonne-Reveau et al., 2011; Ooi et al., 2002; Tauszig et al., 2000) for proposed involvement of Tolls 8, 7 and 9 in immunity). Other *Drosophila* Toll homologs have been implicated primarily in development and brain plasticity (Anthoney et al., 2018; Li et al., 2020b; Li and Hidalgo, 2021; Lindsay and Wasserman, 2014; McIlroy et al., 2013; Paré et al., 2014; Ward et al., 2015).

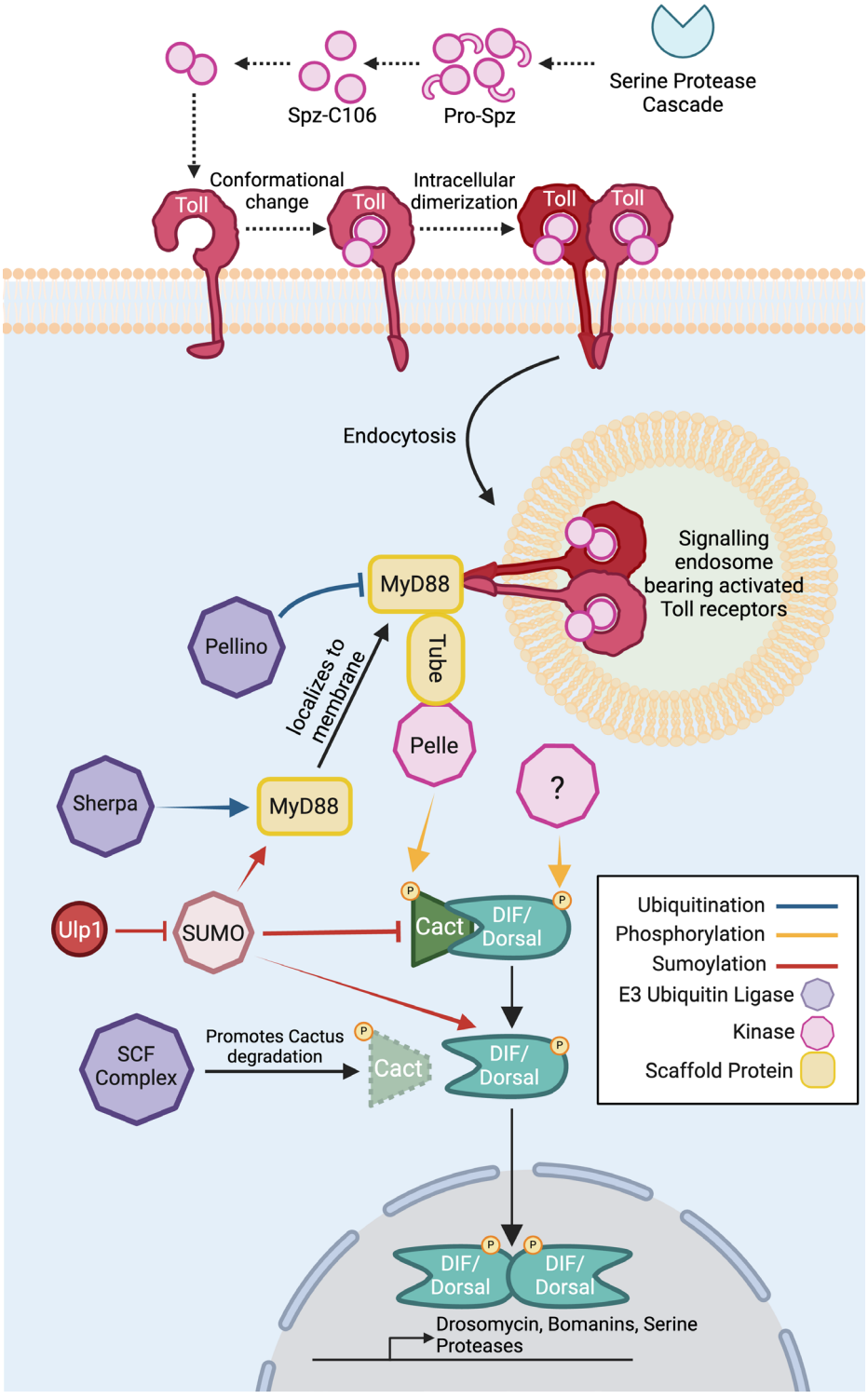
Mutations in genes encoding the canonical components of the Toll pathway (Spz, Toll, MyD88, Tube, Pelle, Dif/Dorsal) in flies cause high susceptibility to Gram-positive bacteria and fungi, as well as many other microbes including some Gram-negative bacteria and viruses (Ferreira et al., 2014; Lau et al., 2003; Lemaitre et al., 1996; Zambon et al., 2005). Over the years, many other proteins have been identified that are involved directly or indirectly in Toll pathway activation. This includes proteins that mediate endocytosis of the Toll receptor, a process that is essential for activation of this signal cascade (Huang et al., 2010; Lund et al., 2010) (see **Box 6**, Immunity and the endocytic machinery, page 99); several E3 ubiquitin ligases; and sumoylation enzymes, which can have both positive and negative regulatory effects at different levels of the Toll pathway (**Figure 11**, see **Supplementary list 1**). Finally, new evidence suggests that the intracellular Toll pathway may be independently activated by cGMP produced by the receptor-type guanylate cyclase Gyc76 in response to bacterial infection (Iwashita et al., 2020; Kanoh et al., 2021).

B. The humoral Imd pathway

The Imd pathway is activated by DAP-type peptidoglycans produced by Gram-negative bacteria and a subset of Gram-positive bacteria (*e.g., Bacillus sp.*) (Aggarwal and Silverman, 2008; Kleerebezem et al., 2010; Lemaitre et al., 1995b; Lemaitre and Hoffmann, 2007; Mengin-Lecreulx and Lemaitre, 2005; Royet et al., 2005). Binding of peptidoglycan to receptors of the PGRP family (PGRP-LC, PGRP-LE) initiates an intracellular signaling cascade whose components share homology with both the TNFα-Receptor and TLR pathways of mammals. This ultimately results in cleavage and phosphorylation of Relish, an NF-κB factor that includes a self-inhibiting ankyrin domain (Hedengren et al., 1999; Stoven et al., 2000). The Imd pathway regulates the expression of many genes encoding effectors such as antibacterial peptides, serine proteases, and transferrin (De Gregorio et al., 2002b). Imd-deficient flies are viable but display acute susceptibility to Gram-negative bacterial infection (Lemaitre et al., 1995b; Leulier et al., 2000; Ryckebusch et al., 2024). While the Imd pathway was initially described for its regulation of the antibacterial response, it has now been implicated in many other processes such as apoptosis, cell competition, delamination, regulation of digestive enzymes, and synaptic plasticity (Combe et al., 2014; Georgel et al., 2001; Harris et al., 2015; Meyer et al., 2014; Zhai et al., 2018a, 2018b).

i) Imd recognition

While PRRs of the Toll pathway can bind bacteria and fungi, PRRs of the Imd pathway are activated by peptidoglycan fragments that are released from below the protective LPS


Serine Protease Cascade
Pro-Spz
Spz-C106
Conformational change
Intracellular dimerization
Toll
Toll
Toll
Toll
Endocytosis
Signalling endosome bearing activated Toll receptors
MyD88
Tube
Pelle
Pellino
localizes to membrane
Sherpa
MyD88
?
Ulp1
SUMO
Cact
DIF/ Dorsal
SCF Complex
Promotes Cactus degradation
Cact
DIF/ Dorsal
DIF/ Dorsal
DIF/ Dorsal
Drosomycin, Bomanins, Serine Proteases
Ubiquitination
Phosphorylation
Sumoylation
E3 Ubiquitin Ligase
Kinase
Scaffold Protein

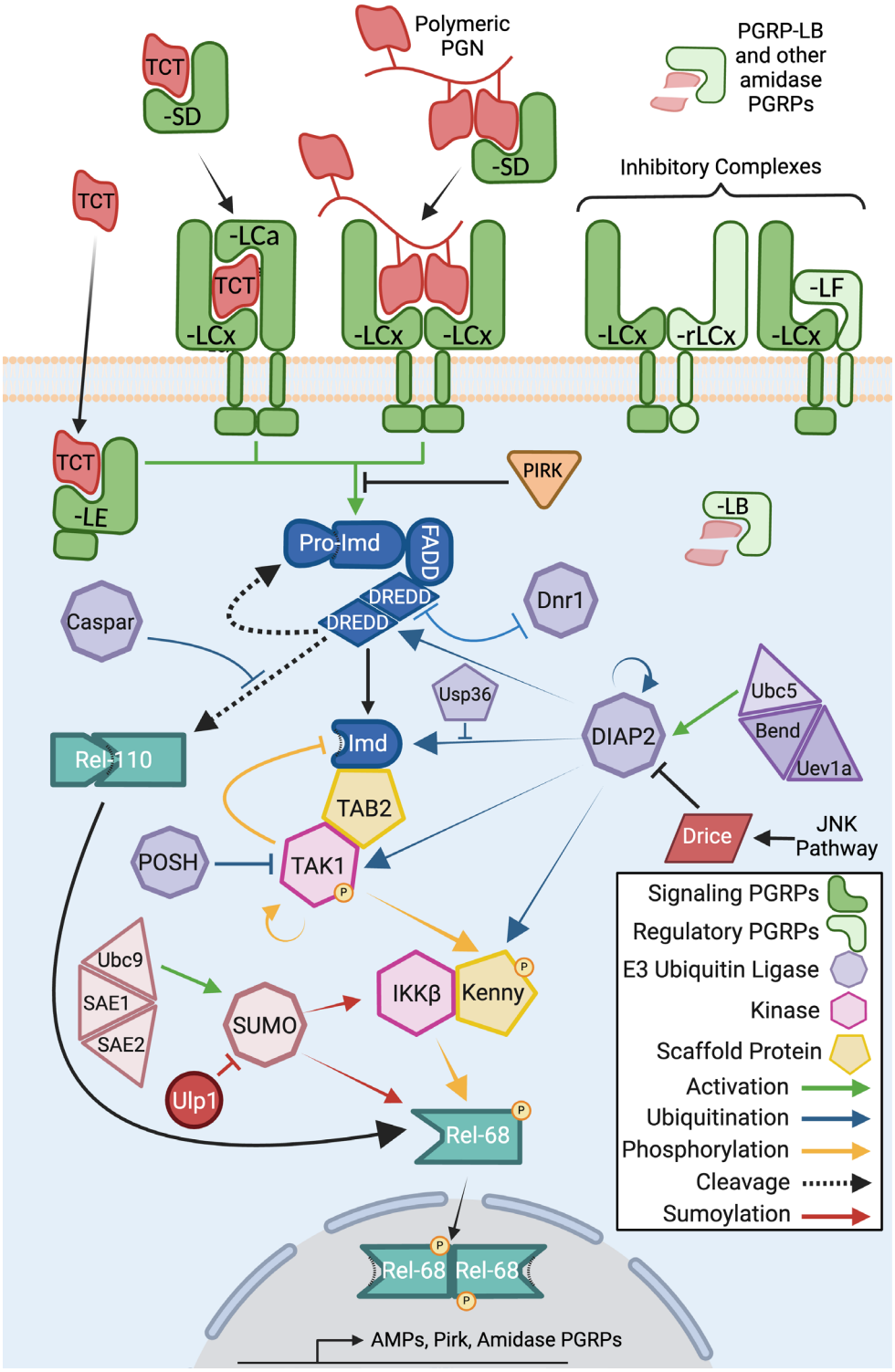
layer by Gram-negative bacteria or from the surface of *Bacillus* during division or upon death. The Imd pathway is activated by extracellular peptidoglycan through PGRP-LC, a transmembrane receptor with three active isoforms, -LCx, -LCa and -LCy, that differ in their PGRP domain (Choe et al., 2005, 2002; Gottar et al., 2002; Rämet et al., 2002b) (**Figure 12**). The major isoform PGRP-LCx has a PGRP domain that can bind DAP-type peptidoglycan, while PGRP-LCa and -LCy function as co-receptors (Chang et al., 2006, 2005; Kaneko et al., 2004; Lim et al., 2006; Stenbak et al., 2004). Functional and structural studies have shown that homodimers of PGRP-LCx are activated by polymeric peptidoglycan, while PGRP-LCx/LCa heterodimers bind peptidoglycan monomers called tracheal cytotoxin (TCT, **Box 2**). TCT is the terminal unit of Gram-negative bacterial peptidoglycans released upon cell division and is not found in Gram-positive bacteria (Mengin-Lecreulx and Lemaitre, 2005). TCT is produced by live bacteria and can be considered an alarmin that signals active danger, more so than polymeric peptidoglycan which is released by dead bacteria (Neyen et al., 2012; Pradeu et al., 2024). Consistent with this, TCT tends to activate a stronger and more persistent immune response than polymeric peptidoglycan (Neyen et al., 2016).

PGRP receptor homologs also function as negative regulators of signaling. Regulatory isoforms (rPGRP-LC) of PGRP-LCx, -LCy and -LCa with distinct intracellular domains adjust Imd pathway activity by forming non-productive complexes and promoting endocytic removal of PGRP-LC from the membrane (Neyen et al., 2016). Rapid endosomal recycling of PGRP-LCx by rPGRP-LC and degradation of polymeric peptidoglycan may explain why polymeric peptidoglycan elicits a shorter response compared to TCT, which is sensed by PGRP-LCx/a (Neyen et al., 2016) (see **Box 6**). PGRP-LF is a transmembrane protein with two PGRP domains that cannot bind peptidoglycan but interacts with and negatively regulates PGRP-LC. Loss of PGRP-LF function leads to signal independent activation of the Imd pathway (Basbous et al., 2011; Maillet et al., 2008; Persson et al., 2007; Tavignot et al., 2017). PGRP-LF mutants are viable but short lived, and display

Figure 11 The Toll signaling pathway
Schematic of Toll receptor activation and intracellular signal transduction leading to gene transcription. Activation of the Toll receptor through Spatzle binding and dimerization triggers endocytosis of the receptor and subsequent signaling events. Scaffold proteins MyD88 and Tube and the kinase Pelle localize to the membrane in a process promoted by the E3 ubiquitin ligase Sherpa (Galindo et al., 1995; Kanoh et al., 2015; Sun et al., 2004). The E3 ligase Pellino ubiquitinates MyD88 in a fashion that promotes its proteasomal degradation and modulates Toll pathway activity (Ji et al., 2014). Pelle phosphorylates Cactus, leading to its degradation and release of Dif and Dorsal transcription factors (Daigneault et al., 2013; Ip et al., 1993; Lemaitre et al., 1995a). Dif and Dorsal are phosphorylated by an unknown kinase. The E3 ubiquitin ligase SCF complex promotes Toll signaling by enhancing Cactus degradation and promoting release of the Dif and Dorsal transcription factors (Khush et al., 2002). Sumoylation affects several steps of the intracellular Toll cascade and has both positive and negative regulatory effects at different steps (Chiu et al., 2005; Hegde et al., 2022, 2020; Koltun et al., 2017; Paddibhatla et al., 2010). Most components of the Toll pathway were identified for their role in embryonic dorsoventral patterning, and later shown to have roles in immunity and other functions (Belvin and Anderson, 1996; Hashimoto et al., 1988; Horng and Medzhitov, 2001; Lemaitre et al., 1996; Nusslein-Volhard and Wieschaus, 1980; Tauszig-Delamasure et al., 2002). Canonical components of the Toll pathways include Spatzle, Toll, MyD88, Tube, Pelle, Cactus, Dif and Dorsal. Figure created with BioRender.com, CC-BY-NC-ND.

constitutive NF-κB/Imd activation specifically in ectodermal tissues, leading to genitalia and tergite malformations (Tavignot et al., 2017). Peptidoglycan sensing by PGRP-LC is also shaped by two secreted PGRPs, PGRP-LB and -SD. In contrast to PGRPs that function as pattern recognition receptors (PGRP-LC, -SD, -LE, and -SA), PGRP-LB retains a PGRP domain with amidase-type enzymatic activity that cleaves the peptide bridge from the glycan backbone (**Box 2**). This cleavage by PGRP-LB converts DAP-type peptidoglycan into non-immunostimulatory fragments, dampening Imd pathway activation (Zaidman-Rémy et al., 2006). In contrast, PGRP-SD, a true PRR, binds DAP-type peptidoglycan and promotes Imd pathway activation by either sequestering it from PGRP-LB or delivering it to PGRP-LC at the membrane (Iatsenko et al., 2016; Leone et al., 2008). As these two PGRPs are themselves regulated by the Imd pathway, they establish positive (PGRP-SD) and negative (PGRP-LB) feedback loops, fine-tuning immune reactivity of the Imd pathway. Other amidase PGRPs, notably PGRP-SC2 and -SC1a/b, may also

Figure 12 The Imd signaling pathway
Schematic of the Imd signaling pathway. Tissue-specific regulators of Imd pathway activity (e.g., Trabid, LUBEL) are not shown. Peptidoglycan binding results in clustering of PGRP-LC (transmembrane) or PGRP-LE (intracellular) receptors. PGRP-SD is a secreted recognition receptor that promotes DAP-type peptidoglycan sensing by PGRP-LC (Iatsenko et al., 2016). Association of cRHIM domains on the PGRP-LE receptor or the intracellular portions of PGRP-LC trigger amyloid fibril formation in association with the cRHIM domains of Imd, and result in recruitment of FADD and DREDD (Kleino et al., 2017; Kleino and Silverman, 2014). Ubiquitination of DREDD by DIAP2 is required for cleavage of both Relish and Imd (Meinander et al., 2012). Imd cleavage exposes an IBM (IAP-binding motif) which recruits DIAP2 (Paquette et al., 2010). DIAP2 ubiquitinates itself, Imd, TAK1, and Kenny (IKKγ) in addition to DREDD, and generally functions to increase association between signaling proteins. Ubiquitination by DIAP2 allows Imd to recruit the TAK1 kinase through TAB2, a structural protein. TAK1 phosphorylates Kenny and itself to promote pathway activity, while TAK1 phosphorylation of Imd promotes a change in ubiquitination (K63 → K43, perhaps mediated by dUsp36) that enhances proteasomal degradation of Imd, generating inhibitory feedback. Phosphorylation of Kenny activates the IKK complex, leading to phosphorylation of Relish by IKKβ. Sumoylation of IKKβ also promotes IKK complex activity. Cleavage of Relish to produce Rel-68 allows translocation to the nucleus, while phosphorylation is required for full transcriptional activity. Imd pathway activity is extensively regulated at the receptor, signaling, and transcriptional levels. Amidase PGRPs with tissue-specific expression patterns cleave peptidoglycan to reduce receptor stimulation (Charroux et al., 2018; Costechareyre et al., 2016; Paredes et al., 2011; Zaidman-Rémy et al., 2006). Some ubiquitin editing events promote proteasomal degradation of signaling intermediates. Relish promotes transcription of the positive regulator PGRP-SD and negative regulators including amidase PGRP-LB and Pirk. Pirk disrupts amyloid fibril formation and signaling by PGRP receptors. JNK signaling also increases Drice caspase activity, which suppresses DIAP2 activity and Imd signaling in the gut (Kietz et al., 2022). Canonical members of the Imd pathway include positive regulators PGRP-SD, PGRP-LC, PGRP-LE, Imd, DIAP2, FADD, DREDD, TAK1, TAB2, IKKβ (ird5), Kenny (IKKγ) and Relish, and negative regulators Pirk and PGRP-LB, which has both cytosolic and extracellular isoforms. Note that TAK1 and TAB2 also function in the JNK pathway, such that pattern recognition through upstream Imd induces low-level JNK activation. Compiled with data from: (Erturk-Hasdemir et al., 2009; Fukuyama et al., 2013; Guntermann et al., 2009; Kaneko et al., 2006, p. 200; Kietz et al., 2022; Kleino et al., 2017; Lhocine et al., 2008; Neyen et al., 2016; Paquette et al., 2010; Park et al., 2004; Silverman, 2000; Stoven et al., 2003; Zhou et al., 2005). Figure created with BioRender.com, CC-BY-NC-ND.


Polymeric PGN
PGRP-LB and other amidase PGRPs
TCT
-SD
Inhibitory Complexes
-LCa
-LCx
-rLCx
-LF
-LE
-LB
PIRK
Pro-Imd
FADD
DREDD
Caspar
Dnr1
Usp36
Ubc5
Bend
Uev1a
DIAP2
Imd
Rel-110
TAB2
TAK1
POSH
Drice
JNK Pathway
Ubc9
SAE1
SAE2
SUMO
Ulp1
IKKβ
Kenny
Rel-68
AMPs, Pirk, Amidase PGRPs
Signaling PGRPs
Regulatory PGRPs
E3 Ubiquitin Ligase
Kinase
Scaffold Protein
Activation
Ubiquitination
Phosphorylation
Cleavage
Sumoylation

regulate the Imd pathway by degrading peptidoglycan in specific tissues (Bischoff et al., 2006; Costechareyre et al., 2016; Guo et al., 2014; Paredes et al., 2011). PGRP-LA encodes multiple isoforms and clusters with PGRP-LC and -LF in the genome, but unlike these genes the precise function for PGRP-LA is not clear (Gendrin et al., 2013). Studies done in mosquitoes and *Drosophila* however suggest a role for PGRP-LA in regulating Imd pathway activity in epithelia such as the trachea and gut (Gao et al., 2020; Gendrin et al., 2017, 2013).

The Imd pathway can also be activated Intracellularly by binding of monomeric peptidoglycan (TCT[5]) to the intracellular sensor PGRP-LE, which recruits Imd as PGRP-LC does to initiate downstream signaling (Kaneko et al., 2006; Takehana et al., 2004, 2002). The mechanisms by which TCT accesses the cytosol are not yet fully understood, but the SLC46 family transporter CG8046 has been shown to facilitate translocation of TCT and promote its recognition by PGRP-LE in the gut (Paik et al., 2017). A cytoplasmic form of PGRP-LB can down-regulate PGRP-LE activation by degrading intracellular peptidoglycan, similar to its extracellular counterpart (Charroux et al., 2018). While PGRP-LC is the main sensor regulating the systemic immune response, PGRP-LE dominates in the midgut (Bosco-Drayon et al., 2012; Neyen et al., 2012) (see **Figure 28**). PGRP-LE may also contribute to immune activation and autophagy in response to bacteria that invade the cytoplasm, such as *Listeria* (Yano et al., 2008). Although the Imd pathway has been linked to autophagy (Liu et al., 2018; Nandy et al., 2018; Tsapras et al., 2022; Tusco et al., 2017), the involvement of PGRP-LE in the control of autophagy has not received direct follow up. The Imd pathway can also be activated through a number of alternative mechanisms (**Box 3**).

ii) Imd signaling

Peptidoglycan binding induces clustering of PGRP-LC (**Box 2**) or PGRP-LE, initiating a complex intracellular signaling cascade that involves the recruitment of Imd, FADD and the DREDD caspase, which cleaves Imd. Imd binds the ubiquitin ligase DIAP2 (**Box 4**) and leads to activation of the TAK1/TAB2 complex, which also participates in the JNK pathway (Elrod-Erickson et al., 2000; Georgel et al., 2001; Kaneko et al., 2004; Kleino and Silverman, 2014; Leulier et al., 2002, 2000; Naitza et al., 2002; Silverman et al., 2003; Stoven et al., 2000; Takaesu et al., 2000; Vidal et al., 2001). The MAP3K[6] TAK1 then phosphorylates Kenny (IKKγ), which together with IKKβ (ird5) forms the IKK complex (Erturk-Hasdemir et al., 2009; Lu et al., 2001; Rutschmann et al., 2000b; Silverman, 2000). Cleavage of Relish by DREDD allows it to translocate to the nucleus, while phosphorylation of Relish by IKKβ fully potentiates its ability to transactivate Imd-mediated genes (Erturk-Hasdemir et al., 2009).

During Imd pathway activation, intracellular cRHIM domains of clustered PGRP-LC or PGRP-LE proteins form amyloid fibrils that recruit Imd and activate downstream signaling (Kleino et al., 2017). An inducible negative regulator, Pirk, disrupts these amy-

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5 While it is clear that TCT (tracheal cytotoxin, a DAP-type peptidoglycan monomer with an anhydro bond) can strongly activate PGRP-LE and the Imd pathway, other DAP-type peptidoglycan monomers (lacking the anhydro bond) appear to be less potent inducers (Stenbak et al., 2004).

6 MAP3Ks are Mitogen-Activated Protein Kinase Kinase Kinases. MAPKs or MAP kinases are serine/threonine-specific protein kinases which are often sequentially activated: MAP3Ks phosphorylate MAP2Ks that in turn phosphorylate MAPKs, which in turn activate transcription factors including AP-1 (see **Figure 18**).

Box 3 Alternative modes of Imd pathway activation

Much is still unknown of mechanisms activating the Imd pathway. For example, we do not know the identity of ligand or elicitor that mediates strong activation of the Imd pathway during septic infection with fungi (Lemaitre et al., 1997), or alternatively if this is due to the presence of contaminants or the injury itself. The role of PGRP-LCy remains unknown. Kosakamoto and collaborators found that neither heat-killed *Gluconobacter* bacteria nor smaller secreted molecules (<10kDa) in the culture medium were immunogenic when fed to flies, but the fraction of supernatant containing large molecules (>10kDa) was highly immunogenic, similar to feeding with live bacteria (Kosakamoto et al., 2020). This suggests that the molecule(s) responsible for activating Imd in the gut in response to *Gluconobacter* are large proteins such as proteases. Moreover, reports have suggested that in addition to PRR-mediated recognition of DAP-type peptidoglycan, the Imd pathway can be activated by proteolytic cleavage of PGRP-LC extracellular domain (Schmidt et al., 2007). One study found that infection with both *B. subtilis* and *S. aureus* (Gram-positive bacteria with DAP- and lysine-type peptidoglycan respectively) resulted in cleavage of the PGRP-LC-GFP extracellular domain, which accumulated in the extracellular space around bacteria (Vaz et al., 2019). Similarly, in *Drosophila* S2 cells an allergen-derived cysteine protease from dust mites can activate the Imd pathway through cleavage of PGRP-LC (Warmbold et al., 2013). Finally, it has been proposed that activation of Imd signaling in the gut triggers hemocyte-mediated accumulation of hemolymph polyols, which upregulate the matrix metalloprotease Mmp2 and lead to cleavage of the PGRP-LC ectodomain at the surface of fat body cells, activating systemic Imd signaling (Yang et al., 2019). While many studies confirm that ectodomain-deleted PGRP-LC acts as a constitutive activator of the Imd pathway (Choe et al., 2005; Maillet et al., 2008; Warmbold et al., 2013), it remains to be seen whether cleavage of PGRP-LC is a significant factor contributing to Imd activation *in vivo*.

The RhoGTPase Rac2 may also directly activate Imd when modified by bacterial toxins similar to activation of immunity through RIP kinases in humans (Boyer et al., 2011). In addition, one study showed that the Imd pathway can be activated in enteroendocrine cells of the midgut by microbiota-derived acetate (Kamareddine et al., 2018). As previously mentioned, Relish can also undergo alternative activation by cGAS-STING (see **Figure 8**). This mode merges with the canonical Imd pathway at the level of IKKβ and does not involve upstream components of the pathway such as Imd. cGAS-STING regulates a set of STING-regulated genes (Srgs) independent of PGRP-LC-Imd-Relish target genes in the fat body (Goto et al., 2018). Future studies are required to better characterize alternative modes of Imd pathway activation beyond the well-established roles of monomeric and polymeric DAP-type peptidoglycans.

Box 4 Modulation of the Imd pathway by ubiquitination and sumoylation

The intracellular Imd pathway is modulated by multiple ubiquitination and sumoylation events with complex positive and negative regulatory effects (Aalto et al., 2019; Handu et al., 2015; Meinander et al., 2012; Paquette et al., 2010; Prakash et al., 2021; Tang et al., 2021; Tusco et al., 2017). Ubiquitination and sumoylation result in covalent attachment of small protein 'tags' to target proteins. Ubiquitination typically either increases binding and recognition by other proteins and facilitates pathway activity by promoting protein-protein interactions, or alternately targets proteins for proteasomal degradation, resulting in a suppressive effect. Increased protein-protein interactions upon ubiquitination are often mediated by ubiquitin-binding Zinc Finger (ZnF) domains. Ubiquitinated proteins can also form aggregates in association with the *Drosophila* p62 protein Ref(2)P, which targets them for autophagy and degradation, similar to mammalian p62 (e.g., (Lindmo et al., 2008)). p38 signaling is also involved in autophagosomal degradation of ubiquitinated protein aggregates, which may include intermediates in immune signaling (Belozerov et al., 2014; Ryan et al., 2021).

Some ubiquitin ligases such as DIAP2, which modifies multiple components of the Imd pathway including itself, are essential for Imd pathway activation (Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006; Zhou et al., 2005). Many additional ubiquitin ligases and ubiquitinases modify Imd pathway activity (Caspar, Dnr1, Usp36, LUBEL, POSH, Trabid, CYLD), Toll pathway activity (Sherpa, Pellino), or both (SCF complex), some in a tissue-specific manner (Aalto et al., 2023). The ubiquitin ligase POSH is required for both Imd and JNK pathway activity (Tsuda et al., 2005, Zhang et al., 2010). Sequential ubiquitin editing of a single target by multiple proteins can fine-tune activity within an immune pathway to restore homeostasis following immune challenge (Chen et al., 2017).

Sumoylation similarly modulates pathway activity, primarily by regulating cell-surface localization of proteins, or by modifying transcription factor activity by affecting protein stability and protein-protein interactions. Sumoylation of IKKβ is required for full Imd pathway activity (Fukuyama et al., 2013). All three *Drosophila* NF-κB transcription factors (Dif, Dorsal, and Relish) are sumoylated, but the effects of these modifications are not yet well understood (Hegde et al., 2020; Tang et al., 2021). Immune roles of proteins involved in ubiquitination and sumoylation must be interpreted with caution, as they often participate in a multitude of processes and can have complex effects when mutated that may indirectly affect immune function.

loid fibrils and disconnects Imd from PGRP receptors to attenuate Imd pathway signaling (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). Multiple systems prevent overactivation of the Imd pathway. In addition to the previously mentioned negative regulators PGRP-LB, Pirk, and PGRP-LF, there are also conditional or tissue specific negative regulators of Imd such as Dnr1, Caspar, Trabid, Ubiquitin-specific proteases (Usp36/Scny, USP2 and USP34/Puf), CYLD and the amidase PGRPs PGRP-SC2/PGRP-SC1A/1B (Engel et al., 2014; Foley and O'Farrell, 2004; Guntermann et al., 2009; Kim et al., 2006; Thevenon et al., 2009; Tsichritzis et al., 2007; Costechareyre et al., 2016; Paredes et al., 2011). Processes such as some forms of ubiquitination that promote rapid proteasomal degradation of Imd pathway intermediates (**Box 4**) also prevent immune overactivation, which can have widespread adverse effects. The existence of many negative regulators at each step of the Imd pathway indicates that this pathway must be tightly controlled to avoid tissue damage, similar to the TNF-R pathway (Aggarwal and Silverman, 2008). Imd signaling is fine-tuned by several ubiquitination and sumoylation events, which may be tissue-specific (**Box 4**).

C. Cross talk between Toll and Imd pathways

Use of specific gene readouts revealed that Toll and Imd are separate pathways that can be selectively activated by different classes of microbes: natural infection with entomopathogenic fungus activates only Toll, while natural infection with Gram-negative bacteria activates mostly Imd (Basset et al., 2000; Lemaitre et al., 1997). Septic injury activates both pathways, but relative strength of activation depends on the characteristics of the introduced microbe. Thus, selective action of these pathways provides a degree of specificity to the systemic immune response (Lemaitre et al., 1997) (**Box 5**). The Imd pathway regulates many genes with an early acute phase profile and faster kinetics than Toll-mediated genes (De Gregorio et al., 2002b; Lemaitre et al., 1997; Rutschmann et al., 2000a). Although subsets of genes that are specific to one of the two pathways exist, many immune genes receive input from both pathways to differing extents. This cross-regulation can be due to several factors. At the promoter level, genes appear to contain NF-κB binding sites with different specificities for combinations of the Dorsal, Dif, and Relish transcription factors (Senger et al., 2006). Regulation by Dorsal, Dif, and Relish heterodimers also remains possible (Tanji et al., 2010), which could explain some complex expression patterns (**Figure 13**). Binding sites near NF-κB sites for transcription factors such as the GATA factor Serpent, the homeobox transcription factor Caudal, or Deaf1 may modify NF-κB affinity or independently shape general or tissue-specific expression patterns of both Toll and Imd-regulated genes (Busse et al., 2007; Choi et al., 2008; Engstrom et al., 1993; Kadalayil et al., 1997; Kappler et al., 1993; Önfelt Tingvall et al., 2001b; Petersen et al., 1999; Reed et al., 2008). Studies of the nuclear IκB Charon/Pickle have produced somewhat contradictory results, but this protein may interact with the histone deacetylase dHDAC1 to selectively repress activity of Relish homodimers and skew transcriptional output (Morris et al., 2016) or promote Relish association with certain NF-κB binding sites (Han et al., 2020; Ji et al., 2016). Some processes such as sumoylation, SCF complex activity, and endocytosis influence both Toll and Imd pathways with variable effects (Huang et al., 2010; Khush et al., 2002; Tang et al., 2021) (**Box 4**, **Box 6**).

Many genes encoding components of the Imd and Toll pathways are themselves induced upon infection, modulating the immune response. Immunity genes are also under hormonal control, notably by ecdysone, the master hormone controlling molting

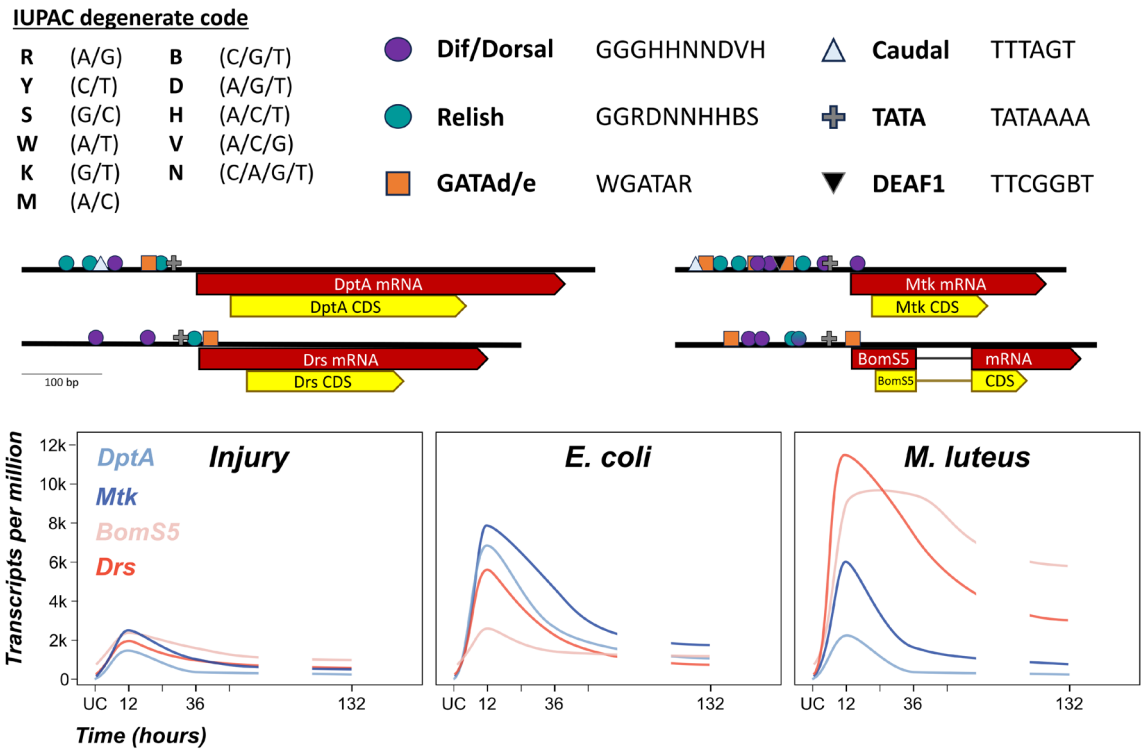

Figure 13 Immune gene promoters integrate Toll- and Imd-pathway activity
The Toll and Imd NF-κB transcription factors Dif, Dorsal, and Relish are the primary regulators of the systemic immune response. However, many additional regulators contribute to the ultimate expression pattern of immune genes. Provided are annotations of cis-regulatory elements found in the upstream region of four representative effector genes, regulated mostly by the Toll pathway (*BomS5*), mostly by the Imd pathway (*Diptericin A*) or partially by both pathways (*Metchnikowin* or *Drosomycin*) (Clemmons et al., 2015; Lemaitre et al., 1997; Levashina et al., 1998). Annotations were built from literature synthesis and manual curation (Busse et al., 2007; Copley et al., 2007; Dearolf et al., 1989; Hanson et al., 2021; Reed et al., 2008; Reichhart et al., 1992; Ryu et al., 2004; Senger et al., 2006). Gene expression patterns upon clean injury or septic infection with a Gram-negative (*E. coli*) or Gram-positive (*M. luteus*) bacteria are approximations from (Troha et al., 2018). Overall, the proportion of NF-κB binding sites for each pathway correlates broadly with inducibility by respective pathways. Gene induction is commonly reported in the literature as relative fold change compared to unchallenged. However, due to differences in basal expression of genes, this obscures the realized expression of these genes relative to one another, shown here as transcripts per million.

and metamorphosis (Meister and Richards, 1996; Nunes et al., 2021). Ecdysone affects Imd pathway-mediated AMP expression by regulating both PGRP-LC and GATA factors (Keith, 2023; Rus et al., 2013). Ecdysone also affects fat body maturation which can strongly impact protein production for both pathways (Ligoxygakis et al., 2002a). All of these factors contribute to the complexity of the systemic immune response, where microbes elicit specific gene expression profiles that extend beyond the classical Gram-negative versus Gram-positive dichotomy (Troha et al., 2018) (**Box 5**).

While the bulk of host defense peptides produced during systemic infection are secreted by the fat body, hemocytes are thought to provide a small contribution. Use of gene reporters and single cell RNAseq studies have found that a distinct class of plasmatocytes seems to specialize in AMP production (Cattenoz et al., 2021, 2020; Hultmark and Andó, 2022). Hemocytes may contribute primarily by supplying effectors locally to specific sites or tissues. Although immune responses in the hemocytes and fat body both rely on Toll and Imd, there are differences in transcriptional output between these two tissues and according to life stage (Vaibhvi et al., 2022). Other tissues such as Malpighian tubules might also contribute to the systemic antimicrobial response (Davies et al., 2012).

Not surprisingly, the Toll and Imd pathways interact with many other signaling pathways including Hippo (Liu et al., 2016; Yang et al., 2024) and JNK (Boutros et al., 2002; Li et al., 2020b; Silverman et al., 2003). Liu and colleagues found that the Cactus kinase Pelle can promote Hippo pathway activity in the fat body, resulting in direct Yorkie-dependent suppression of *cactus* transcription and increased Toll activity. Toll pathway activation in the fat body also suppresses growth and nutrient storage through insulin signaling (Roth et al., 2018) (see Hemocytes are a central metabolic hub, page 104). Some studies indicate that Toll activity contributes to JNK-mediated cell death by promoting ROS production (Li et al., 2020b; Wu et al., 2015). As the Imd and JNK pathways share several components including the TAK1/TAB2 complex, infection transiently activates JNK signaling through upstream components of Imd (Boutros et al., 2002; De Gregorio et al., 2001; Silverman et al., 2003) (**Figure 12**, **Figure 18**). Overactivation of Imd signaling leads to apoptosis in several contexts (e.g., (Georgel et al., 2001; He et al., 2017; Paredes et al., 2011; Ryu et al., 2008; Shibata et al., 2013; Zhai et al., 2018a)), but can also suppress JNK activity through upregulation of DIAP1 during development (Tavignot et al., 2017). Some components of the Toll and Imd pathways also contribute to other processes such as cell competition (Alpar et al., 2018; Germani et al., 2018.; Katsukawa et al., 2018; Meyer et al., 2014), that appear to be separate from their roles in immunity.

Box 5 On the Gram-positive/Toll Gram-negative/ Imd dichotomy

In the early nineties, the concept of innate immunity was defined by the fact that it lacked specificity and memory (Pradeu et al., 2024). However, later work in *Drosophila* revealed the existence of two signaling pathways, Imd and Toll, that when disrupted produce acute susceptibility to different classes of microbes: Gram-positive bacteria and fungi for Toll (Lemaitre et al., 1997, 1996; Rutschmann et al., 2002), and Gram-negative bacteria for Imd (Lemaitre et al., 1995b; Leulier et al., 2000; Rutschmann et al., 2000b). The use of reporter genes, notably *Diptericin A* for Imd and *Drosomycin* for Toll*, revealed that these pathways can similarly be selectively activated by different classes of microbes.

* While *Diptericin* is tightly regulated by the Imd pathway, *Drosomycin* also receives minor early input from the Imd pathway in addition to Toll. Today, some may prefer to use *Bomanins* (e.g., *BomS1, BomBc3*) as Toll reporters as they are not similarly cross-regulated by Imd. Note that these host defense peptides are good reporters for the systemic immune response, but may not reflect the activity of these pathways in other tissues such as epithelia, due to the existence of tissue specific regulators or signaling cascades (Neyen et al., 2014; Troha and Buchon, 2019).

This effect is more apparent when using natural infection routes, as injury activates both pathways to a certain extent. This led to a simplified conception of Toll and Imd pathway activation: Toll is activated by Gram-positive bacteria and fungi, and Imd by Gram-negative bacteria. Subsequent characterization of microbial elicitors (DAP- or Lysine-type peptidoglycans, β-1,3 glucans, microbial proteases) complicated this early dichotomy by showing that: (**i**) Gram-negative bacteria and DAP-type peptidoglycan also stimulate the Toll pathway (Leulier et al., 2003; Vaz et al., 2019); (**ii**) some Gram-positive bacteria have DAP-type peptidoglycans that can stimulate both Toll and Imd pathways; (**iii**) many Gram-negative bacteria produce proteases that can activate the Toll pathway; (**iv**) accessibility and concentration of elicitors influence signaling (Leulier et al., 2003; Vaz et al., 2019); and (**v**) infection route influences sensing. Some clear distinctions do remain: for example, the strongest elicitor of the Imd pathway is monomeric peptidoglycan (TCT), which does not activate the Toll pathway (Kleino and Silverman, 2014). Today, we know that immune recognition is highly complex, and that each microbe or even each strain can elicit a unique response. Because of this, the classical dichotomy of Gram-positive/Toll and Gram-negative/Imd has been characterized as misleading by some authors in recent years. We choose to continue using this framing as it is conceptually useful as long as we are aware of the complexity behind it, as simplifications often are.

D. Post-transcriptional regulation of Toll and Imd immune responses

In recent years, we have learnt a lot about the mechanisms regulating the systemic antimicrobial response and the NF-kB mediated transcriptional response in particular. Findings increasingly show that regulatory steps take place at both the post-transcriptional and post-translational levels, and that these are critical to mount an efficient systemic immune response.

i) Post-transcriptional regulation by genome-encoded RNAs

Regulatory RNAs including microRNA (miRNA), long non-coding RNA (lncRNA) and circular RNA (circRNA) have been shown to regulate the products of transcription in various ways, typically by regulating gene expression, protein processing, or protein activity (Mattick and Makunin, 2006).

Several miRNAs[7] regulate the systemic antimicrobial response by targeting transcripts encoding signaling components of the Toll and Imd pathways as well as antimi-

7 Micro RNAs (miRNA) are small, single-stranded, non-coding RNA molecules containing 21 to 23 nucleotides that pair to complementary sequences in mRNA molecules and silence them in various ways including, i) cleavage of the mRNA strand into two pieces, ii) destabilization of the mRNA by shortening its poly(A) tail, or iii) by reducing translation of the mRNA into proteins.

crobial peptides (Abbas et al., 2023; Atilano et al., 2017; Huang et al., 2024; Li et al., 2017; Moure et al., 2022). Some miRNAs of the miR-959-962 cluster negatively regulate Toll pathway activity by binding the 3'UTR of the Tube, Dorsal, or Toll transcripts (mRNAs) to suppress their expression (Li et al., 2021; Vodala et al., 2012). Similarly, *Drosophila* miR-317 negatively regulates Dif-RC, one of the four Dif transcripts. Flies transiently overexpressing *miR-317* have poor survival while $miR317^{KO}/+$ heterozygous flies have better survival than wild-type during Gram-positive bacterial infection (Li et al., 2017, 2019). An emerging concept is that miRNAs might be secreted through extracellular vesicles (EVs) in plants and animals to accomplish cross-taxa RNAi and silence virulence factor genes encoded by pathogens. In *Anopheles* mosquitoes, both let-7 and miR-100 miRNAs silence virulence-related genes of the entomopathogenic fungus *Beauveria* (Wang et al., 2021). This concept of host-encoded pathogen-targeting RNAi has not yet been extended to *Drosophila*.

Several lncRNAs[8] have similarly been implicated in the systemic immune response of *Drosophila* (Moure et al., 2022). Zhou et al. proposed that the immune inducible lncRNA-CR46018 and lncRNA-CR11538 interact with the Toll pathway by targeting the transcription factors Dif and Dorsal, or by competing with Dif and Dorsal to bind to AMP promoter regions (Zhou et al., 2021a, 2021b). A similar interaction was described for lncRNA-CR33942 in modulating Relish binding to Imd-regulated AMP promoters, although in this case lncRNA binding facilitated AMP expression (Zhou et al., 2022). lncRNAs may also function in the immune response to viruses. One study reported that an lncRNA (VINR) accumulates due to the action of the *Drosophila C virus* viral suppressor of RNA silencing (VSR). VINR interacts with Cactin and prevents its ubiquitin proteasome-dependent degradation, promoting AMP expression through a non-canonical pathway. Knockdown of VINR or Cactin increased host susceptibility to bacterial and viral infections (Zhang et al., 2020a).

Circular RNAs[9] have also been implicated in *Drosophila* host physiology and the immune response. The circular RNA circATP8B(2) binds to the Duox NAD-BD domain in the cytosol to regulate Duox activity, impacting both ROS production and susceptibility to viruses in the *Drosophila* gut (Liang et al., 2024). The circularization of some RNAs also allows translation to produce encoded peptide products. An example in *Drosophila* is the case of the circRNA CircSfl which is encoded by the *sulfateless* gene, and rescues both the fecundity and lifespan of insulin mutant flies by producing a small Sulfateless sub-peptide product (Weigelt et al., 2020). In neurodevelopment, circEct4/Edis (Ect4-derived immune suppressor), encodes a functional peptide, Edis-p, that inhibits proteolytic processing of the immune transcription factor Relish, preventing overactivation of the immune response (Liu et al., 2022a; Xiong et al., 2022). Some genes previously annotated as lncRNAs may ultimately be protein-coding circRNAs with different

8 Long non-coding RNAs (lncRNA) were initially defined as genes encoding transcripts of more than 200 nucleotides that are not translated into proteins. However, continued studies showed that many of these lncRNAs are actually circular RNAs that encode small proteins or micro-peptides. Thus, lncRNAs are now defined as a class of RNA molecules of more than 200 nucleotides that have no or limited coding capacity.

9 Circular RNAs (circRNAs) are the latest addition to the noncoding and regulatory RNA collection, characterized as covalently closed RNA loops generated by "head-to-tail" backsplicing events. Some genes initially annotated as lncRNAs are now understood to be circRNAs.

putative mechanisms of activity. The budding field of circRNA biology is therefore an exciting and unexplored direction of research.

These examples show that gene regulation does not end with production of the primary transcript. It should be said that reports of the impacts of miRNAs and lncRNAs in *Drosophila* immunity have generally used complex genetic tools that did not always control for host genetic background; further study is needed to understand the effect size and importance of these interactions. Additionally, early automated approaches to lncRNA annotation sometimes discounted peptides with <100 codons of open reading frame, and so a recent shift in bioinformatic prediction has expanded the list of putative short protein-coding genes (Guerra-Almeida et al., 2021). The *Drosophila* lncRNA CR44404 serves as a valuable example that care is needed when evaluating putative lncRNAs. Initially, lncRNA CR44404 was interpreted to regulate immune-metabolic interactions at the post-transcriptional level (Valanne et al., 2019a). However, CR44404 is now understood to encode an Imd-regulated peptide called IBIN that bears some resemblance to Metchnikowin (Hanson, 2022; Valanne et al., 2019b), which is also induced in the nervous system (Ebrahim et al., 2021).

In addition to regulation by non-coding RNAs, some antimicrobial peptides can be regulated at the post-transcriptional level in other ways. Some AMP genes encode transcripts containing AU-rich elements (AREs) in their 3'-untranslated region (UTR) that affect mRNA stability via Tis11-mediated mRNA degradation, a process inhibited by p38 MAPK (Lauwers et al., 2009; Wei et al., 2009). The early acute expression profile of Cecropin A1 (compared to other AMP genes) might be explained by differences in mRNA stability associated with these AU-rich elements.

ii) Post-translational regulation of AMPs

Production of an effective antimicrobial response also requires the translation, post-translational modification (e.g., glycosylation, amidation, cleavage), and secretion of antimicrobial peptides, steps that have not yet been fully characterized for most AMPs. The product of the inducible gene *Bombardier* is thought to maturate or shuttle mature Bomanins for secretion into the hemolymph (Lin et al., 2019). When Bomanins are induced but fail to be secreted into the hemolymph, flies suffer reduced survival to even heat-killed infections, suggesting an autotoxic cost when correct processing of immune peptides fails. Many AMPs are also regulated post-translationally by the nibbling off of dipeptidylpeptidase motifs (e.g., XA/XP) from AMP precursor proteins, and/or through cleavage at Furin cleavage sites (Hanson et al., 2021) (see **Table 1**), both of which are required for secretion of mature AMPs into the hemolymph (Hanson and Lemaitre, 2020). Post-translational modifications of AMPs can further impact their potency. One example is the glycosylation of Drosocin, whose unglycosylated protein isoform displays just a fraction of the antimicrobial activity compared to mono- or disaccharide O-glycosylated Drosocin (Bulet et al., 1996).

The many steps involved in the production of host defense peptides explain why the Toll and Imd pathways not only induce immune genes, but also genes that favor translation and secretion of immune peptides. This includes the PolyA binding protein Pab2, the eukaryotic initiation factor 4E-BP (Thor) that favors 5' cap-independent translation of some antimicrobial peptide genes (Bernal and Kimbrell, 2000; De Gregorio et

al., 2001; Vasudevan et al., 2017), and the transcription factor CrebA which upregulates genes that support production of secretory vesicles. Impairing any of these processes affects resistance to infection by preventing full deployment of antimicrobial defenses (Darby et al., 2023). For instance, loss of CrebA during infection triggers endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), which contributes to infection-induced mortality (Troha et al., 2018).

These studies reflect the nuanced ways in which the intermediates and effectors of humoral immunity are regulated, either by impacting their initial expression, or post-transcriptionally affecting their translation, processing, or secretion. It is clear that in order to gain a comprehensive understanding of how humoral immune signaling produces the realized defense response, it will be necessary to study regulatory mechanisms beyond direct activity of transcription factors.

5
Systemic immunity: Effectors

Studies of the Toll and Imd pathways have, until recently, mostly focused on the first phases of the immune response: recognition, signaling, and transcription. In contrast, how immune effectors directly shape host resistance downstream of these pathways was poorly characterized, owing to technical difficulties in targeting small genes through random mutagenesis. Fortunately, the development of the CRISPR-Cas9 gene editing approach has recently allowed studies that shed light on how effectors individually or collectively contribute to host defense.

A. Host defense peptides

Antimicrobial peptides (AMPs) are small, positively charged effectors that exhibit microbicidal activities against bacteria or fungi (Hanson and Lemaitre, 2020; Imler and Bulet, 2005). Being cationic, they tend to bind to membranes of microorganisms, which are more negatively charged (Brown and Hancock, 2006). Many AMPs disrupt membrane integrity by forming pores, though some target specific intracellular microbial processes, as exemplified by Drosocin, which inhibits translation (Koller et al., 2023; Mangano et al., 2023) or Metchnikowin, which targets the iron-sulfur subunit of succinate–coenzyme Q reductase (Moghaddam et al., 2017).

Eight families of inducible AMPs are currently known in *D. melanogaster*: the antifungals Drosomycin (7 genes) (Fehlbaum et al., 1994), Baramicin A (Hanson et al., 2021; Huang et al., 2023), and Metchnikowin (Levashina et al., 1995); Cecropins (4 genes (Kylsten et al., 1990)) and Defensin (Dimarcq et al., 1994), which have both antibacterial and some antifungal activities *in vitro*; and Drosocin (Bulet et al., 1996; Charlet et al., 1996), Attacins (4 genes (Hedengren et al., 2000)) and Diptericins (2 genes (Hedengren et al., 2000; Wicker et al., 1990)), which primarily exhibit antibacterial activity (Hanson and Lemaitre, 2020; Imler and Bulet, 2005) (**Table 1**). In addition, the *Drosophila* genome encodes many other host defense peptides such as Daisho (2 genes), Bomanins (12 genes) and Buletin, for which overt antimicrobial activity *in vitro* has not yet been demonstrated, although functional studies have shown that they are important *in vivo* to survive microbial infection (Clemmons et al., 2015; Cohen et al., 2020b; Hanson et al., 2022). This list is far from exhaustive, and many putative effectors downstream of Toll and Imd pathways remain uncharacterized (**Table 1**). At least eight uncharacterized genes encoding secreted peptides have features of host defense peptides, including Edin (Vanha-aho et al., 2015, 2012), Listericin (Goto et al., 2010), IM18, IBIN, CG45045, CG33493, CG43920 and GNBP-Like3 (see **Table 1**). Moreover, some AMP genes (BaraA, Drc, AttA, AttB, AttC, DptB, Def) produce several peptides that may have distinct functions through

Table 1 List of immune peptides.
Summary of known and predicted features of immune effector genes and the peptides that they produce, including gene regulation, protein maturation, peptide structural predictions, and antimicrobial characteristics. In some cases, genes are presented as being similar to existing gene families (e.g., Att/Dpt-like, Mtk-like), either for sequence similarity or evolutionary ancestry reasons**.** Cleavage motifs annotated are dipeptidylpeptidase (DPase: XA/XP motifs) and Furin (Furin: RXXR, often RX[R/K]R). Major, moderate, and minor annotations reflect the strength of Imd or Toll pathway regulation on gene expression. **Activity *in vitro*** describes results of studies done on peptides of the family, but these demonstrations are often limited to a few microbes, and may not mean that all genes in a family or all mature peptide products of a gene have been tested. Similarly, **activity *in vivo*** often does not distinguish between distinct sub-peptides of a gene, and may be limited to only a subset of microbes. An extended table 1 on *Drosophila* antimicrobial host defense peptides is available at https://www.epfl.ch/labs/lemaitrelab/lemaitre-lab/resources/.

Gene family	Genes	Mature peptides	Cleavage	Imd pathway	Toll pathway
Defensin	*Def*	2	DPase, Furin	major	
Attacin	*AttA, AttB, AttC, AttD*	2	DPase, Furin	major	minor
Diptericin	*DptA, DptB*	1-2	DPase, Furin	major	
Attacin-like / Diptericin-like	*CG33493, Edin*	1-2	DPase (Edin), Furin	major (Edin)	
Drosocin	*Dro*, Buletin	2	DPase, Furin	major	moderate
IM18	*CG33706*	1	DPase	major	
Cecropin	*CecA1, CecA2, CecB, CecC*	1	DPase	major	
Metchnikowin	*Mtk*	1	DPase	major	moderate
Metchnikowin-like (putative)	*Mtkl, CG43920, IBIN, CG45045*	1	DPase (Mtkl, CG43920)	major	minor (Mtkl)
Drosomycin	*Drs, Drsl1, Drsl2, Drsl3, Drsl4, Drsl5, Drsl6*	1	DPase	minor (Drs)	major (Drs)
Daisho	*Dso1, Dso2*	1	DPase		major
Baramicin	*BaraA1, BaraA2*	5	DPase, Furin	minor	major
Bomanin	*BomS1, BomS2, BomS3, BomS4, BomS5, BomS6, BomT1, BomT2, BomT3, BomBc1, BomBc2, BomBc3*	1	DPase		major, minor (BomT1, BomS6), other (BomS4)
CG4269	*CG4269*	1	DPase	minor	
Listericin	*Listericin*	1-2(?)	DPase, Furin(?)	minor	

Ancestry	Activity in vitro	Activity in vivo	References
Animalia	G+	G+	Dimarcq et al., 1994; Tzou et al., 2000; Touré et al., 2023a; Hanson, 2022
Insecta	G-	G-	Åsling et al., 1995; Hedengren et al., 2000; Rabel et al., 2004; Hanson et al., 2019b
Diptera	G-	G-	Wicker et al., 1990; Reichhart et al., 1992; Unckless et al., 2016; Barajas-Azpeleta et al., 2018; Hanson et al., 2019b; Hanson et al., 2023
Insecta			Vanha-aho et al., 2012; Hanson, 2022
Drosophila / Insecta	G-	G-	Bulet et al., 1996; Tzou et al., 2000; Hanson et al., 2019b; Hanson et al., 2022; Koller et al., 2023; Mangano et al., 2023
Diptera			Uttenweiler-Joseph et al., 1998; Hanson (thesis)
Insecta	G-, F	G-, F	Kylsten et al., 1990; Samakovlis et al., 1990; Tryselius et al., 1992; Ekengren and Hultmark, 1999; Carboni et al., 2022
Diptera	G+, F	F	Levashina et al., 1995, 1998; Hanson et al., 2019a; Hanson et al., 2019b; Moghaddam et al., 2017
Diptera			Valanne et al., 2019b; Tattikota et al., 2020; Ebrahim et al., 2021; Hanson, 2022
Drosophilidae	F (Drs)	F (Drs)	Fehlbaum et al., 1994; Jiggins and Kim, 2005; Chakrabarti et al., 2016; Hanson et al., 2019b
Drosophilidae	F	F	Cohen et al., 2020b
Drosophilidae	F	F	Hanson et al., 2021; Hanson et al., 2022; Huang et al., 2023
Drosophilidae		G+, F	Uttenweiler-Joseph et al., 1998; Clemmons et al., 2015; Lindsay et al., 2018; Hanson et al., 2019b; Lin et al., 2019; Cohen et al., 2020b; Xu et al., 2023b
Arthropoda			De Gregorio et al., 2002b; Hanson (thesis)
Drosophilidae	G+		Goto et al., 2010; Hanson (thesis)

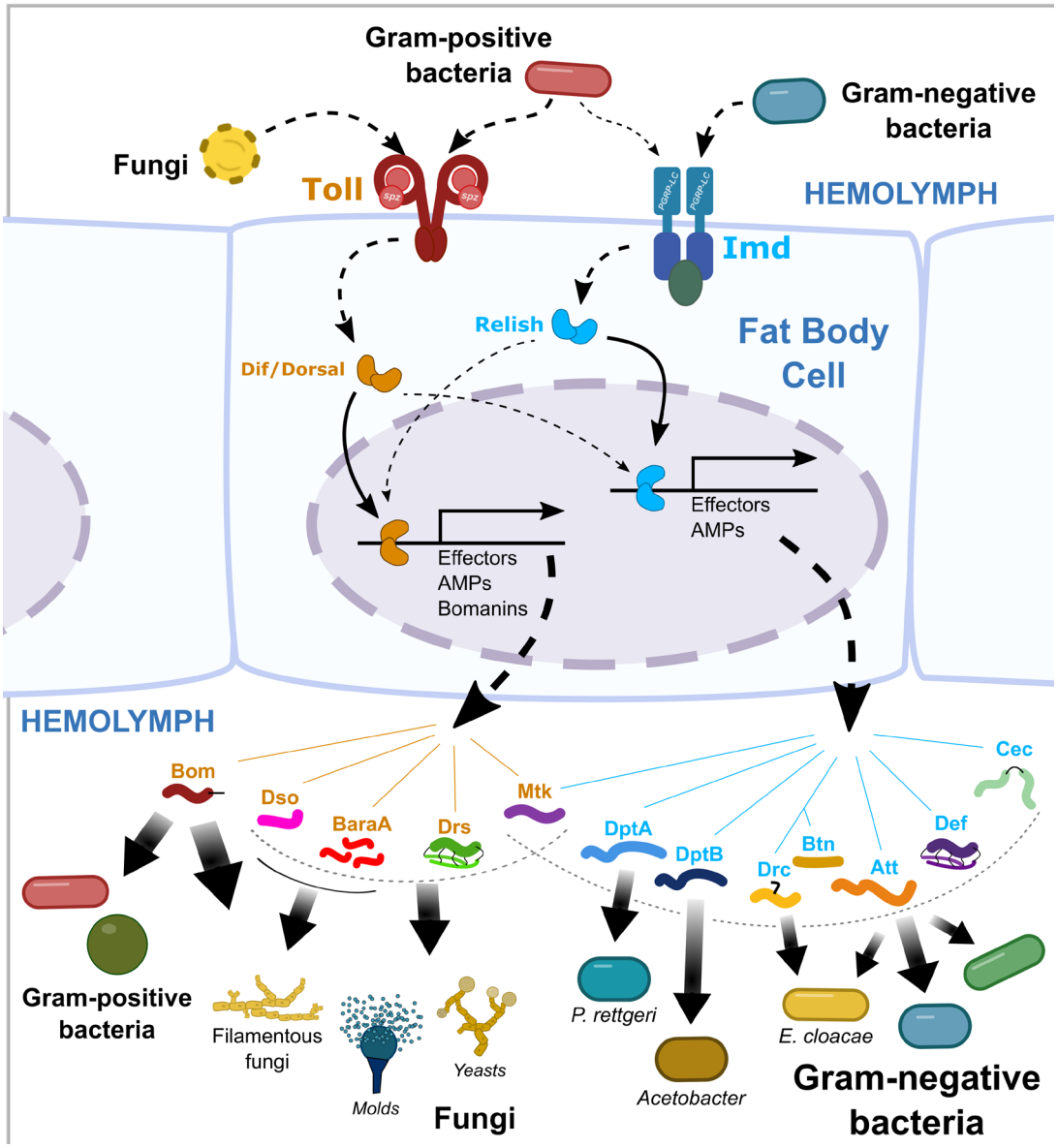

Figure 14 The logic of the systemic immune effector peptide response
The Toll and Imd pathways regulate different subsets of effector peptides, although some target genes can be activated by either pathway (Hanson and Lemaitre, 2020; Imler and Bulet, 2005). The susceptibility of Toll and Imd pathway mutants can be explained by the effectors they control, notably antibacterial peptides for Imd, and antifungal peptides and Bomanins for Toll (Clemmons et al., 2015; Hanson et al., 2019b). Many effector peptides are induced simultaneously upon infection, and in some cases their collective action contributes to microbial control. However, in multiple cases, single effector genes have key importance for defense against specific pathogens (bold block arrows). Many additional immune effector peptides are induced to a similar extent as those shown here, but have not yet been formally characterized (see **Table 1** and **Supplementary list 2**). Adapted from (Hanson and Lemaitre, 2020).

Furin cleavage of a single precursor (Hanson et al., 2022, 2021; Huang et al., 2023; Rabel et al., 2004). This leads to a total of at least 38 putative host defense peptide genes, many of which encode multiple peptides, that are induced upon systemic infection.

Although most *Drosophila* defense peptide-encoding genes are strongly induced in the fat body downstream of the Toll and Imd pathways in response to systemic infections, many show specific and complex patterns of expression in tissues such as the tracheae, gut, salivary glands or reproductive tracts (Ferrandon et al., 1998; Önfelt Tingvall et al., 2001a; Reichhart et al., 1992; Samakovlis et al., 1990; Tzou et al., 2000)(see Gut and Epithelial Immunity, page 109). Use of fly lines lacking host defense peptide genes has revealed that Imd-regulated antibacterial peptides (Diptericins, Drosocin, Attacins and Cecropins) are the major contributors to elimination of Gram-negative bacteria (Carboni et al., 2022; Hanson et al., 2019b). Similarly, Toll regulated AMPs (Drosomycin and Metchnikowin), and host defense peptides (Bomanin, Daisho, Baramicin A) contribute to resistance to systemic infection by fungi (Clemmons et al., 2015; Cohen et al., 2020b; Hanson et al., 2021; Huang et al., 2023). Thus, the susceptibility of mutants of the Toll and Imd pathways to different sets of microbes not only reflects specificity at the level of recognition but can now also be tied directly to the activities of downstream effectors (**Figure 14**). Use of single and compound mutants reveals that many of these AMPs function additively or synergistically against specific microbes (Hanson et al., 2019b). A surprise has been that the classic *Drosophila* AMPs do not contribute noticeably to defense against Gram-positive bacteria *in vivo* (Carboni et al., 2022; Hanson et al., 2019b; Touré et al., 2023b), despite *in vitro* studies finding potential activity (Dimarcq et al., 1994; Ekengren and Hultmark, 1999). This could be due to impaired membrane disruption by the thick cell walls of Gram-positive bacteria protected by teichoic acids (Arias-Rojas et al., 2023; Attieh et al., 2020; Kamar et al., 2017) (**Box 2**), or dynamics of infection *in vivo* that prevent bacterial exposure to host antimicrobial peptides (Touré et al., 2023a).

A further revelation in recent years is that AMPs have highly specific host-microbe interactions where a single AMP determines most of the AMP-mediated host resistance against particular pathogens. This specificity is best exemplified by the Diptericin gene family, where two members (Diptericin A and B) encode microbe-specific defenses against *Providencia rettgeri* and *Acetobacter* bacteria respectively (Hanson et al., 2023; Hanson et al., 2019b; Unckless et al., 2016). This specificity is also found for other *Drosophila* AMPs and AMP-like genes such as Drosocin/*Enterobacter cloacae*, Daisho/*Fusarium* fungi, Attacin/*Serratia marcescens* (Cohen et al., 2020b; Hanson et al., 2022)(Brian Lazzaro, personal communication). Drosocin likely sequesters bacterial ribosome release factors, arresting ribosome function (Koller et al., 2023; Mangano et al., 2023). Its specificity could reflect a particular binding affinity for the *E. cloacae* ribosome, or alternately a propensity for *E. cloacae* to take up Drosocin through the action of uptake permeases including the ABC transporter SbmA (Krizsan et al., 2015). Crucially, such specificity reveals the critical role of a single peptide among multiple immune effectors to resist infection, but we are yet to determine the mechanistic basis of peptide-microbe specificity. Analyzing the basis of specificity might give clues to the "Achilles' heel" of various pathogens (Hanson, 2024). Novel screens that use pathogenic microbes found in the fly's natural ecology are a promising arena to reveal additional effector-microbe specificities.

An exciting new concept is that some inducible host defense peptides may not be microbicidal, but rather protective for the host against virulence factors (e.g.,

proteases, toxins) common to pathogens (Huang et al., 2023; Xu et al., 2023a). As discussed above, a major component of Toll-mediated defense against Gram-positive bacteria and fungi are the Bomanins, a family of 12 genes in three forms (short, tailed, and bicipital) (Clemmons et al., 2015). Proper secretion of short-form Bomanins requires another Toll-inducible gene, *bombardier* (Lin et al., 2019). The precise mechanisms of Bomanin-mediated defense remain unclear. Single *Bomanin* genes may be multifunctional, or different Bomanins may operate in a pathogen-specific manner. For instance, hemolymph deficient for short-form Bomanins lacks killing activity against *Candida* yeast (Lindsay et al., 2018), suggesting Bomanins have direct antifungal activity. However, a recent report convincingly showed that *Aspergillus fumigatus* filamentous fungus kills Bomanin-deficient flies due to reduced tolerance to its toxins. Expression of *Bomanin Short 6* ubiquitously or in the nervous system protected flies against toxin injection independent of infection, supporting a role for Bomanins in tolerance of toxin-mediated damage (Xu et al., 2023a). A similar role in tolerance has been suggested for Baramicin A (Huang et al., 2023). Among all the immune-induced AMPs, Attacin D (AttD) uniquely lacks a signal peptide and is not secreted. A recent pre-print shows that *Attacin* D (AttD) is induced by the Imd pathway in Malpighian tubules and its over-expression is associated with cell death (Oi et al., 2024). Of note, another immune induced protein downstream of the Toll pathway (De Gregorio et al., 2002b), Ninjurin A, (NijA) also has a role in induction of non-apoptotic cell death (Broderick et al., 2012). The idea that some immune effectors downstream of Toll and Imd pathways contribute to cell death is appealing, as elimination of infected cells is a conserved host defense mechanism (Pradeu et al., 2024). The physiological roles of Attacin D and Ninjurin A in host defense await further characterization.

B. Transferrin and other putative effectors

Nutritional immunity is a mechanism that combats pathogens through sequestration of nutrients required for pathogen growth, notably iron (Núñez et al., 2018; Pradeu et al., 2024). In *Drosophila*, septic infection induces the expression of two transferrin genes, *Tsf1* and *Tsf3* (Skaar, 2010) as well as the iron binding protein *Zip89B* (De Gregorio et al., 2001). A recent study showed that Tsf1 sequesters iron from the hemolymph to the fat body upon infection, and flies mutant for Tsf1 are susceptible to infection by *Cunninghamella bertholletiae* fungi and *Pseudomonas aeruginosa* bacteria (Iatsenko et al., 2020). These studies demonstrate that nutritional immunity is key in surviving certain infections in *Drosophila.*

In *Drosophila*, six genes encode proteins that carry a domain structurally related to the mammalian complement factor C3 family, named the thioester-containing proteins (TEPs). While Tep5 is a pseudogene and Tep6 (also called Macroglobulin complement-related, Mcr) lacks the thioester motif and is a component of epithelial septate junctions (Batz et al., 2014), TEPs 1-4 encode signal peptides and are expressed in immune tissues, indicating a potential role in host defense (Bou Aoun et al., 2011; Dostálová et al., 2017). Tep2 and Tep4 appear to be regulated by the Imd and Toll pathways (De Gregorio et al., 2001), and Tep1 by the JAK-STAT pathway upon systemic infection (Irving et al., 2005; Lagueux et al., 2000). Studies in mosquitoes have revealed a key role of Tep1 in defense against the malaria parasite *Plasmodium falciparum.* Similar to the complement factor C3b, Tep1 binds to the ookinete surface, and by recruiting LRM1/APL1 proteins

induce killing and/or melanization of the ookinete (Blandin et al., 2004; Povelones et al., 2016). The functions of *Drosophila* TEPs are less well-characterized, and the LRM1/APL1 proteins that function with Tep1 in mosquitoes are not found in *Drosophila*. Nevertheless, mutations of these TEPs in *Drosophila* revealed roles in both humoral and cellular immunity, as they promote both Toll pathway activation and phagocytosis of Gram-positive bacteria (Dostálová et al., 2017). Additionally, Tep4 acts as an opsonin that promotes phagocytosis of ingested *P. aeruginosa* (Haller et al., 2018) and some TEPs protect against nematode (Arefin et al., 2014; Castillo et al., 2013; Tafesh-Edwards and Eleftherianos, 2023a) and parasitoid wasp infections (Bou Aoun et al., 2011; Dostálová et al., 2017). These studies collectively suggest that TEPs bind to microbes and facilitate immune reactions, contributing to effector-mediated immunity.

Finally, systemic infection triggers the expression of many protease inhibitors from the Serpin and Kunitz families, some of which may block the entry or virulence effects of pathogen proteases (De Gregorio et al., 2001; Kress et al., 2004). Many secreted immune effectors remain to be characterized (see **Supplementary List 1**).

C. Metabolic adaptation associated with systemic antimicrobial responses

In addition to immunity, the fat body and hemocytes have important roles in metabolism and storage. Mobilizing the immune system to fight infection requires massive reprogramming of these tissues to fuel the production of defense peptides (Clark et al., 2013). Consistent with this, Toll- and Imd-mediated immune responses interact with host metabolism (Bland, 2022; Dionne, 2014; Lee and Lee, 2018)(see also Hemocytes are a central metabolic hub, and **Figure 27**, page 105). Systemic infection also suppresses glycolytic and basal metabolic pathways (Clark et al., 2013; De Gregorio et al., 2001), and is usually accompanied by loss of glycerides and carbohydrate stores (Davoodi et al., 2019; DiAngelo et al., 2009; Dionne et al., 2006; Martínez et al., 2020; Roth et al., 2018). Activation of the Toll pathway in larvae similarly results in reduced triglyceride storage and synthesis in the fat body and reallocation of resources to phospholipid synthesis to deal with increased vesicle production required for effector secretion, a switch that is triggered by high levels of AMP secretion and mediated by the Kennedy pathway (Martínez et al., 2020). Toll activation also suppresses insulin signaling through reduced Akt[10] phosphorylation (Roth et al., 2018) and chronic activation inhibits larval growth (DiAngelo et al., 2009). Both Toll and Imd pathways have been shown to impact lipid metabolism (Davoodi et al., 2019; Molaei et al., 2019; Roth et al., 2018). Thus, alteration of host metabolism can have a profound effect on the immune response. As pathogens rely on specific host metabolites, dietary or metabolic changes can be detrimental or beneficial to the host depending on the infecting pathogen (Bland, 2022). As the fat body also provisions oogenesis, notably through the production of yolk, trade-offs occur between reproduction and immunity (Gordon et al., 2022; Gupta et al., 2022) (see Consequences of mating on immunity, page 122).

10 The Akt kinase is a component of the insulin growth factor pathway that functions downstream of the product of Pi3K92E and is activated by phosphatidylinositol binding and phosphorylation. Its phosphorylation is used a read-out of the insulin pathway.

6

Systemic immunity: Melanization

Melanization is an arthropod-specific immune mechanism resulting in the rapid deposition of the black pigment melanin at wound or infection sites and concomitant production of microbicidal reactive oxygen species (Cerenius et al., 2008; Marieshwari et al., 2023; Tang, 2009)[11]. This elegant effector process produces not only microbicidal activity, but also hardens clots with melanin polymer plugs that prevent blood loss, akin to the mechanical function of mammalian fibrin scabs. Melanization is central to many immune reactions such as wound healing, nodulation[12], and encapsulation, and involves specialized crystal cell hemocytes that rupture in a caspase-dependent manner to release clotting and melanization factors.

A. Melanization: Enzymatic pathway and microbicidal activity

Melanization relies on the activation of phenoloxidase (PO) enzymes, which catalyze critical steps resulting in melanin polymerization. Phenoloxidases are copper-containing enzymes related to invertebrate hemocyanins which transport oxygen, and insect laccases which sclerotize and tan the cuticle (Cerenius et al., 2008; Marieshwari et al., 2023). There are three POs in *Drosophila*, all of which are involved in immune reactions (Asano and Takebuchi, 2009; Dudzic et al., 2015; Nam et al., 2008). PO1 and PO2 are produced by crystal cells as zymogens called prophenoloxidases (PPOs) that are converted into active POs when cleaved at the N-terminus by serine proteases of the Toll-PO SP cascade. Two CLIP domain serine protease homologs, cSPH35 and cSPH242, act as co-factors in activation of PPO1 by the Toll-PO cascade (Jin et al., 2023). In contrast, PO3 is produced in an active form by lamellocytes and is therefore likely regulated at the transcriptional level (Nam et al., 2008) (see Encapsulation, and **Figure 26**, page 100).

Phenoloxidases are sticky enzymes that bind to self and non-self tissues, generating microbicidal reactive oxygen species (ROS) and toxic secondary compounds. *In vitro* experiments reveal that phenoloxidases have broad spectrum bactericidal activity, and

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11 The melanization reaction described here is an arthropod specific immune reaction. It is distinct although related to the deposition of melanin during cuticle formation that results in body pigmentation, which involves other enzymes. The immune melanization reaction is thought to take place at injury sites, in the hemolymph, around large parasites (encapsulation), on some abnormal tissues (melanotic tumors) and in some epithelia such as the hindgut and trachea.

12 Nodulation is the aggregation of invading pathogens by hemocytes and secreted materials (Miller et al., 1994; Satyavathi et al., 2014). While nodulation has been observed in other insects, this process has not formally been characterized in *Drosophila*. However, related processes such as hemocyte degranulation, clotting and agglutination are found in *Drosophila* (Matskevich et al., 2010; Theopold et al., 2004).

contribute to formation of large melanized bacterial aggregates (Zhao et al., 2007). The aromatic amino acid tyrosine and its derivatives are the precursors of melanin (Nappi et al., 2009; Tang, 2009) (**Figure 15**). Phenoloxidases contribute to the initial step of melanin synthesis by transforming tyrosine into DOPA (l-3,4-dihydroxyphenylalanine) by hydroxylation, and to the late stage of the pathway by converting phenols to quinones that polymerize to form melanin. Surprisingly, the blackening and microbicidal effects of the melanization cascade are not always linked (Dudzic et al., 2019). Although a mutation in the serine protease Hayan leads to the almost complete loss of blackening in adult flies, *Hayan* mutants are not as susceptible as *PPO1,PPO2* double mutant flies are to *S. aureus* infection. In contrast, *Sp7* mutant flies do not survive *S. aureus* infection, despite almost wild-type levels of cuticle and hemolymph blackening. This suggests that it is not the blackening *per se* that is involved in the control of *S. aureus*, but rather other events downstream of PO activity such as ROS production (Dudzic et al., 2019; Ramond et al., 2021).

Melanization is more than the deposition of melanin, as it involves the production of ROS and other toxic compounds. DHI (5,6-dihydroxyindole) conversion to melanin via intermediates indole-semiquinone and indole-5,6-quinone produces reactive oxygen species (H_2O_2, O_2^-) and cytotoxic molecules (Zhao et al., 2007) through the Fenton reaction (Dolezal, 2023) (**Figure 15** and **Box 9**). During the Fenton reaction, ROS are converted to highly reactive hydroxyl radicals (OH^-) in the presence of proteins containing copper (such as PO) or iron (such as peroxidase). Hydroxyl radicals participate in lipid peroxidation, which can damage pathogens and parasites but also host tissues (see Protection from ROS, page 87). Note that *Drosophila* lacks a homolog of NADPH-quinone reductase (NQO) which catalyzes conversion of DHI to melanin in mammals (Vasiliou et al., 2006). Dopamine, which is produced by Dopadecarboxylase (Ddc), spontaneously forms melanin in the presence of iron ions (Zhao et al., 2007). ROS are also produced by host enzymes such as NADPH oxidase (Nox) or dual oxidase (Duox) (see **Box 9**).

The precise role of melanin itself is not fully understood, but it may aggregate bacteria, form a physical barrier around parasites, or scavenge ROS to limit diffusion and damage to the host. Mutations affecting the melanization cascade can lead to more extensively disseminated infections, indicating that this cascade has a role in restricting pathogen spread (Ayres and Schneider, 2008). While hemolymphatic POs are post-transcriptionally regulated, enzymes involved in the melanization reaction including Dopadecarboxylase (Ddc), Pale, Punch and Dhpr are regulated at the transcriptional level by the Imd and JNK pathways upon infection (De Gregorio et al., 2001; Silverman et al., 2003). Reporters reveal that Ddc is produced in the epidermis around wound sites, and is regulated by the MAP kinase p38c (Davis et al., 2008) (see **Figure 18**).

B. Regional and functional specialization of prophenoloxidases

Immune melanization can occur in the hemolymph, but also in clots, at wound sites, around parasites, and in various tissues, usually in association with cell death. Use of mutations affecting each of the POs alone or in combination revealed that both PPO1 and PPO2 contribute to hemolymph melanization (Binggeli et al., 2014; Dudzic et al., 2015; Neyen et al., 2015; Rizki et al., 1980). PPO1 provides an immediate source of phenoloxidase activity, while PPO2 is stored as crystalline inclusions in the specialized crystal

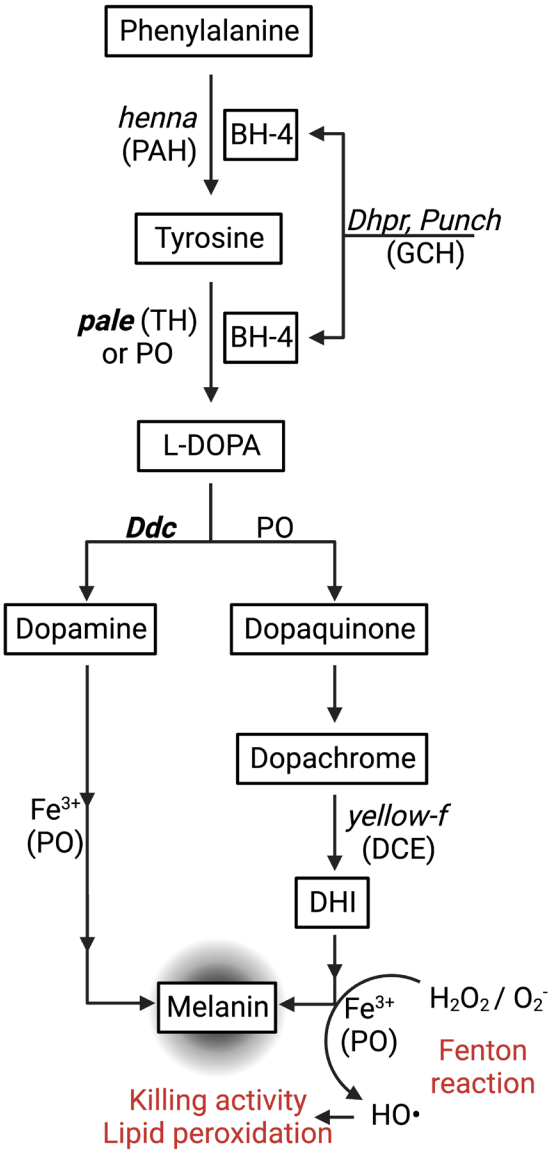


Figure 15 Metabolic pathway associated with the melanization reaction
Schematic representation of the melanization cascade. *Drosophila* genes involved in the melanization pathway are indicated in *italics*; genes upregulated by wounding or infection are in **bold** (from (De Gregorio et al., 2001)). Phenoloxidases (PO) activated by the Toll-PO SP serine protease cascade catalyse several steps in melanin production. PAH, phenylalanine hydroxylase; Dhpr, dihydropteridine reductase; GCH, GTP cyclohydroxylase; BH-4, tetrahydrobiopterin; TH, tyrosine hydroxylase; Ddc, dopadecarboxylase; DCE, dopachrome conversion enzyme; DHI, 5,6-dihydroxyindole. *yellow-f* is a paralog of the *Drosophila yellow* gene involved in body pigmentation. Compiled with data from (De Gregorio et al., 2001; Dolezal, 2023; Nappi et al., 2009; Tang, 2009). Created with BioRender.com, CC-BY-NC-ND.

cell hemocytes and functions as premade reserves deployed at a slightly later stage (see Systemic immunity: Cellular response, page 91). Indeed, PPO2-deficient flies have crystal cells that contain no crystals (Binggeli et al., 2014). The exact localization of PPO1, which may be present in the cytosol or crystal inclusions of crystal cells and/or in the hemolymph, is not fully established but a PPO1-GFP fusion shows that this PPO is present in larval crystal cells (B.L. unpublished). Thus, both PPO1 and PPO2 are produced by crystal cells, consistent with the observation that *lozenge*-deficient flies that lack this hemocyte type fail to melanize (Rizki et al., 1980, 1985; Rizki and Rizki, 1974; Warner et al., 1974). Melanization of capsules generated by larval lamellocytes is mediated by PPO2 released from crystal cells and PPO3 produced by lamellocytes (Dudzic et al., 2015). PPO3 lacks a signal peptide and is constitutively active (Dudzic et al., 2015; Nam et al., 2008). It may not be secreted, but instead involved in the melanization of lamellocytes themselves (Dudzic et al., 2015; Nam et al., 2008) (see Encapsulation, and **Figure 26**, page 100). Thus, differences in spatial localization, immediate or late availability, and mode of activation underlie the functional diversification of the three *Drosophila* PPOs, each of which have non-redundant but overlapping functions (Dudzic et al., 2015).

PPO1,PPO2 double mutant flies that lack hemolymphatic POs are susceptible to large wounds and to infection by many bacterial and fungal pathogens, revealing the role of melanization in the wound response and infection (Binggeli et al., 2014). Against certain infections such as low-dose *Staphylococcus aureus,* melanization can be the main factor determining survival, more so than transcriptional activation of the Toll or Imd pathways or presence of plasmatocytes (Dudzic et al., 2019; Ryckebusch et al., 2024). This is consistent with the high susceptibility of *S. aureus* to ROS (Gonzalez et al., 2013; Ramond et al., 2021). *PPO2,PPO3* deficient larvae that cannot produce melanized capsules are also susceptible to wasp infestation (Dudzic et al., 2015; Rizki and Rizki, 1990). Fascinatingly, PPOs can also function externally: they are present in molting fluid and help prevent colonization of the freshly-molted cuticle by fungal spores (Zhang et al., 2017). These studies reveal key roles of melanization in host defense. A recent study has convincingly shown that crystal cells contribute to oxygen transport through PPO2 protein phase transition (Shin et al., 2024) similar to crustacean hemocyanins, pigments with homology to PPOs that transport oxygen in crustaceans (Coates and Costa-Paiva, 2020). Shin and colleagues demonstrated that crystal cells, attracted by H_2O_2, move to sessile patches to collect oxygen from the trachea. This process is expected to be crucial in hypoxic conditions, particularly in oxygenation of the fat body, which is poorly connected to the tracheal system. In support of this, PPO2 deficient larvae are susceptible to hypoxia (Shin et al., 2024).

C. Crystal cell rupture: A pyroptosis-like cell death?

The mechanism that restricts melanization to localized areas is not well known, but likely relies on (**i**) spatial inhibition of the serine protease cascade by serpins and, (**ii**) localized delivery of PPOs by crystal cells. Sequestration of PPO1 and PPO2 in crystal cells separates them from substrates in the hemolymph, and their requirement for activation by serine proteases prevents spontaneous activation and toxicity to host tissues. Indeed, mutations leading to constitutive activation of the phenoloxidase pathway such as serpin mutations are very detrimental to flies (Charron et al., 2008; De Gregorio et al., 2002a; Scherfer et al.,

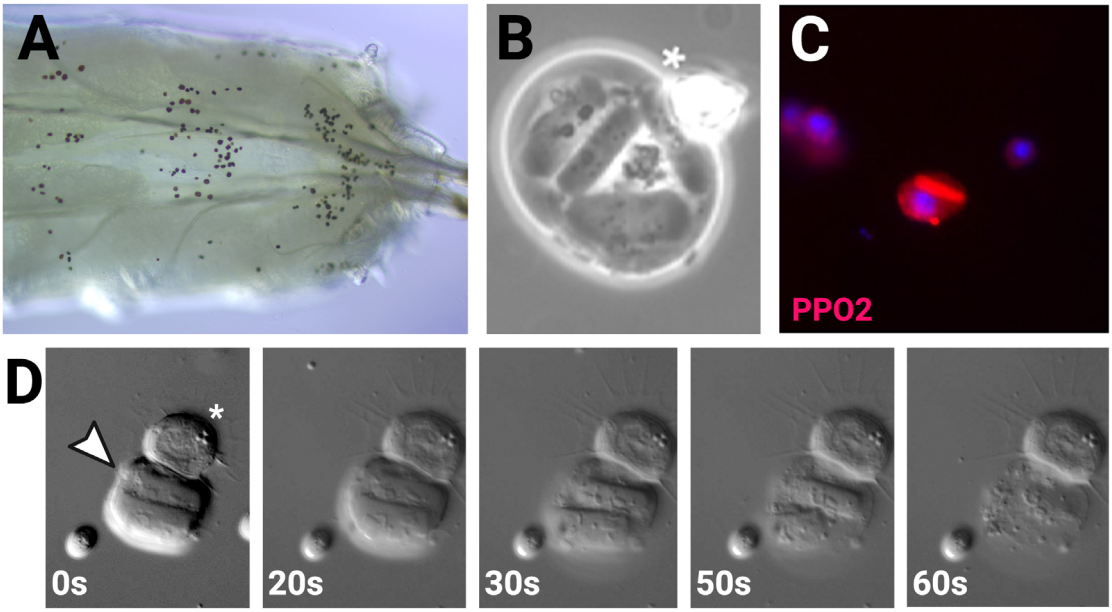


Figure 16 Crystal cells in Drosophila melanogaster
A Posterior end of a larva that has been heated to induce crystal cell rupture and melanization, showing crystal cells adherent underneath the cuticle (Photograph, B. Lemaitre). **B** Light micrograph of a crystal cell; asterisk indicates an adherent fat body fragment. Crystals can be seen as regular rod-shaped structures within the cell (from (Bidla et al., 2007)). **C** Crystal cell stained with PPO2 antibody, showing fluorescent PPO2 crystals within the cell (from (Binggeli et al., 2014)). **D** Time series of crystal cell rupture and PPO2 crystal dissolution (from (Bidla et al., 2007)). Arrowhead indicates crystal cell with rod-shaped crystal inclusions; asterisk indicates a plasmatocyte. Crystal cells rapidly rupture and dissociate upon bleeding, making them difficult to capture or manipulate *ex vivo*.

2008). Conventionally reared[13] larvae and adults have more cuticular sessile crystal cells and produce more PPO, respectively, than age-matched axenic individuals, indicating an impact of the microbiota on hematopoiesis (Benoit et al., 2017). Although its precise role has not been determined, the larvae and adults deficient for the odorant binding protein Obp28A fail to produce crystal cells and have a melanization defect (Benoit et al., 2017).

Crystal cells migrate to wounds and undergo a special form of programmed cell death that results in membrane swelling and cell rupture, releasing PPOs, which lack signal peptides, into the hemolymph (Dziedziech and Theopold, 2021) (**Figure 16**). This process requires the caspase inhibitor DIAP1, the initiator caspase Dronc and the effector caspase Dcp-1 as well as a component of the apoptosome (Dziedziech and Theopold, 2021). It also involves JNK activation by the TNF-related factor Eiger and ROS (Bidla et al., 2007). As in other contexts, ROS likely activates JNK to trigger a caspase cascade

13 Conventionally raised or reared animals refers to animals kept in standard lab conditions with their indigenous microbiota, as opposed to axenic (germ free) or gnotobiotic (reconstituted microbiota) animals.

that induces crystal cell rupture. *Drosophila* crystal cell rupture has similarities to pyroptosis, a programmed cell death pathway that leads to the release of cytokines through membrane pores (Dziedziech and Theopold, 2021). Crystal cell activation is not only induced upon wounding or infection, but by cell surface exposure of negatively charged phospholipids normally confined in the inner layer of the membrane (e.g., phosphatidylserine), which can occur during apoptosis or stress (Bidla et al., 2009). PO activation can be induced by heating (**Figure 16A**) and occurs spontaneously in crystal cells of larvae that carry the gain-of-function *Black cells (PPO1)* mutation (Neyen et al., 2015; Rizki et al., 1980).

7

Systemic wound and stress responses

Drosophila has an open circulatory system and must quickly seal wounds to prevent hemolymph loss and pathogen entry (George and Martin, 2022; Theopold et al., 2004). Wound healing has been studied using assays including large and small punctures, pinching, internal tissue damage using genetically directed apoptosis, and laser ablation in embryos, larvae, pupae, and adults. Although different life stages and tissues affect results, wound healing involves both local and systemic reactions. There is extensive cross talk between immune and repair processes: several factors involved in wound healing and clotting contribute to host defense against pathogens, and several of these are regulated by the Toll and Imd immune pathways (De Gregorio et al., 2002b).

A. Local epithelium repair

The first signal following wounding is an influx of calcium into damaged cells at the wound edge (Razzell et al., 2013; Shannon et al., 2017) (**Figure 17**). This calcium flash spreads across several cell diameters and is dependent on innexins, suggesting transcellular signaling through gap junctions (George and Martin, 2022). A second independent calcium release takes place in more distal cells through activation of the Methuselah 10 G-coupled receptor (Mthl10) by Growth Blocking Peptides[14] Gbp1 and Gbp2, that are themselves activated by proteases released at the injury site (O'Connor et al., 2021). These calcium flashes activate the NADPH oxidase Duox, generating H_2O_2 that stimulates a transcriptomic response and promotes migration of neighboring hemocytes to the wound site (Juarez et al., 2011; Moreira et al., 2010)(see **Box 9**).

Wound repair in the embryonic epithelium involves the contraction of an actomyosin "purse string" in the edge of cells closest to the wound, that acts like stitches to close the wound. Without cell division, cells at the edge of the wound extend dynamic filipodia and lamellipodia that meet to heal the gap, a process involving the small GTPases Rho, Rac, and Cdc42 and integrins (Park et al., 2018; Wood et al., 2002; Wood and Martin, 2017). In larvae and pupae, these cells fuse to form a syncytium, which improves wound re-epithelization compared to diploid cells due to pooling of resources (Galko and Krasnow, 2004; White et al., 2023). More distant cells begin to change shape and intercalate to restore epithelium organization (**Figure 17**).

14 Growth Blocking Peptides (Gbps) are insect-specific cytokines initially identified in Lepidoptera (Matsumoto et al., 2012). They are induced by various stresses through the JNK pathway and can trigger calcium flashes and cell spreading *in vitro* (Ono et al., 2024; Tsuzuki et al., 2012).

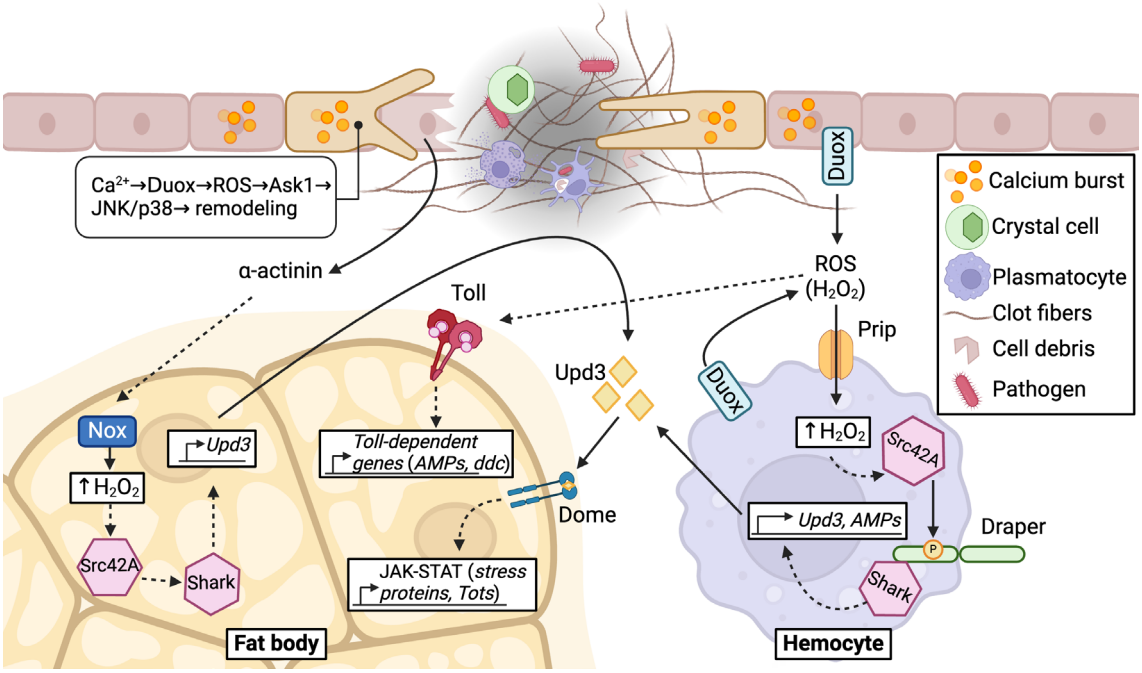


Figure 17 The local and systemic wound responses
The wound site produces signals such as DAMPs (e.g., α-actinin) and ROS that co-ordinate interrelated responses in multiple tissues during wound healing, referred to as the systemic wound response (SWR). Calcium flux in cells near the wound site activates Duox which produces ROS, which activates JNK and p38 signaling required for cellular remodeling that repairs the wound site. Upd3, a ligand of the JAK-STAT pathway that contributes to expression of stress proteins and tissue repair, is produced through processes involving ROS generated by the oxidases Nox and Duox in the fat body and hemocytes, respectively. Upd3 production by the hemocytes in response to septic injury also promotes renewal of the gut epithelium (Chakrabarti and Visweswariah, 2020). ROS also activates Toll signaling through an unknown mechanism and increases expression of genes that resist infection (AMPs), and melanize the wound site (Ddc), promoting clotting. ROS production through Duox at the wound site primes hemocytes to migrate to the wound, which is mediated by an uncharacterized chemoattractant signal. Hemocytes at the wound site contribute clotting and melanization factors in addition to phagocytosis of pathogens and debris. Compiled with data from (Chakrabarti and Visweswariah, 2020; Gordon et al., 2018; Shannon et al., 2017; Srinivasan et al., 2016; Wood and Martin, 2017). Figure created with BioRender.com, CC-BY-NC-ND.

Reactive oxygen species (ROS) induced upon injury play a signaling role, activating a transcriptomic program largely through the JNK and p38 pathways (Lesch et al., 2010; Patterson et al., 2013) (**Figure 18**). Reporter proteins for Puckered (Puc) and Misshapen (Msn) reveal that JNK is activated in a ring around the wound. This pathway involves the successive activation of the JN4K Misshapen (Msn), the JN3K Slipper (Slpr), the JN2K Hemipterous (Hep), and the JNK Basket (Bsk) to induce the dimeric Jun/Fos (AP-1) transcription factor. The JNK pathway induces expression of genes en-

coding cytoskeletal proteins and the metalloproteases Mmp1 and Mmp2, which remodel the basement membrane separating epithelial cells from the hemolymph (Stevens and Page-McCaw, 2012). Re-epithelialization requires a strict balance between *de novo* production and degradation of extracellular matrix. Blocking the JNK pathway prevents epithelial repair, revealing the key role of this pathway in wound healing (Rämet et al., 2002a). Activation of JNK and p38 pathways by ROS may be mediated by a ROS sensitive kinase or phosphatase such as the MAP3K Ask1 (Santabárbara-Ruiz et al., 2019; Serras, 2022). The p38 pathway negatively regulates the JNK pathway to prevent excessive activation leading to apoptosis. In the gut, the p38 target gene MK2 down-regulates JNK (Seisenbacher et al., 2011).

The Toll pathway also contributes to cell adhesion and cytoskeletal rearrangements that lead to epidermal sealing in late-stage embryos (Capilla et al., 2017; Carvalho et al., 2014). This pathway is activated by an unidentified protease downstream of Duox-generated H_2O_2. It regulates the expression of *pale* and *Dopadecarboxylase* (*Ddc*), enzymes involved in melanization cascades. p38c also regulates *Ddc*, showing that multiple pathways integrate wound healing signals to orchestrate expression of the repair program (Davis et al., 2008). Another wound healing pathway involves the activation of Stitcher receptor tyrosine kinase, which through the downstream effectors Drk, Src42a, and ERK, induce formation of the actin ring, re-epithelization, and the Grainy Head transcription factor. Grainy Head is critical to repair protective cuticle layers of the wounded epidermis, regulating expression of wound repair genes such as *Ddc* and *pale* (Wang et al., 2009). Wound healing is also accompanied by antioxidant responses mediated by the Nrf2 pathway and DNA repair by GADD45 (Stramer et al., 2008; Weavers et al., 2019).

Hemocytes are thought to clean up the wound by phagocytosing debris, and by restoring extracellular matrix. Local production of antimicrobial peptides by hemocytes might also help prevent infection. In embryos, only mature macrophages that have taken up apoptotic corpses move towards wounds (Weavers et al., 2016a). Mathematical modelling demonstrates that the speed of the chemotactic signal coming from the wound travels much slower than H_2O_2, indicating that an uncharacterized alternate factor attracts hemocytes to the wound (Weavers et al., 2016b). However, H_2O_2 remotely primes hemocyte migration, which is transduced through a Src42a-Draper-Shark-mediated signaling axis (Evans et al., 2015) (**Figure 17**). Surprisingly, fat body cells can exhibit 'hemocyte behavior' upon injury in pupae, migrating to plug the wound and phagocytose wound debris (Franz et al., 2018). In larvae, circulating plasmatocytes encountering the wound attach to it without the need for chemoattractants (Babcock et al., 2008; Pastor-Pareja et al., 2008). In larvae but not embryos, injury produces a scab composed of debris crosslinked by melanization to protect the underlying epithelium as it heals (Galko and Krasnow, 2004).

B. Clotting

Coagulation or clotting is the formation of an insoluble matrix that stops bleeding, promotes wound healing, and protects against infection (Dushay, 2009; Theopold et al., 2014) (**Figure 19**). Clotting has primarily been studied in larvae using *ex vivo* and proteomic approaches (Scherfer et al., 2006; Karlsson et al., 2004). The larval clot involves both plasma factors produced by the fat body (Fondue, lipoproteins Lipophorin I and II, hexamerins and possibly Gelsolin) and by hemocytes (Transglutaminase, Hemolectin,

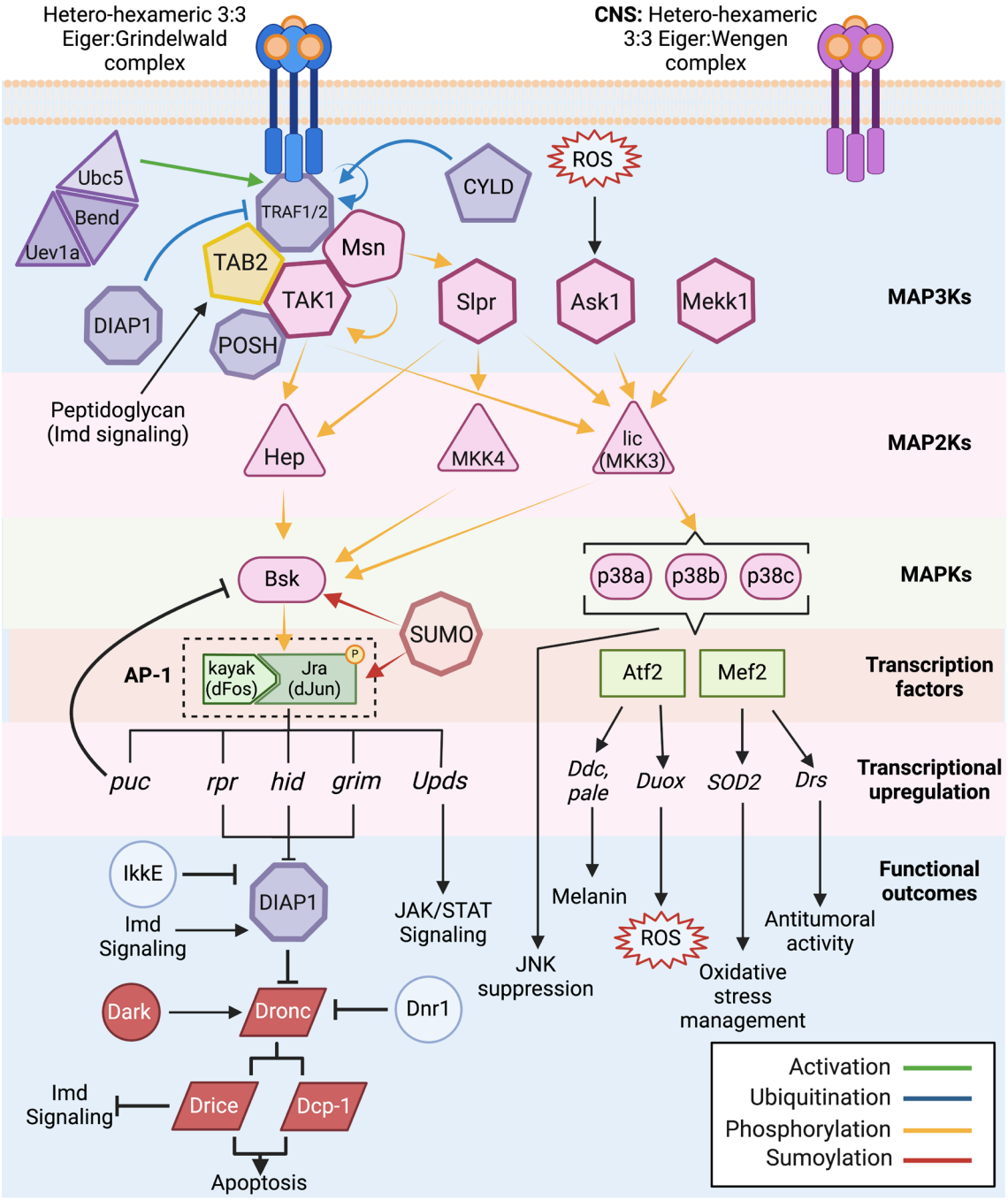

Hetero-hexameric 3:3 Eiger:Grindelwald complex
CNS: Hetero-hexameric 3:3 Eiger:Wengen complex
Ubc5
Bend
Uev1a
TRAF1/2
CYLD
ROS
TAB2
Msn
TAK1
POSH
DIAP1
Slpr
Ask1
Mekk1
MAP3Ks
Peptidoglycan (Imd signaling)
Hep
MKK4
lic (MKK3)
MAP2Ks
Bsk
p38a
p38b
p38c
MAPKs
SUMO
AP-1
kayak (dFos)
Jra (dJun)
P
Atf2
Mef2
Transcription factors
puc
rpr
hid
grim
Upds
Ddc, pale
Duox
SOD2
Drs
Transcriptional upregulation
IkkE
Imd Signaling
DIAP1
JAK/STAT Signaling
Melanin
ROS
Antitumoral activity
JNK suppression
Oxidative stress management
Functional outcomes
Dark
Dronc
Dnr1
Imd Signaling
Drice
Dcp-1
Apoptosis
Activation
Ubiquitination
Phosphorylation
Sumoylation

Figure 18 The Drosophila MAPK pathways
Schematic of *Drosophila* JNK and p38 MAPK (Mitogen-Activated Protein Kinase) pathways. MAP3Ks initiate JNK or p38 signaling in developmental processes or in response to a variety of stresses such as UV damage, high osmolarity, heat shock, ER stress, or loss of cell apico-basal polarity. The MAP3K Ask1 appears to be directly activated by ROS (Santabárbara-Ruiz et al., 2019). Additional MAP3Ks with minor or poorly studied roles (Wallenda, TAK1-like 1, TAK1-like 2) are not shown. Specificity in this pathway is strongly reliant on temporal and spatial expression of kinases, such that functions and interactions of kinases may differ greatly depending on tissue and developmental stage. Recent evidence shows that the Grindelwald receptor (TNF-R homolog) mediates apoptotic functions of the JNK pathway, whereas the Wengen homolog functions predominantly in the central nervous system (CNS) (Colombani and Andersen, 2023). The *Drosophila* Wengen cytoplasmic domain is unique with no sequence homology to any mammalian TNFR family members, and lacks both the expected TRAF-binding domain and death domain (Colombani and Andersen, 2023). JNK activity is spatially restricted by low diffusibility of the TNF homolog Eiger. JNK controls expression of many genes required for cytoskeletal components, and is strongly activated in cells undergoing migration, wound healing, or shape change (Boutros et al., 2002; Galko and Krasnow, 2004; Rämet et al., 2002a). Imd and JNK activity are interrelated: (**i**) they share the TAK1/TAB2 complex, allowing JNK to be activated by peptidoglycan upstream of Imd (Hua et al., 2022); (**ii**) they share the ubiquitin ligase POSH, which also has essential scaffolding roles (Tsuda et al., 2005; Zhang et al., 2010); (**iii**) Relish activity upregulates *DIAP1* to suppress JNK, and can negatively affect developmental processes (Tavignot et al., 2017) (**iv**) JNK activity suppresses DIAP2 activity through Drice to attenuate Imd pathway activity (Kietz et al., 2022); and (**v**) overactivation of JNK resulting in tissue damage activates Imd signaling. AP-1 binding may also displace Relish to downregulate expression of certain genes including AMPs (Kim et al., 2007). Many of the results tying JNK directly to immunity in *Drosophila* are marred by the fact that widely used mutants of *eiger* bore a secondary mutation of the phagocytic receptor *NimC1* (Kodra et al., 2020). p38 MAPKs (p38a, p38b, p38c) have somewhat overlapping functions in development, but appear to have more specific roles in stress responses. p38 signaling may positively regulate the stability of some AMP mRNAs through AU-rich elements (AREs) (Wei et al., 2009). p38 signaling is also involved in autophagosomal degradation of ubiquitinated protein aggregates, which may include intermediates in immune signaling (Belozerov et al., 2014; Ryan et al., 2021). Pathway compiled with data from: (Andersen et al., 2015; Chakrabarti et al., 2014; Chen et al., 2010; Geuking et al., 2009; Karkali and Panayotou, 2012; Krautz et al., 2020; Kuranaga et al., 2002; La Marca and Richardson, 2020; Mathew et al., 2011; Nishida et al., 2021; Patel et al., 2019; Primrose et al., 2007; Seisenbacher et al., 2011; Sekine et al., 2011; Tafesh-Edwards and Eleftherianos, 2020; Zhuang et al., 2006). Figure created with BioRender.com, CC-BY-NC-ND.

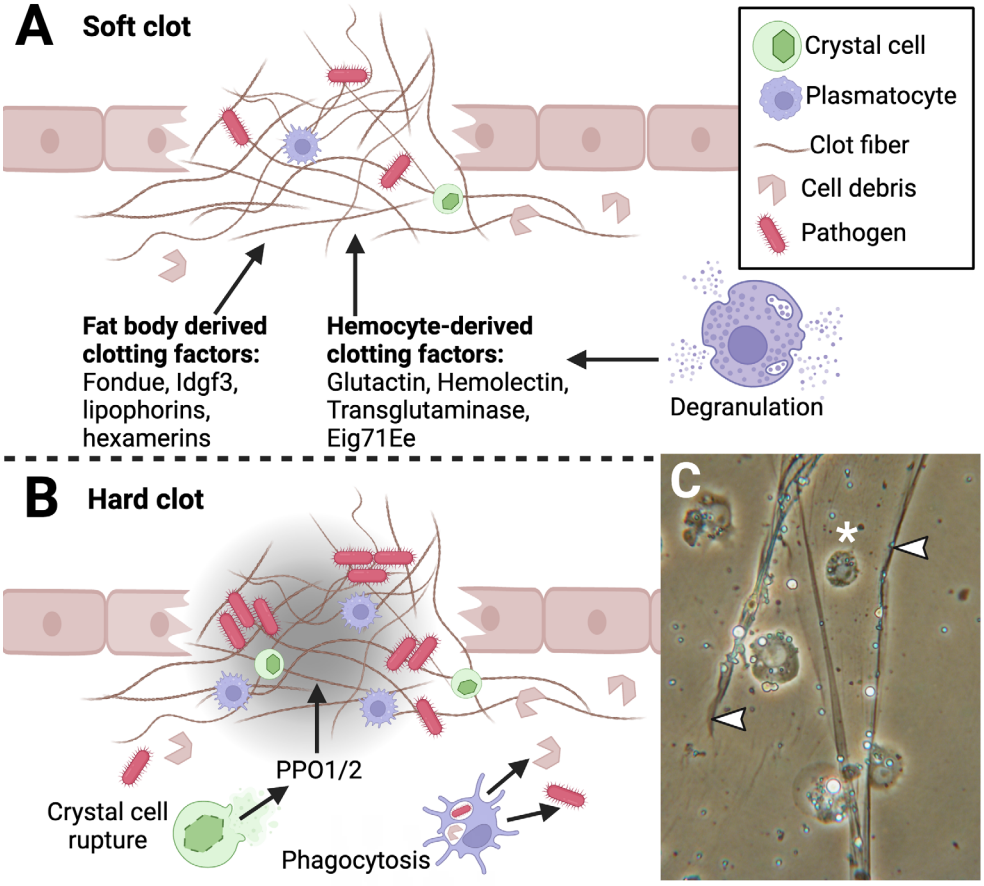


Figure 19 Clotting in Drosophila
A Soon after wounding, a soft clot forms. Clot fibers incorporate both hemolymph- and hemocyte-derived factors. Transglutaminase released from the hemocytes through exosomes cross-links the soft clot, stabilizing it and immobilizing pathogens (Dziedziech et al., 2020; Schmid et al., 2019; Theopold et al., 2014). **B** Phenoloxidases released from crystal cells harden the clot and deter pathogen growth. Phagocytosis removes debris from the wound site and reduces pathogen dissemination. **C** Micrograph of a *Drosophila* clot with fibers (**arrowheads**) and incorporated plasmatocytes (**asterisk**). Photo courtesy of Ulrich Theopold, Stockholm University. Figure created with BioRender.com, CC-BY-NC-ND.

Eig71Ee and prophenoloxidases) (Scherfer et al., 2004, 2006, 2008; Karlsson et al., 2004; Korayem et al., 2004). An unknown signal induces degranulation of plasmatocytes, releasing Hemolectin (a protein that includes a von Willebrand factor domain) and Eig71Ee, which interact with plasma factors Lipophorin and Fondue, a clot structural protein with multiple repeats rich in glycine, alanine, and glutamine. These proteins are then crosslinked by Transglutaminase at lysine and glutamine residues, forming a soft clot composed of fibers and trapped hemocytes (Lindgren et al., 2008) (**Figure 19A**). Transglutaminase is the only *Drosophila* clotting factor that is conserved in vertebrates,

sharing homology with Factor XIIIa (Wang et al., 2010). This protein does not have a signal peptide and is thought to be secreted by exosomes (Dziedziech et al., 2019). The chitin binding protein IDGF3 is also required for efficient clotting and wound healing. Its mode of action is not yet known, but it may localize the clotting reaction by promoting interactions between the clot and cuticle, or function as a damage sensor to activate downstream programs (Kucerova et al., 2016). The primary soft clot is then hardened by melanization through PPO1 and PPO2 to generate a stronger mature clot (Bidla et al., 2005) (**Figure 19B**).

Larvae have hydrostatic support and must rapidly prevent hemolymph loss, making clotting especially important at this life stage. Hemolymph from *fondue* or *hemolectin* loss-of-function mutants fails to aggregate beads and forms long, atypical strands (Bajzek et al., 2012; Chang et al., 2012). The clotting reaction is thought to be reduced or absent in adults, as the hard cuticle provides a rigid scaffold that restricts hemolymph leakage. Clotting-defective adult flies display only mild susceptibility to injury and subtle immune defects (e.g., Binggeli et al., 2014; Lindgren et al., 2008; Nam et al., 2012), which may suggest that clotting and melanization are somewhat redundant in terms of sealing the wound. Beyond preventing hemolymph loss, clots entrap bacteria and promote killing in a process reminiscent of nodulation, an immune process where pathogens are trapped by hemocytes and cross-linking factors (Miller et al., 1994; Satyavathi et al., 2014). Processes similar to nodulation have been reported in *Drosophila* species but are not well characterized. Hemocytes of *Drosophila suzukii* have been shown to form extracellular traps (Carrau et al., 2021). In *Drosophila melanogaster*, the glucan sensor GNBP3 has been reported to agglutinate yeast cells in the hemolymph to produce melanized aggregates, but it is unclear if this is connected to clotting (Matskevich et al., 2010).

Clotting factors are also involved in resistance to entomopathogenic nematodes, which cause wounds with specialized mouthparts and enter the host either via the cuticle or the gut (Arefin et al., 2014; Hyrsl et al., 2011; Kucerova et al., 2016; Wang et al., 2010).

C. The systemic wound response

The wound site functions as a coordinator that generates signals affecting remote organs, referred to as the Systemic Wound Response (SWR) (Lee and Miura, 2014) (**Figure 17**). Integument injury and melanization in adults produces hemolymphatic ROS, leading to JNK activation in neurons that promotes a whole-body cytoprotective program that appears critical in surviving injury (Nam et al., 2012). However, the JAK-STAT pathway is the primary coordinator of the systemic wound response (**Figure 20**). Integument or internal damage triggers expression and secretion of the cytokines Upd2 and Upd3 (and maybe Upd1) by hemocytes, which bind to the Domeless receptor in remote organs such as the gut, muscles and fat body to activate the JAK-STAT pathway (Agaisse et al., 2003; Chakrabarti and Visweswariah, 2020; Pastor-Pareja et al., 2008). This Upd response to wounding remotely controls intestinal stem cell proliferation in the midgut (Chakrabarti et al., 2016; Takeishi et al., 2013), expression of stress proteins such as Turandots by the fat body (Agaisse et al., 2003; Brun et al., 2006; Rommelaere et al., 2024) (see **Figure 21**), and metabolic regulation in muscles (Kierdorf et al., 2020; Woodcock et al., 2015) (see **Figure 27**). Interestingly, activation of the JAK-STAT pathway in muscles by Upds produced in hemocytes stimulates lamellocyte differentiation, revealing an unexpected immune role of muscle tissue (Yang and Hultmark, 2016).

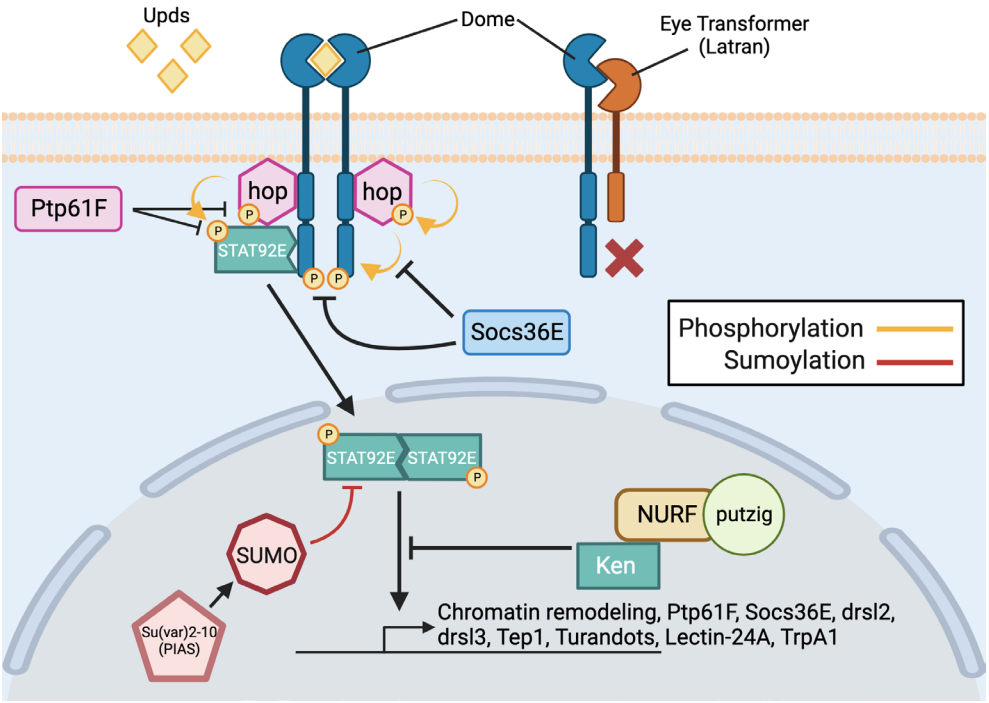


Figure 20 The JAK-STAT signaling pathway
Schematic of the *Drosophila* JAK-STAT signaling pathway. JAK-STAT signaling participates in wound healing, epithelial renewal, resilience and hematopoiesis. Upd cytokines (Upd1, Upd2, Upd3) bind to the Domeless (Dome) receptor, initiating phosphorylation of the STAT92E transcription factor by the Hopscotch (Hop) kinase. Feedback inhibitors fine-tune pathway activity: Socs36E destabilizes Domeless and inhibits Hop kinase activity, while the phosphatase Ptp61F antagonizes Hop and STAT92E phosphorylation. In the nucleus, JAK-STAT activity is further controlled by sumoylation through the Sumo E3 ligase Su(var)2-10 (PIAS) and the DNA-binding protein Ken, which with the NURF complex selectively inhibits expression of some JAK-STAT targets by disrupting STAT92E binding (Kwon et al., 2008). Upon wasp parasitization, upregulation of the inhibitory receptor homolog *eye transformer* (Latran) and downregulation of the active receptor *domeless* decreases JAK-STAT activity and promotes differentiation of lamellocytes (Makki et al., 2010). Pathway inspired by Amoyel et al., 2014; Bina and Zeidler, 2009; Myllymäki and Rämet, 2014; Stec et al., 2013; Valanne et al., 2010. Figure created with BioRender.com, CC-BY-NC-ND.

Several pathways have been shown to regulate Upd ligands in response to wounding. In wounded imaginal discs, Upds are induced by JNK and p38 downstream of Duox-produced ROS (Santabárbara-Ruiz et al., 2015). In the gut, *Upd3* transcription is regulated by multiple pathways, including Hippo, p38, TGF-β/Dpp, and Src (Houtz et al., 2017). In response to integument wounds of adults, Duox-produced ROS enters hemocytes through the aquaporin channel Prip to trigger *Upd3* expression via a Src42A/Draper/Shark pathway (Chakrabarti and Visweswariah, 2020). Finally, injection of actinin in *Drosophila* triggers *Upd3* expression by the fat body via Shark and Src42A, a process that is dependent on activity of the NADPH oxidase Nox (Srinivasan et al., 2016)

(**Figure 17**). Actinin is an intracellular cytoskeletal protein that is released upon injury or cell death and may acts as a Damage Associated Molecular Pattern (DAMP) (Gordon et al., 2018). Wounds, including sterile pinch wounds which damage the epithelium without affecting the overlying cuticle, activate low-level expression of antimicrobial peptide genes through the Toll and Imd pathways in the fat body (Kenmoku et al., 2017; Nainu et al., 2019). This is an example of sterile inflammation, as expression of antimicrobial peptides still occurs upon pinching in germ-free larvae (Nainu et al., 2019; Shaukat et al., 2015). Blocking apoptosis in wing epidermal cells also induces Toll activation via Hayan/Psh in the absence of infection (Ming et al., 2014; Nakano et al., 2023; Obata et al., 2014). Overexpression of *Duox* in hemocytes is also sufficient to activate the Toll pathway in the absence of wounding (Chakrabarti and Visweswariah, 2020). Thus, sterile wounding can activate Toll and Imd pathways to a certain extent, through activity of ROS and possibly proteases. That the JAK-STAT pathway is involved in many processes including stress, resilience and the wound response might explain the multiple mechanisms that lead to the expression of its Upd ligands. Further studies are required to clarify how these multiple pathways intersect in various contexts.

8

Systemic infection: Tolerance mechanisms

Systemic responses to wounds or infection are accompanied by increased activity of proteases, cationic peptides, and ROS that can be deleterious to the host. Consequently, several disease tolerance mechanisms have evolved to attenuate negative impacts of immune or wound effectors. In contrast to wound healing and immunity genes, tolerance genes are induced with late and sustained kinetics, with complex regulation by the Toll, Imd, JNK, JAK-STAT, and p38 pathways (Agaisse et al., 2003; Brun et al., 2006).

A. Protection from ROS

Reactive oxygen species (ROS) production is generic to many stress and immune reactions and must be rapidly detoxified by enzymes such as catalases (see **Box 9**). *Immune regulated catalase* (*IRC*) encodes a secreted catalase that is strongly induced upon infection, and likely acts to control ROS in the hemolymph (Nam et al., 2012; Prakash et al., 2021; Westlake et al., 2024). The *rosy* gene encodes *Drosophila* Xanthine Dehydrogenase/Oxidase (XDH/XOD) which catalyzes the oxidation of xanthine to uric acid, a ROS scavenger. Rosy has a protective role with respect to both ROS and nitric oxide (NO); *rosy* deficient flies have increased susceptibility to bacterial infection (Kim et al., 2001).

Infection and stress also deplete hemolymphatic lipids, which are excreted through the Malpighian tubules. Lipid re-localization is mediated by Materazzi, a stress-induced lipid binding protein. *Materazzi* deficient flies are more susceptible to many stresses, indicating that reduction of hemolymphatic lipids is essential for survival (Li et al., 2020a). This process likely protects hemolymph from damaging effects of ROS by preventing lipid peroxidation[15], and subsequent tissue damage. In addition to xanthine dehydrogenase, ROS may be detoxified by glutathione peroxidase (GST) which is upregulated by wounding and infection, and catalases such as immune regulated catalase (IRC), Jafrac1 and superoxide dismutase (SOD). Hemocytes are also thought to serve a central role in resistance to oxidative stress through JNK-mediated induction of *Upd3* in response to oxidative DNA damage (Hersperger et al., 2023), which presumably promotes tolerance through JAK-STAT signaling. The KEAP1-NRF2 pathway is the principal pathway that protects the host against oxidative stress. Under homeostatic conditions (Gerasimos and Bohmann, 2008), KEAP1 forms part of an E3 ubiquitin ligase, which tightly reg-

15 Lipid peroxidation involves the production of reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These aldehydes can in turn generate more ROS, leading to chain reactions that form protein and DNA-adducts that disrupt function and cause cell death.

ulates the activity of the transcription factor NRF2 by targeting it for proteasome-dependent degradation. Detection of ROS by sensor cysteines of KEAP1 allows NRF2 to escape ubiquitination and translocate to the nucleus, where it promotes an antioxidant transcription program. The gene encoding KEAP1 is induced upon systemic infection, pointing to an important role of this pathway in ROS detoxification during the immune response (De Gregorio et al., 2002b).

B. Filtration and cleaning the hemolymph

Malpighian tubules play an important role in osmoregulation and waste removal, analogous to the mammalian kidney (Chapman et al., 2013; Cohen et al., 2020a). Malpighian tubule activity is under endocrine control by neuropeptides such as Dh44 (Cabrero et al., 2002; Cannell et al., 2016). Interestingly, microarray data have shown that expression of Dh44 is induced upon immune challenge (De Gregorio et al., 2002b), and likely increases tubule filtering activity. The role of Malpighian tubules in immunity is more fully discussed below (see Gut and Epithelial Immunity, page 109).

Scavenging of serpin/proteinase complexes and other secreted proteins from the hemolymph may also be a critical step in the regulation of proteolytic cascades and maintenance of homeostasis (Soukup et al., 2009). This is accomplished by two groups of nephrocytes, the garland cells surrounding the esophagus and pericardial cells flanking the heart. Nephrocytes can also sequester microbiota-derived peptidoglycan from the hemolymph and degrade it inside lysosomes, preventing Toll pathway activation (Troha et al., 2019).

C. Protection of host tissues from antimicrobial peptides

While antimicrobial peptides (AMPs) are protective against pathogens, these cationic peptides can be cytotoxic to host cells in certain contexts. Tracheal cell membranes of *Drosophila* expose high levels of the negatively charged phospholipid phosphatidylserine, sensitizing them to the action of AMPs which are attracted to negatively charged bacterial membranes (Rommelaere et al., 2024). A family of eight stress-induced proteins, the Turandots, protect *Drosophila* host tissues from AMPs, increasing tolerance to stress (Ekengren and Hultmark, 2001, Ekengren and Hultmark, 1999; Rommelaere et al., 2024). Turandots are induced by both immune and stress pathways in the fat body (Agaisse et al., 2003; Brun et al., 2006; Ekengren et al., 2001) and bind to tracheal cells to protect them against AMPs. *In vitro*, Turandot A binds to phosphatidylserine on membranes and inhibits the pore-forming activity of *Drosophila* and human AMPs on eukaryotic cells without affecting microbicidal activity (Rommelaere et al., 2024) (**Figure 21**).

Strikingly, basal Turandot expression by epithelia and the fat body protects the respiratory epithelium during pupariation. During metamorphosis, larval tracheae undergo histolysis and adult tracheae arise from pupal progenitors. Both Turandots and antimicrobial peptides are highly expressed during this stage (Ekengren et al., 2001; Ekengren and Hultmark, 2001; Kappler et al., 1993; Reichhart et al., 1992; Samakovlis et al., 1990; Tryselius et al., 1992), where antimicrobial peptides are thought to play a prophylactic role to prevent infection by bacteria escaping the gut during metamorphosis

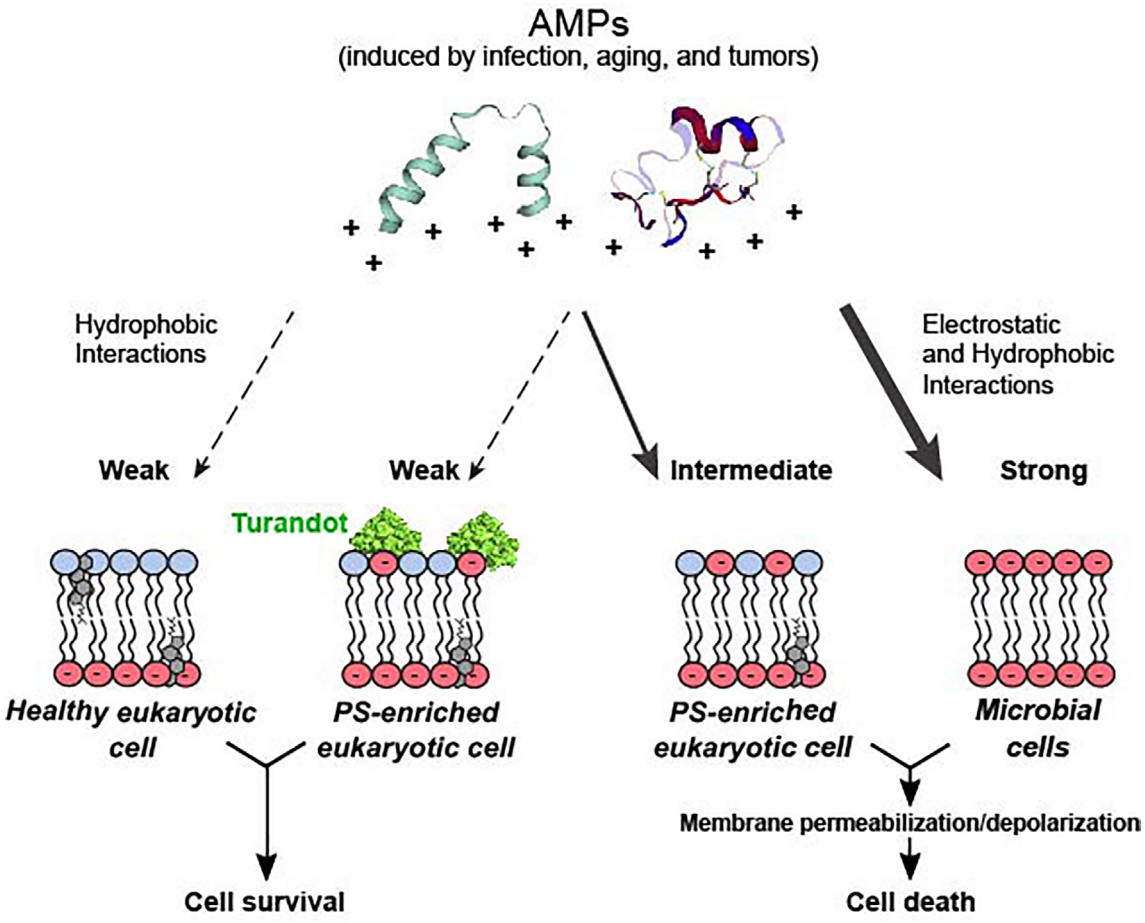


Figure 21 Impact of antimicrobial peptide and Turandot activity on bacteria and host cells
AMPs are small cationic and amphipathic peptides that interfere with the negatively charged membranes of microbes (**far right**). Because of their amphipathic nature and positive charge, AMPs can bind to the membrane and form pores or otherwise disrupt membrane integrity. Eukaryotic cells are usually insensitive to AMPs as their membranes contain cholesterol and are less negatively charged than microbes (**far left**). Recent studies have shown that some eukaryotic cells including certain cancer cells and *Drosophila* tracheal cells expose phosphatidylserine (PS) at the surface, making them more negatively charged (**middle right**). Turandots can bind to the surface of PS-enriched host tissues to mask PS and selectively protect these membranes from the action of cationic AMPs, without disrupting AMP activity against prokaryotic cells (**middle left**) (Hanson and Lemaitre, 2020; Rommelaere et al., 2024).

(Nunes et al., 2021). High Turandot expression during metamorphosis likely protects tracheae from high antimicrobial peptide expression at this stage. The immune response during the four-day process of pupariation has so far received little attention, perhaps due to methodological difficulties in studying this stage.

9

Systemic immunity: Cellular response

Drosophila possess specialized hemocyte types that participate in a wide range of processes including development, immunity, metabolism and wound healing (Honti et al., 2014; Hultmark and Andó, 2022) (**Figure 22**). These incredibly plastic and motile cells perform diverse functions including deposition and remodeling of the extracellular matrix, metabolic regulation, management of oxidative stress, inter-organ signal transduction, and production of immune effectors. The many functions of hemocytes are dependent on their motility and ability to recognize and respond to a variety of signals via cell surface receptors. These processes are fundamentally dependent on vesicle trafficking, which dynamically delivers receptors required for recognition and adhesion to the cell surface and allows remodeling of the cytoskeleton and plasma membrane. Cytoskeletal remodeling is required for the formation of filopodia and lamellipodia involved in hemocyte functions such as motility or phagocytosis.

A. Hematopoiesis

Embryonic hematopoiesis produces several hundred blood cells that proliferate throughout the larval stage to become the ~5000 circulating and resident (sessile) hemocytes present in the third instar (see (Banerjee et al., 2019; Evans et al., 2021)) for extensive review). In the larva, hemocytes are found in three compartments: (**i**) the lymph gland, a central hematopoietic organ that functions as a reservoir that releases hemocytes after parasitic infection or at metamorphosis, (**ii**) circulating in the hemolymph and (**iii**), in sessile[16] patches between the cuticle and muscle layers (Crozatier and Meister, 2007; Evans et al., 2003; Honti et al., 2010; Jung, 2005; Lanot et al., 2000; Makhijani et al., 2011; Makhijani and Brückner, 2012). Depletion of embryonic hemocytes in larvae triggers overgrowth and premature differentiation of lymph gland as a consequence of decreased extracellular matrix production (Monticelli et al., 2024). This indicates a connection between the early (embryonic) and late phases (lymph gland) of hematopoiesis. Hemocytes in the adult fly constitute a mix of embryonic and lymph-gland derived hemocytes, and are largely found in populations adherent to the respiratory epithelia, ostia and heart (Ghosh et al., 2015; Sanchez Bosch et al., 2019). Evidence suggests that no significant hematopoiesis occurs in the adult fly (Boulet et al., 2021; Sanchez Bosch et al., 2019). Indeed, the total number of hemocytes declines throughout adult life, even in

16 Sessile or adherent hemocytes are those attached to tissues rather than free-floating in the hemolymph.

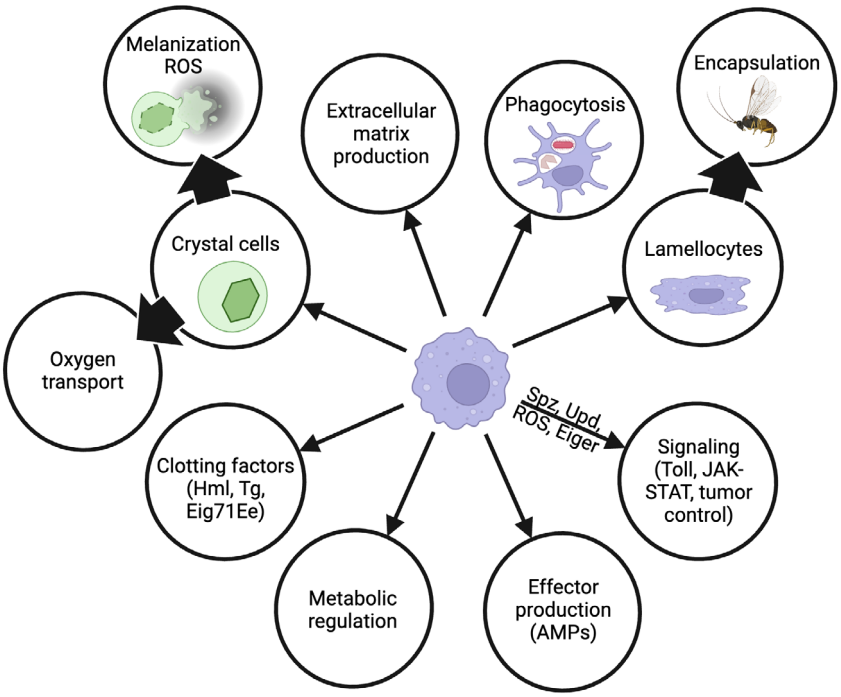


Figure 22 Diverse roles of plasmatocytes
Plasmatocytes can transdifferentiate into crystal cells or lamellocytes, with roles in melanization and oxygen transport or encapsulation, respectively. Plasmatocytes are essential producers of many secreted proteins including components of the extracellular matrix (Fessler et al., 1994; Nelson et al., 1994; Tepass et al., 1994), antimicrobial peptides, ligands of the JAK-STAT and Toll pathways, and clotting factors. Phagocytosis contributes to both pathogen defense and wound healing, while cytokine production by hemocytes contributes to tumor elimination, metabolic regulation, and gut epithelial renewal. Figure created with BioRender.com, CC-BY-NC-ND.

the first week, and decreases more rapidly upon infection suggesting that hemocytes are consumed during defense and not replaced (Mackenzie et al., 2011). However, bacterial challenge does upregulate genes commonly used as markers for hemocytes (*hml, crq*), which can cause the false impression of hemocyte proliferation.

Drosophila larvae and adults have two major hemocyte types: plasmatocytes, which are macrophage-like, and crystal cells, rounded hemocytes which contain crystallized prophenoloxidases (PPO) (Lanot et al., 2000) (see Systemic immunity: Melanization, page 71). In larvae, a third hemocyte type, the lamellocytes, can differentiate from progenitor cells in the lymph gland or from peripherical plasmatocytes in response to wasp infestation or non-self recognition (see Autoimmunity, page 127) (Anderl et al., 2016). *Drosophila* plasmatocytes are plastic and can transdifferentiate into crystal cells or lamellocytes (Anderl et al., 2016; Leitão and Sucena, 2015; Márkus et al., 2009). Single

cell analysis has revealed additional distinct hemocyte populations including AMP-producing plasmatocytes and immature lamellocytes, as well as many less well-defined hemocyte cell states representing either intermediate differentiation stages or various transient functional programs engaged by plasmatocytes (Brooks et al., 2024; Cattenoz et al., 2020; Cho et al., 2020; Coates et al., 2021; Hersperger et al., 2023; Hultmark and Andó, 2022; Tattikota et al., 2020). These studies also reveal a cell type, the primocytes, that are found in circulation and the posterior signaling center[17] (PSC) and may control lamellocyte differenciation (Hultmark and Andó, 2022).

Sessile hemocytes are attached in a segmental pattern to the larval body wall, closely associated with secretory cells called oenocytes and endings of peripheral neurons, which secrete activin-β to regulate hemocyte adhesion (Makhijani et al., 2011; Márkus et al., 2009) (**Figure 23**). Adhesion depends on the interaction between the membrane receptor Eater on the hemocytes and the specialized collagen Multiplexin in the extracellular matrix (Bretscher et al., 2015; Csordás et al., 2020). As a consequence, *eater*-deficient larvae have no sessile hemocytes. Loss of these neuronal microenvironments through mutation of *Dscam1* results in reduced hemocyte numbers (Ouyang et al., 2020). Recruitment to these patches contributes to plasmatocyte proliferation and transdifferentiation to terminal hemocyte types (Leitão and Sucena, 2015). Hemocytes leave the sessile patches and enter circulation upon wasp infestation, infection, or mechanical stimulation of the cuticle (Márkus et al., 2009; Makhijani et al., 2011). The function of the sessile hemocyte patches is not yet established, but it has been proposed that they constitute **i)** a diffuse hematopoietic organ (Márkus et al., 2009; Makhijani et al., 2011), **ii)** storage for easily-deployed hemocytes (Bretscher et al., 2015), **iii)** localized environments allowing neural control of hematopoiesis (Makhijani et al., 2017) or, **iv)** sites where hemocytes contribute to increase oxygenation (Shin et al., 2024).

Hemocyte division and differentiation can occur in all hemocyte compartments, and are influenced by internal (insulin, ecdysone) and external cues (olfactory cues, injury, wasp infestation) (Madhwal et al., 2020; Shim et al., 2013, 2012; Tian et al., 2023). The balance between differentiation and proliferation of hemocytes is essentially controlled by varying levels of JAK-STAT activity (e.g., (Krzemień et al., 2007)), which can be influenced by input from multiple pathways including Toll (Louradour et al., 2017) and Relish (Ramesh et al., 2021). Maintenance and migration of hemocytes relies on the PVR receptor and its ligands PVF2 and PVF3 (Bond and Foley, 2012, 2009; Bruckner et al., 2004; Munier et al., 2002) as well as the FGF receptor Heartless and its ligand Pyramus (Banerjee et al., 2019; Dragojlovic-Munther and Martinez-Agosto, 2013; Ramond et al., 2020a). In larvae, bacterial infection and activation of the Toll or Imd pathways triggers the release of sessile hemocytes into circulation and early dissociation of the lymph gland, increasing the number of hemocytes available for defense. Care should be taken using mutations that trigger hemocyte differentiation, reduce cell adhesion, or cause premature lymph gland rupture, as these processes can lead to changes in the number of circulating hemocytes, but this is often due to loss of sessile hemocytes and not due to genuine change in total hemocyte number.

17 The Posterior Signaling Center (PSC) is a group of cells in the primary lobe of the lymph gland that play a key role in regulating hematopoietic progenitor differentiation. The PSC contributes to the cellular immune response to wasp parasitism, which is triggered by elevated ROS levels and regulated by JAK-STAT and Toll activity (Banerjee et al., 2019; Benmimoun et al., 2015; Evans et al., 2021; Krzemień et al., 2007).

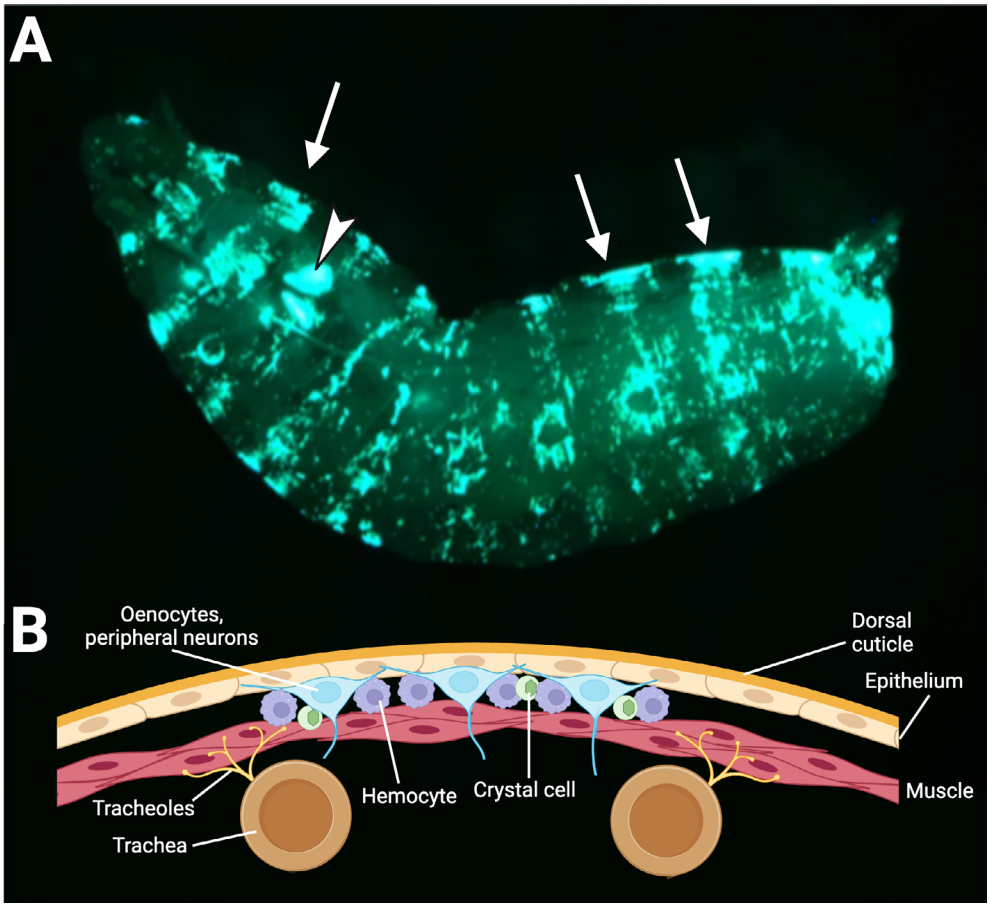


Figure 23 Larval hematopoiesis and hemocyte sessility
A Larva expressing GFP in the hemocytes (*Hml>UAS-GFP*), showing a segmental banding pattern of sessile hemocytes (**arrows**) and lymph gland (**arrowhead**). Scattered hemocytes can also be seen circulating in the third hemocyte compartment, the hemolymph (Evans et al., 2003; Honti et al., 2010; Lanot et al., 2000; Makhijani et al., 2011). Adapted from (Bretscher et al., 2015).
B Schematic of attachment of sessile hemocytes to the body wall. Secretory oenocytes and peripheral neurons are shown in **blue**, hemocytes in **purple**, and crystal cells in **green.** Sessile hemocytes are attached to the internal surface of the larval body wall, forming patches, some of which are closely associated with secretory oenocytes and peripheral nerve endings (Makhijani et al., 2011). Hemocytes continuously exchange between sessile patches and the circulation (Babcock et al., 2008; Bretscher et al., 2015; Makhijani et al., 2011; Márkus et al., 2009; Welman et al., 2010). Figure created with BioRender.com, CC-BY-NC-ND.

B. Phagocytosis

Phagocytosis is a stepwise process consisting of (**i**) particle binding by the phagocyte, (**ii**) internalization of the particle into a phagosome, (**iii**) phagosome maturation, and (**iv**) destruction of the particle following lysosomal fusion, which subjects the particle to enzymatic activity, acidity and ROS (reviewed in (Melcarne et al., 2019a; Ulvila et

al., 2011)). Disruption of any one of these steps typically reduces phagocytic capacity and influences development and immunity. The particle destruction step resulting in pathogen killing is poorly characterized in *Drosophila*, but is thought to involve lysosomal enzymes (DNase II, Stress Induced DNase (SID), Cathepsin) and production of ROS (Brennan et al., 2007; Myers et al., 2018; Seong et al., 2014, 2006).

An ecdysone pulse at the end of the larval stage increases expression of hemocyte cell surface receptors including the Imd receptor PGRP-LC, further shaping the adult immune response (Rus et al., 2013). Hemocytes clear bacteria opportunistically crossing the gut barrier into the hemolymph in homeostatic conditions, preventing infection and widespread immune activation (Braun et al., 1998). Following ingestion, pathogenic *Serratia marcescens* Db11 accumulate in the hemolymph of phagocytosis-impaired adults (Nehme et al., 2007) (see **Box 9**, Systemic immune activation in response to oral infection, page 114). Phagocytosis contributes with other immune processes to combatting infections (Charroux and Royet, 2009; Defaye et al., 2009; Shia et al., 2009, Shinzawa et al., 2009), but only rarely is it the major deciding factor in survival (Elrod-Erickson et al., 2000; Nehme et al., 2011; Ryckebusch et al., 2024). Circadian rhythm also contributes to phagocytic activity: mutation of circadian rhythm genes prevents a burst of phagocytic activity that occurs at night (Stone et al., 2012) and increases sensitivity to certain pathogens (Lee and Edery, 2008; Shirasu-Hiza et al., 2007; Stone et al., 2012).

i) Cell-surface receptors

Phagocytic receptors bind molecules that identify apoptotic cells, pathogens, and other particles as targets for destruction (**Figure 24**). Phagocytic receptors further engage downstream signaling to trigger particle uptake. Phagocytic uptake is complex and involves multiple receptors with both specific and overlapping functions. Many phagocytic receptors of *Drosophila* belong to the Nimrod family, a group of 12 proteins that contain specialized adhesive EGF repeats (NIM repeats). The Nimrod receptors Eater and NimC1 play a key role in phagocytosis of multiple targets. The N-terminal EGF repeats of Eater bind to *Staphylococcus aureus* or *Enterococcus faecalis*, consistent with its essential role in phagocytosis of Gram-positive bacteria (Chung and Kocks, 2011; Kocks et al., 2005; Melcarne et al., 2019b). NimC1 is essential for uptake of latex beads and zymosan (fungus-like) particles. Intriguingly, phagocytosis of Gram-negative bacteria and apoptotic cells is not blocked in *eater* or *NimC1* single mutants, but is abolished in *NimC1;eater* double mutants, revealing key overlapping roles of these receptors (Melcarne 2019, B.L. personal communication). The Nimrod receptors SIMU and Draper, a conserved member of the CED1/MEGF-10 family, bind phosphatidylserine, an eat-me signal found on the surface of apoptotic cells (Kurant et al., 2008; MacDonald et al., 2006; Manaka et al., 2004; Shklyar et al., 2013; Tung et al., 2013). Phagocytosis of apoptotic corpses mediated by these two receptors induces signaling that modifies hemocyte expression profile and migration ability (Brooks et al., 2024; Goethem et al., 2012; Weavers et al., 2016a). Some integrins such as βν and αPS3 function as phagocytic receptors in addition to their roles in hemocyte adhesion and migration (Nagaosa et al., 2011; Nonaka et al., 2013; Shiratsuchi et al., 2012). Integrin βν plays a role in phagocytosis of both apoptotic cells and *S. aureus*. Draper and integrin βν cooperate in defense against *S. aureus* by binding lipoteichoic acid (Hashimoto et al., 2009) and peptidoglycan respectively (Shiratsuchi et al., 2012). Recent evidence suggests the CD36 factor

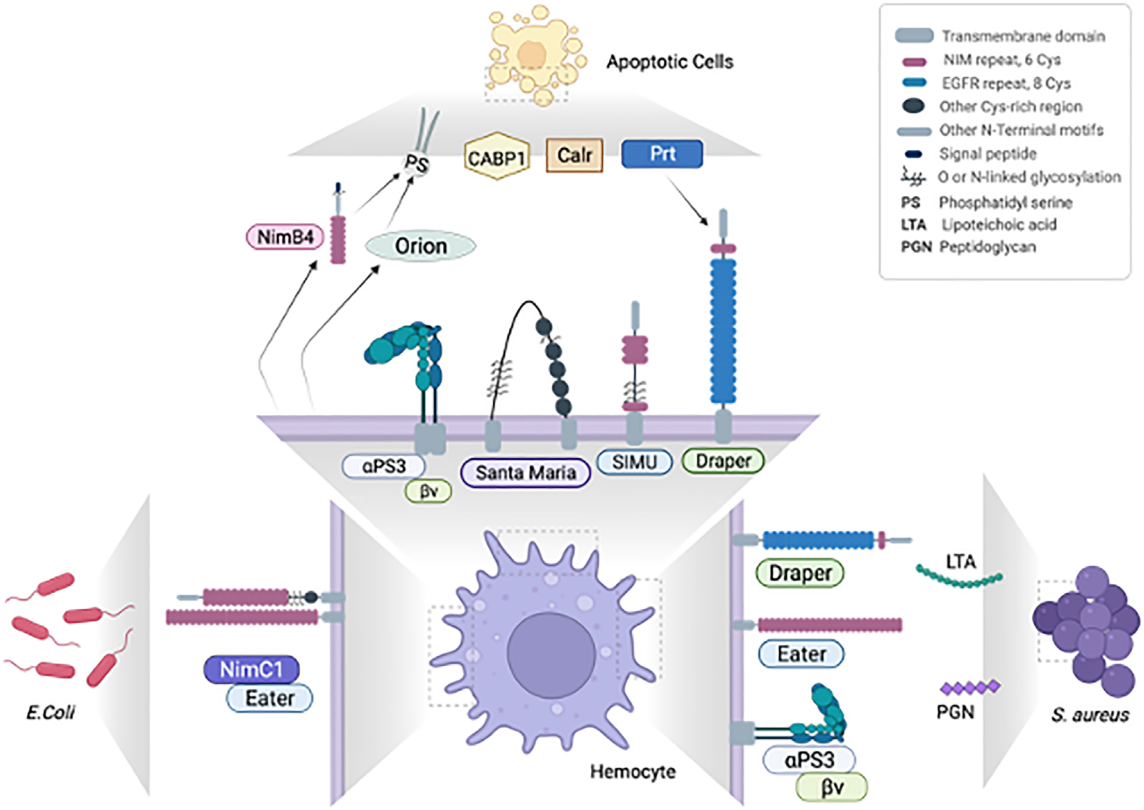


Figure 24 Phagocytic receptors
Hemocyte receptors and opsonins have been implicated in phagocytosis of apoptotic cells (SIMU/NimC4, Draper, βν-integrin, Orion, NimB4, Santa-maria (Ji et al., 2023; Kuraishi et al., 2009; Kurant et al., 2008; MacDonald et al., 2006; Manaka et al., 2004; Nagaosa et al., 2011; Nonaka et al., 2013; Petrignani et al., 2021; Tung et al., 2013)) and bacteria (Draper, NimC1, Eater, βν-integrin (Kocks et al., 2005; Kuraishi et al., 2009; Kurucz et al., 2007; Melcarne et al., 2019b; Shiratsuchi et al., 2012)). Croquemort, a member of the CD36 scavenger receptor family, is involved in phagosome maturation (Guillou et al., 2016; Han et al., 2014). Scavenger Receptor C1 (SR-CI) binds acetylated low density lipoproteins (AcLDLs) and a variety of other ligands, and although studies in cell culture have implicated SR-CI in phagocytosis its roles *in vivo* are unclear (Abrams et al., 1992; Rämet et al., 2001). The early steps of bacterial phagocytosis by hemocytes remain poorly characterized. Phosphatidylserine (PS), Calcium-Binding Protein 1 (CABP1), calreticulin (Calr) and Pretaporter (Prtp) are potential 'eat me' signals exposed at the surface of apoptotic cells (Kuraishi et al., 2009, 2007; Okada et al., 2012; Shklover et al., 2015; Tung et al., 2013; Zheng et al., 2017).

Santa-maria also contributes to phagocytosis of apoptotic cells mediated by SIMU by glia during embryogenesis (E. Kurant, personal communication). There are likely many other receptors that help in the uptake of bacteria or apoptotic cells, and many putative receptors (such as Scavenger Receptors C1-C4, several Nimrod family receptors and CD36 homologs) remain to be characterized.

ii) Opsonins and phagosome maturation

Opsonins are secreted proteins that act as bridging molecules by binding target particles and promoting recognition by phagocytic receptors. They play key roles in particle uptake and engage specific phagosome maturation programs. *Drosophila* opsonins include NimB4, which binds to phosphatidylserine of apoptotic cells to promote uptake by plasmatocytes (Petrignani et al., 2021), and Orion which bridges phosphatidylserine and glial Draper (Ji et al., 2023). These may also include secreted lectins such as Lectin-galC1 (galactin) and other C-type lectins (Ao et al., 2007; Petrignani et al., 2021; Tanji et al., 2006), and other yet-uncharacterized secreted Nimrods (B1, B2, B3) (Melcarne et al., 2019a; Somogyi et al., 2008; Zsámboki et al., 2013).

Proteins involved in cytoskeletal control such as the nonaspanin transmembrane proteins TM9SF4 and TM9SF2 (Bergeret et al., 2008; Perrin et al., 2015), peroxisomes (Di Cara et al., 2017), glutamate transport (Gonzalez et al., 2013), and phagosome maturation such as the *Drosophila* CD36 homolog Croquemort also contribute to phagocytosis (**Figure 25**). Recent evidence shows that Croquemort is not a phagocytic receptor of apoptotic cells and bacteria as initially thought, but is required for phagosome maturation (Guillou et al., 2016). Croquemort contributes to clearance of non-apoptotic cell debris in the central nervous system, lipid metabolism, and may promote phagoptosis of nurse cells in the ovaries (Brown and Neher, 2012; Etchegaray et al., 2012; Meehan et al., 2016; Timmons et al., 2016; Woodcock et al., 2015). Disruption of the stepwise phagosome maturation process results in phagocytic defects at late time points as bloated phagocytes are unable to continue taking up pathogens from the hemolymph, which also sensitizes the fly to infection (Kuo et al., 2018; Moy and Cherry, 2013; C.-O. Wong et al., 2017b). The p38 MAPK pathway also contributes to sequestration of some bacteria in phagosomes to promote disease tolerance (Shinzawa et al., 2009).

Phagosomes undergo a maturation process comprised of stepwise fusion with endosomes and lysosomes that add enzymes to the phagolysosomal compartment, which are required for particle degradation and bactericidal activity (**Figure 25**). Little is known of the hydrolases contributing to particle destruction in *Drosophila* phagosomes, but these likely include cathepsins (Kocks et al., 2003). Acidification of the phagosome lumen to a final pH of 4.5-5, which is required for enzyme activity and particle degradation, is accomplished by proton-pumping vacuolar ATPase (V-ATPase) (Cheng et al., 2005; Philips, 2005). Fusion events involve sequential recruitment of small GTPases of the Rab family (Kinchen and Ravichandran, 2008; Li et al., 2009; Nieto et al., 2010) and the HOPS (Homotypic Fusion and Protein Sorting) complex (Akbar et al., 2011; Kinchen and Ravichandran, 2008; Nickerson et al., 2009). Phagosome maturation may involve different proteins and processes depending on their cargo. For example, the calcium-permeable cation channel Amo, the *Drosophila* homolog of mammalian *pkd2*, is required for acidification of apoptotic-cell containing phagosomes downstream of SIMU (Brooks et al., 2024). Additional processes including glutamate transport and a nuanced intracellular ROS response are also required to regulate and maintain endosome processing (Gonzalez et al., 2013; Myers et al., 2018). Interestingly, mutations in the Imd pathway can impair phagocytosis in the long term by preventing upregulation of *NimC1* and *Eater* through Relish (Wong et al., 2017b). Disruption of the endocytic machinery can also have strong effects on phagocytosis and activation of signaling pathways by affecting receptor localization (**Box 6**).

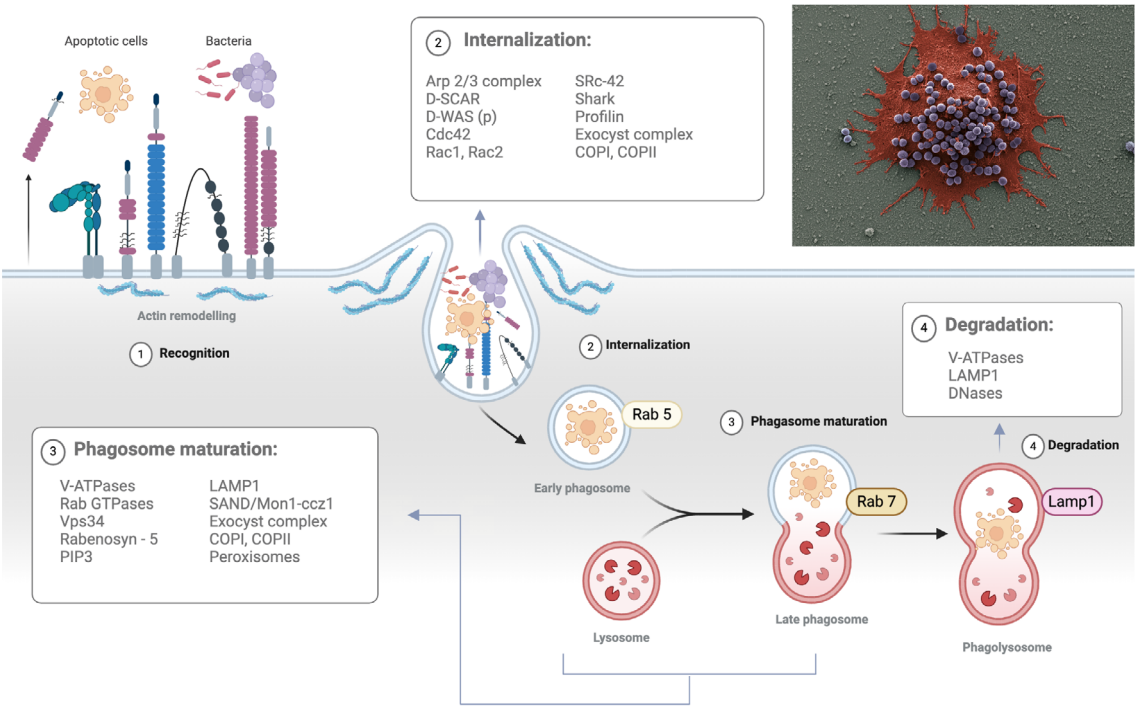


Figure 25 Phagosome maturation

Phagocytosis and phagosome maturation consist of a stepwise process where distinct proteins are recruited to the phagosome at each stage (shown in boxes). **1** Target particle recognition by cell surface receptors on *Drosophila* professional phagocytes (plasmatocytes) triggers F-actin branching at the engulfment site and formation of a phagocytic cup. **2** Actin polymerization progressively extends protrusions around the particle that ultimately fuse at the leading edges to generate a new phagosome (Agaisse et al., 2005; Avet-Rochex et al., 2007; Pearson et al., 2003; Philips, 2005; Stroschein-Stevenson et al., 2005; Stuart et al., 2007, 2005; Ulvila et al., 2011). **3** The phagosome undergoes maturation through a series of fission and fusion events with cellular organelles (early endosomes, late endosomes, lysosomes). The GTPase Rab5 is a key regulator of initial fusion events (Agaisse et al., 2005; Cheng et al., 2005; Horn et al., 2014; Peltan et al., 2012; Philips, 2005; Yousefian et al., 2013), while Rab7 is needed for late phagosome-lysosome fusion. **4** Phagosome maturation produces a highly acidic phagolysosome where target particles are digested (Akbar et al., 2011; Garg and Wu, 2014; Yousefian et al., 2013). During this last step, the phagolysosome acquires enzymes required for degradation including DNAses and proteases (Cheng et al., 2005; Di Cara et al., 2017; Kocks et al., 2003; Mukae et al., 2002; Myers et al., 2018; Philips, 2005; Seong et al., 2014, 2006). Inspired by (Melcarne et al., 2019a). **Inset:** scanning electron micrograph of plasmatocyte (stained in red) from a third instar *Drosophila* larva engulfing *S. aureus* bacteria (from Melcarne et al., 2019a with permission).

Box 6 Immunity and the endocytic machinery

Disruption of the endocytic machinery may produce complex immune phenotypes as many components function in multiple fundamental cellular processes including phagocytosis, autophagy, and activation and attenuation of signaling pathways. Receptor localization is important to regulate pathway activation in both Imd (Neyen et al., 2016) and Toll signaling (Huang et al., 2010). Mutations affecting the HOPS complex proteins Vps16B (*full-of-bacteria, fob*) or Vps33B cause specific defects in maturation of bacteria-containing phagosomes. Furthermore, *Vps33B* mutants experience lethal over-activation of Imd signaling in response to heat-killed bacteria due to an inability to process endosomes bearing internalized PGRP-LC receptors, which accumulate in intracellular compartments (Akbar et al., 2016, 2011). The result of Akbar (2016) suggests that PGRP-LC is capable of signaling from the endosomal membrane and requires processing through multivesicular bodies (MVBs) to attenuate signaling (Akbar et al., 2016).

Mutation of another HOPS complex component, Deep orange (Vps18), constitutively activates Toll signaling in larvae (Schmid et al., 2016). Endocytosis of the Toll receptor is required to activate signaling (Huang et al., 2010; Lund et al., 2010) and is dependent on the ESCRT-0 complex (Hrs, Mop, Stam) (Huang et al., 2010) which processes ubiquitinated cargo for sorting in MVBs (Lund et al., 2010; Rusten et al., 2006) and is also involved in endocytosis and degradation of the Toll negative regulator Necrotic (Soukup et al., 2009). Conversely, disruption of the class III PI3 kinase complex (Vps15/ird1, Vps34) involved in MVB sorting and autophagic clearance of ubiquitinated protein aggregates constitutively activates the Toll pathway, and may also simultaneously suppress Imd signaling (Lindmo et al., 2006; Schmid et al., 2016; Wu et al., 2007).

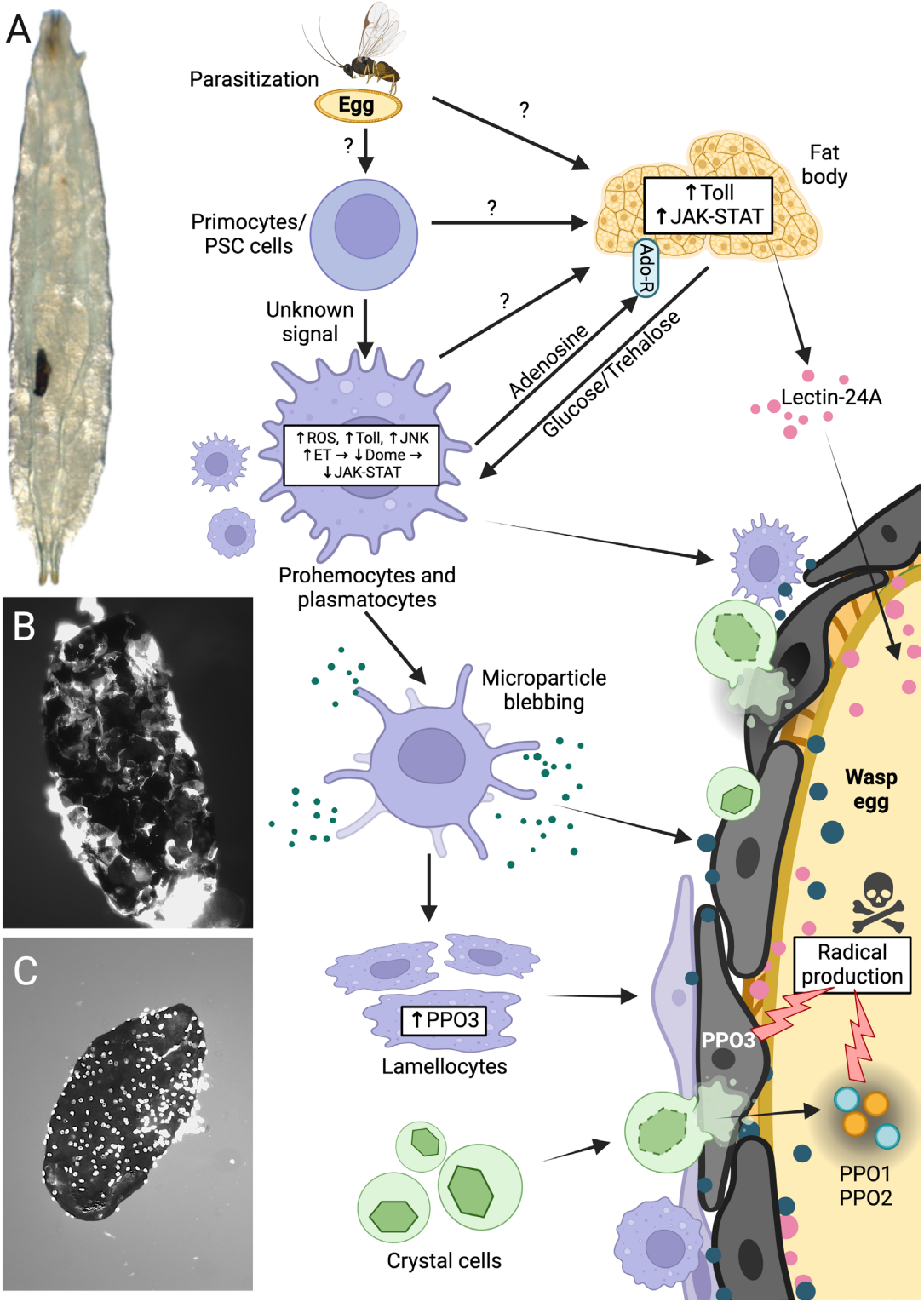
RNAi of components of the ESCRT-I and -II complexes prevents removal of PGRP-LC from the plasma membrane and extends Imd signaling but does not affect amplitude (Neyen et al., 2016), suggesting that a failure to process PGRP-LC through the MVB pathway maintains it in an active signaling state. Further studies may produce a deeper understanding of the nuanced effects of receptor localization and processing on immune signaling. Finally, it is important to note that deciphering direct versus indirect impacts of genes influencing host defense is often a challenge in the genetic dissection of the immune system. As an illustration of this, mutations affecting Deep orange (Vps18) impair the ecdysone response needed for maturation of the fat body and indirectly suppress Imd signaling in larvae (Meister and Richards, 1996), in addition to constitutively activating Toll signaling (Schmid et al., 2016).

C. Encapsulation

Encapsulation is the process by which lamellocytes neutralise material within the larval body cavity that is too large to be removed by phagocytosis (Dolezal, 2023; Kim-Jo et al., 2019; Lefèvre et al., 2012). Encapsulation protects against eggs of parasitoid wasps in the wild, but also attacks tumorous or damaged self-tissue within the larval body cavity (see Autoimmunity, page 127). Encapsulation of wasp eggs is thought to occur sequentially: first, humoral factors bind the eggs, followed by circulating plasmatocytes that begin to transdifferentiate into lamellocytes, and finally lamellocytes released by rupture of the lymph gland (**Figure 26**). Effective neutralization of wasp eggs requires recruitment of lymph gland hemocytes (Louradour et al., 2017). Lamellocytes adhere to the foreign object in layers and melanize, forming a tight capsule that isolates and bombards the encapsulated object with toxic reactive oxygen species produced by lamellocyte-exclusive PPO3, and PPO2 from crystal cells (Dudzic et al., 2015; Rizki and Rizki, 1994; Vass and Nappi, 2000)(see Systemic immunity: Melanization, page 71). Polymerized melanin chains can also form a physical barrier around parasites that trap ROS and direct it towards invaders (Nappi et al., 2009).

Lamellocytes are derived from both the peripheral hemocytes and the larval lymph gland. Differentiation of lamellocytes from both of these populations is thought to be controlled by primocytes, a subset of cells with a distinct transcriptional profile found in circulation and in the posterior signaling center (PSC) of the lymph gland (Hultmark and Andó, 2022). Signals from multiple pathways (JNK, PVR, JAK-STAT, Toll) contribute to differentiation of lamellocytes, which have a unique transcriptional profile and strongly express JNK pathway genes (Cattenoz et al., 2021, 2020; Cho et al., 2020; Csordás et al., 2021; Evans et al., 2022; Hirschhäuser et al., 2023; Hultmark and Andó, 2022; Irving et al., 2005; Krzemień et al., 2007; Morin-Poulard et al., 2013; Sorrentino et al., 2004; Tattikota et al., 2020; Tokusumi et al., 2009, 2018; Zettervall et al., 2004; Zhang et al., 2023).

Figure 26 Encapsulation
Schematic of the *Drosophila* larva encapsulation response against eggs of the parasitoid wasp *Leptopilina boulardi*. Egg oviposition triggers lamellocyte differentiation and fat body Toll activation, likely through 'missing-self' recognition mechanisms such as a lack of N-glycosylation on the surface of the wasp egg. The fat body produces factors such as Lectin-24A that opsonize the wasp egg and promote encapsulation. Differentiating lamellocytes increase Toll and JNK activity, ROS generation, and expression of the JAK-STAT inhibitor *eye transformer* (ET), leading to reduced JAK-STAT activity (see **Figure 20**). Inhibiting these changes prevents lamellocyte differentiation. Lamellocyte differentiation is energetically costly and requires glucose release from fat body glycogen stores in response to an adenosine signal generated by the hemocytes (see **Figure 27**). As they differentiate, early lamellocytes release microparticles exposing hemomucin and phosphatidylserine (PS), which stick to the encapsulation target and act as nucleation sites for hemocyte attachment and melanization. Lamellocytes become thin and flat, increase expression of PPO3, and adhere to the wasp egg in layers. Crystal cells also adhere to the capsule and release PPO1/2 through rupture. Activity of these prophenoloxidases produces highly toxic oxygen radicals that kill the wasp egg (see **Figure 12**). Inspired from (Dolezal, 2023). **Insets: A** wasp egg viewed through translucent *Drosophila melanogaster* larva; capsules stained with **B** Hoechst and **C** phalloidin in *Drosophila yakuba* larvae. Photos courtesy Shubha Govind, Todd Schlenke. Figure created with BioRender.com, CC-BY-NC-ND.


A
Parasitization
Egg
?
?
Primocytes/
PSC cells
?
↑Toll
↑JAK-STAT
Fat
body
Ado-R
Unknown
signal
?
Adenosine
Glucose/Trehalose
Lectin-24A
↑ROS, ↑Toll, ↑JNK
↑ET → ↓Dome →
↓JAK-STAT
Prohemocytes and
plasmatocytes
B
Microparticle
blebbing
Wasp
egg
C
↑PPO3
Lamellocytes
Radical
production
PPO3
PPO1
PPO2
Crystal cells

During differentiation, microparticles (small extracellular vesicles) are released by budding of the hemocyte plasma membrane as they begin differentiation into lamellocytes. The microparticles, which attach to the encapsulation target and to other lamellocytes, expose hemomucin, which can bind coagulation proteins including lipophorin through transglutaminase crosslinking and is thought to glue the hemocytes together, and phosphatidylserine, which can recruit hemocytes and potently induce melanization (Bidla et al., 2009; Rizki and Rizki, 1979; Rizki and Rizki, 1983; Theopold and Schmidt, 1997). Lamellocyte adhesion requires the βPS integrin Myospheroid (Mys) (Irving et al., 2005), which also mediates hemocyte migration (Comber et al., 2013). Humoral factors also contribute to encapsulation: Lectin-24A is released from the fat body and coats parasite eggs, greatly potentiating encapsulation and killing rates (Arunkumar et al., 2023). The *Lectin-24A* gene, controlled by the JAK-STAT and Toll pathways, is induced upon wasp infection with stronger expression in the posterior region of the larval fat body (Wertheim et al., 2005; Zhou et al., 2024). Interestingly, Dorsal rather than Dif seems to be the major regulator of the humoral immune response to parasites (Zhou and Day et al., 2024).

Little is known of the initial mechanisms that recognize foreign bodies and lead to encapsulation (**Figure 26**). Self-tissue can be also encapsulated under certain conditions: loss of surface protein N-glycosylation on host tissues triggers encapsulation in the presence of lamellocytes (Mortimer et al., 2021), and disrupted basement membranes exposing phosphatidylserine are also sufficient to recruit hemocytes and produce melanization (Diwanji and Bergmann, 2020; Kim and Choe, 2014; Mortimer et al., 2021; Pastor-Pareja et al., 2008; Rizki, 1960). This suggests that patrolling hemocytes identify intact basement membrane as self, while parasitoid eggs are recognized via a missing-self mechanism (Mortimer et al., 2021; Pradeu et al., 2024) (see Autoimmunity, page 127). Wounding caused by oviposition may induce differentiation of a few lamellocytes which then survey the hemocoel for incorrectly glycosylated surfaces (Márkus et al., 2005; Mortimer et al., 2021; Rizki and Rizki, 1974; Leitão et al., 2024), and trigger massive differentiation of lamellocytes upon recognition. Alternatively, secreted or transmembrane receptors may exist that directly recognize wasp antigens and initiate lamellocyte formation through an unknown pathway (Arunkumar et al., 2023). Consistent with this latter hypothesis, oil beads coated in wasp extracts become more melanized when injected into larvae than uncoated beads or beads coated with Drosophila extracts (Leitão et al., 2024). In an example of a host-pathogen arms race, wasps have evolved many strategies that target aspects of the host immune response to promote survival of their eggs within *Drosophila* larvae (**Box 7**).

D. Hemocytes in signal transduction and local repair

Hemocytes express complete Toll and Imd pathways and are sources of immune effectors including AMPs and PPOs (see Systemic Immunity: Melanization, page 71) (e.g., Dudzic et al., 2019, 2015; Sanchez Bosch et al., 2019), and additionally act in signal transduction in a number of processes (**Figure 22**). Hemocytes trigger intestinal stem cell proliferation following systemic wounding through the release of Upd3 (Chakrabarti et al., 2016). Hemocytes also link oral bacterial infection to systemic fat body expression of antimicrobial peptides in larvae (Basset et al., 2000; Charroux and Royet, 2009; Foley and O'Farrell, 2003) (see Systemic immune activation in response to oral infection, page 118). In response to infection, *Spatzle* is strongly upregulated in the hemocytes, and hemocyte-secreted Spatzle is thought to act as a cytokine to activate the Toll pathway in the fat body (Irving et al., 2005; Ming et al., 2014; Parisi et al., 2014; Shia et al., 2009; Tattikota et al., 2020). In addition to

Box 7 Wasps target the *Drosophila* immune system

Parasitoid wasps can inject discrete particles and a number of proteins that target the *Drosophila* larval immune response to protect the wasp egg. The nature of these particles and venom protein constituents are not well understood. Venom proteins include the SERCA-type calcium pump of *Ganaspis* that antagonizes host hemocyte calcium signaling to inhibit encapsulation (Mortimer, 2013); the 40 kDa surface/spike protein SSp40 of *Leptopilina heterotoma*, which has similarities to the IpaD/SipD family of *Shigella* and *Salmonella* enteric pathogen proteins and promotes lysis of host lamellocytes (Heavner et al., 2017); and the RhoGAP protein of *L. boulardi* that modulates the host actin cytoskeleton (Colinet 2009). The venom glands of *Leptopilina* spp. produce membrane-bound extracellular vesicles (EVs, also called venosomes or virus-like particles, VLPs) (Chiu et al., 2006; Heavner et al., 2017; Morales et al., 2005; Ramroop et al., 2021; Wan et al., 2020); these vesicles have not been reported in *Ganaspis* venom (Chiu et al., 2001). *Leptopilina heterotoma* vesicles have been shown to enter and affect the viability of both plasmatocytes and lamellocytes (Chiu and Govind, 2002; Ramroop et al., 2021). The vesicles of *L. boulardi* enter larval hemocytes through the endocytic pathway and affect lamellocyte shape or cause cell lysis that disrupts encapsulation (Wan et al., 2020). The venom of *Leptopilina boulardi* contains serpins that inhibit melanization (Colinet et al., 2009). *Leptopilina victoriae* virulence factors target and counteract progressive surface N-glycosylation of hemocytes transforming into lamellocytes to decrease efficacy of capsule formation (Mortimer et al., 2012). Multiple wasp species target the JAK-STAT signaling pathway, which regulates lamellocyte differentiation (Brantley et al., 2024). Finally, some species like *Asobara tabida* inject eggs with a sticky chorionic surface that adheres to host tissues, preventing encapsulation by hemocytes (Prevost et al., 2005). These studies illustrate the variety of ways in which parasitoids disrupt host immunity to promote egg survival.

its signaling role in hemocytes, ROS produced by hemocytes may be microbicidal against certain pathogens (Shaka et al., 2022; Sekihara et al., 2016).

Hemocytes contribute to wound healing and tumor neutralization (Araki et al., 2019; Chakrabarti and Visweswariah, 2020; Fogarty et al., 2016; Parisi et al., 2014) (see Systemic wound and stress responses, page 77 and Immunity in tumor control, page 129). They also produce and deposit extracellular matrix, which is important in maintaining self/non-self distinctions and preventing autoimmune activation (see Autoimmunity, page 127) (Fessler et al., 1994; Goto et al., 2001; Irving et al., 2005; Lunstrum et al., 1988; Nelson et al., 1994). An interesting aspect of hemocytes is their ability to act locally in contact with specific tissues (Van De Bor et al., 2015). For example, hemocytes can bind to tissues and target cells for apoptosis by expressing Eiger, which binds the TNF receptors Grindelwald or Wengen on target cells (Araki et al., 2019; Fogarty et al., 2016; Parvy et al., 2019).

Hemocytes are essential for embryogenesis and metamorphosis, which involve major tissue remodeling (Charroux and Royet, 2009; Defaye et al., 2009; Ghosh et al., 2020; Lanot et al., 2000; Sampson et al., 2012; Stephenson et al., 2022). Although metamorphosis can be completed successfully even when the great majority of these hemocytes are ablated (Charroux and Royet, 2009; Defaye et al., 2009), complete deletion of

hemocytes with a strong Hemolectin driver (e.g., *Hml-Gal4*; *UAS-Bax*) causes pupal lethality (Stephenson et al., 2022). Pupae with reduced hemocyte numbers over-activate other immune programs including melanization and AMP production in response to microbes released from the gut during remodeling of the digestive tract, and under conventional rearing conditions the majority do not survive metamorphosis (Arefin et al., 2015; Charroux and Royet, 2009; Defaye et al., 2009; Glittenberg et al., 2011; Shia et al., 2009) (see Protection of host tissues from antimicrobial peptides, page 88).

E. Hemocytes are a central metabolic hub

Hemocytes are central regulators and major consumers of metabolic stores. Hemocytes closely link JAK-STAT activity, insulin signaling, immunity, and lipid metabolism in a number of important ways. Under homeostatic conditions, hemocytes promote normal insulin signaling and growth, and facilitate lipid storage in the larval fat body through PDGF/VEGF signaling (Cox et al., 2021). In adults, hemocyte-derived Upd3 promotes normal levels of JAK-STAT signaling in muscles that is essential for healthy metabolism (Kierdorf et al., 2020). As they are metabolically demanding, the number of hemocytes is reduced under nutrient-deficient conditions (Dolezal et al., 2019; Ramond et al., 2020a). This is in part mediated by the adipokine NimB5, which is secreted from the fat body in nutrient-poor conditions and binds to hemocytes to reduce hemocyte proliferation, freeing up resources for development and growth (Ramond et al., 2020b). Blocking NimB5 results in hemocyte proliferation, energy depletion and eventual death of larvae raised on a poor diet (Ramond et al., 2020b). Conversely, a chronic lipid-rich diet in *Drosophila* induces overproduction of Upd3 by macrophages, causing JAK-STAT mediated insulin insensitivity and reduced lifespan (Woodcock et al., 2015).

Hemocyte activation in response to wasp parasitization in larvae or infection in adult flies incurs a huge metabolic cost that draws on stored energy in the fat body to meet hemocyte nutritional demand (Bajgar et al., 2015; Dolezal et al., 2019) (**Figure 27**). Differentiation of lamellocytes, which is required for proper encapsulation, is energetically costly (Bajgar et al., 2015). Hemocyte activation triggers a metabolic switch to aerobic glycolysis (Bajgar et al., 2015; Bajgar and Dolezal, 2018; Krejčová et al., 2019), a process that provides energy more quickly than oxidative phosphorylation, but at much lower efficiency. Increased aerobic glycolysis is accompanied by suppression of anabolic enzymes and upregulation of glycolytic processes that mobilize fat body nutrient stores, resulting in hyperglycemia (increased circulating glucose and trehalose). Increased circulating sugars are consumed by hemocytes during the immune response (Bajgar et al., 2015). This metabolic switch is initiated by adenosine produced by the hemocytes (Bajgar et al., 2015); later on in infection, aerobic glycolysis is inhibited by adenosine inhibitor ADGF-A also produced by the hemocytes (Bajgar and Dolezal, 2018). This metabolic switch is required for lamellocyte differentiation and effective resistance to certain bacterial infections in adult flies (Bajgar et al., 2015; Bajgar and Dolezal, 2018).

Aerobic glycolysis in adult flies in response to *Streptococcus* infection is mediated by Hypoxia inducible factor 1α (HIF1α) and lactate dehydrogenase (LDH) in hemocytes (Krejčová et al., 2019). In mammals, NF-κB activation through Toll or TNF-R signaling stabilizes HIF1α to promote aerobic glycolysis (Jung et al., 2003; Siegert et al., 2015). Parasitoid wasp infestation of larvae promotes secretion of Upd ligands from hemocytes that increase JAK-STAT and subsequently insulin (TOR) signaling in muscles, which

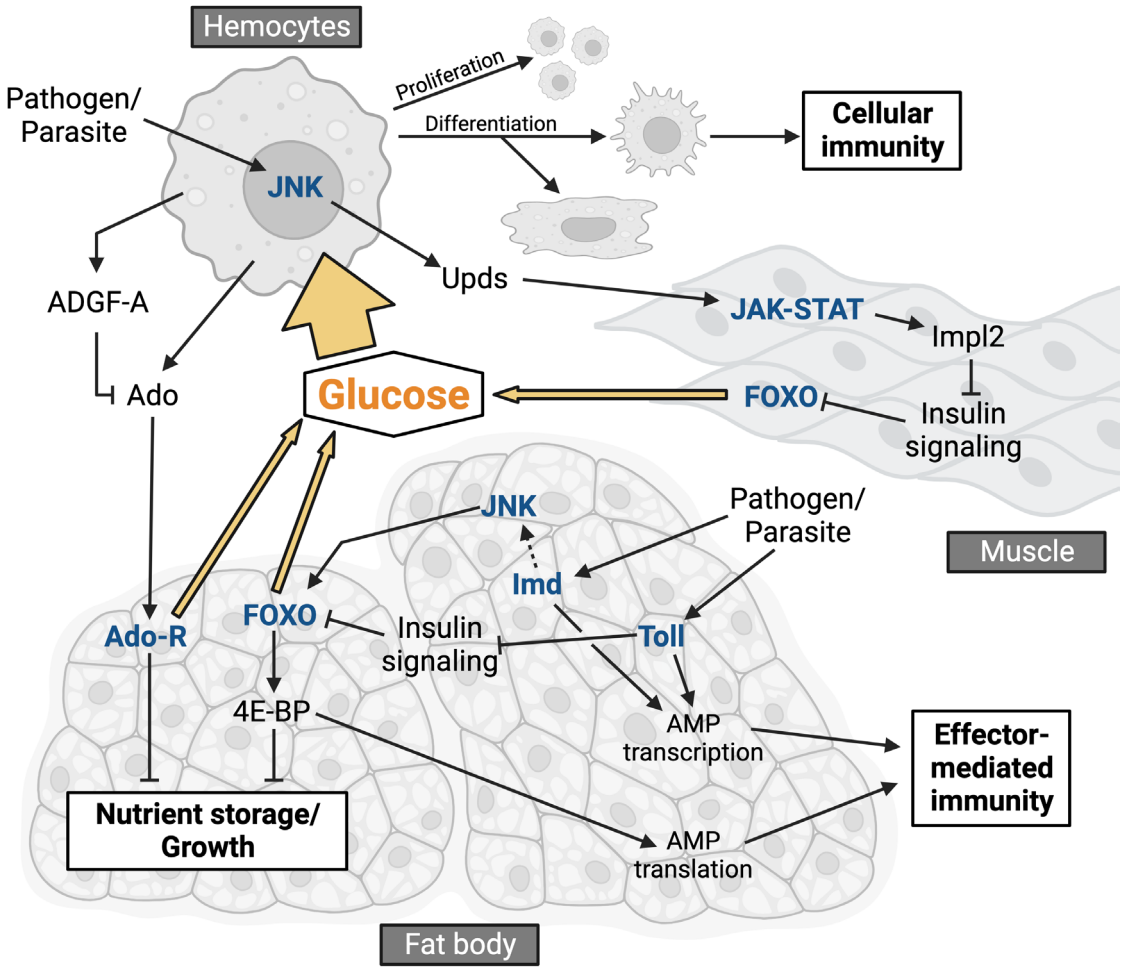


Figure 27 Metabolic reprogramming upon infection or parasitization
Hemocyte activation and differentiation is highly energetically costly. Infection or parasitization induces a metabolic switch where energy stores are redirected away from growth and homeostasis towards defense (the 'privileged immune system'). Activated lamellocyte precursors release adenosine (Ado), which binds the adenosine receptor (AdoR) on the fat body, inhibiting anabolic processes and slowing down larval development. Late in the parasitization response, hemocytes also release the Ado inhibitor ADGF-A to attenuate resource stealing by the immune system (Dolezal et al., 2019). Glycogen stores in the muscles are also mobilized to provide energy for lamellocyte differentiation. Upd ligands produced by the hemocytes activate JAK-STAT in the muscles, increasing expression of the insulin inhibitor *ImpL2* and increasing free glucose. Infection also promotes the release of stored nutrients to support immune function. Toll or JNK activation suppresses insulin signaling, increasing free glucose and upregulating the FOXO target *4E-BP*, which promotes cap-independent translation of genes including some AMPs while reducing cap-dependent translation of targets such as anabolic enzymes (see Metabolic adaptation associated with systemic antimicrobial responses, page 69). This biases resources towards translation of immune proteins. Compiled with data from: (Bland, 2022; Dolezal et al., 2019; McMullen et al., 2023; Roth et al., 2018; Vasudevan et al., 2017). Figure created with BioRender.com, CC-BY-NC-ND.

are a major glycogen store in the larva (Yang et al., 2015; Yang and Hultmark, 2017). Surprisingly, these authors found that blocking JAK-STAT or insulin signaling in muscles reduced circulating sugars and impaired lamellocyte differentiation in response to wasp parasitization, indicating that the muscles act as a source of energy to fuel hemocytes. Late in infection, insulin signaling is suppressed. The metabolic switch in macrophages is a response that is conserved in mammals and uses homologous processes, making *Drosophila* an attractive model to study this phenomenon.

The metabolic switch can also have maladaptive effects in certain infectious scenarios, as an increase in circulating glucose or intermediates of aerobic glycolysis may benefit certain pathogens (Bajgar and Dolezal, 2018; Passalacqua et al., 2016). Similarly, accumulation of lipid droplets triggered by Upd3-mediated JAK-STAT signaling in hemocytes in response to *Mycobacterium marinum* infection promotes intracellular survival and proliferation of this pathogen (Péan et al., 2017). Accumulation of lipid droplets also transiently occurs in hemocytes phagocytosing tumorous tissue (Mari et al., 2023); the significance of this is currently unknown. Lipid droplets and the proteins they sequester, including histones, may have conserved roles in bacterial resistance (Anand et al., 2012; Bosch et al., 2021; Bosch and Pol, 2022; Stephenson et al., 2021; Tang et al., 2021), viral immunity (Monson et al., 2021), and ROS detoxification (Wang et al., 2023). The roles of lipid droplets and trafficking in immunity are exciting avenues to explore further (Harsh et al., 2019).

Box 8 Immune priming in *Drosophila*

Immune priming is a widespread phenomenon among arthropods describing improved survival of previously-infected individuals compared to naïve controls upon re-infection (Pradeu et al., 2024; Prakash et al., 2023; Sadd and Schmid-Hempel, 2006; Tang et al., 2023). Arthropods lack adaptive immune programs such as somatic recombination of B- and T-cell receptor genes and differentiation of memory cells common to vertebrates, so the innate mechanisms underlying improved survival upon re-infection in *Drosophila* have been of great interest since their discovery (Boman et al., 1972; Cooper and Eleftherianos, 2017; Kurtz, 2005; Pham et al., 2007). Many early studies of insect priming lacked conceptual precision and appropriate controls, or were done in very artificial settings, with unclear *in natura* relevance (Hauton and Smith, 2007). Some early results proposed mechanisms similar to vertebrate immune memory, such as production of 'antibody-like' proteins from the hypervariable *Dscam1* locus, which has the potential to encode thousands of isoforms (Watson et al., 2005). However in *Drosophila* at least, Dscam1 isoforms invariably contain a transmembrane domain (Celotto and Graveley, 2001), are not upregulated following infection (Armitage et al., 2014), and appear to have roles in hemocyte proliferation rather than opsonization (Ouyang et al., 2020). Recent evidence suggests that trans-generational immune priming can occur against viruses but not bacteria in *Drosophila,* although the mechanisms behind this are not yet well understood (Mondotte et al., 2020; Radhika and Lazzaro, 2023) (see The antiviral response, page 31).

Recently, the diverse mechanisms underlying priming in insects have been conceptually clarified (Pradeu et al., 2024; Pradeu and Du Pasquier, 2018; Tang et al., 2023). Priming in *Drosophila* can be broadly grouped into four categories: (**i**) a persistent

low-level infection that continuously stimulates the immune system; (**ii**) the perdurance of effectors or activated hemocytes from a primary challenge persist, increasing baseline resistance against subsequent infection (Uttenweiler-Joseph et al., 1998); (**iii**) a shift in basal immunity leaves the fly in an 'anticipatory' state of immune readiness, enabling stronger or more rapid responses upon secondary challenge (Chakrabarti and Visweswariah, 2020; Fuse et al., 2022; Mulcahy et al., 2011); (**iv**) a primary challenge shifts baseline physiology such that subsequent infection induces a different set of genes (Cabrera et al., 2023; Fuse et al., 2022) (**Figure Box 8**). Simple wounding, challenge with heat-killed pathogens, or low-virulence infections that are cleared can also have a persistent priming effect (Aymeric et al., 2010; Chakrabarti and Visweswariah, 2020; Christofi and Apidianakis, 2013; Fuse et al., 2022; Pham et al., 2007).

In *Drosophila*, immune priming, far from being a general property of the immune system, requires specific circumstances to occur (Acuña Hidalgo and Armitage, 2022). The success of priming may depend on whether the immune mechanisms stimulated by the primary pathogen are effective defenses against the secondary pathogen. Priming is somewhat dose-specific, as too high of an initial dose of pathogenic bacteria either weakens or kills the fly, while a low dose or weak initial pathogen may not have a sufficient priming effect (Boman et al., 1972; Cabrera et al., 2023) (see **Box 1**). Priming is also dependent on infection route, as oral infection may protect against subsequent oral infections, but not septic infections (Liehl et al., 2006; Mulcahy et al., 2011). As the rapidity of the immune response is a key factor in determining survival against some pathogens (Duneau et al., 2017a; Park et al., 2005), a higher basal immune state or residual effectors such as AMPs in the hemolymph can effectively increase resistance against re-infection; for Gram-negative bacteria, AMPs are specifically important for resistance (Hanson et al., 2019b). In contrast, a recent study found that priming against *E. faecalis* (a Gram-positive bacterium) relied on metabolic effects promoting tolerance (Cabrera et al., 2023). Some residual effectors could similarly promote immune tolerance, such as Turandots (Rommelaere et al., 2024) or Bomanins (Xu et al., 2023a) (see Protection of host tissues from antimicrobial peptides, page 88).

The priming effect against most pathogens has been found to require hemocytes in some capacity (Aymeric et al., 2010; Cabrera et al., 2023; Chakrabarti and Visweswariah, 2020; Fuse et al., 2022; Pham et al., 2007), suggesting that mechanisms such as hemocyte differentiation or metabolic reprogramming may be central to priming effects (Cabrera et al., 2023; Fuse et al., 2022). Many studies show a requirement for Toll or Imd pathways for priming, but these effects are often not attributable to effector activation through these pathways (Aymeric et al., 2010; Cabrera et al., 2023; Christofi and Apidianakis, 2013; Pham et al., 2007; Prakash et al., 2021). Therefore, investigating non-canonical roles of NF-κB signaling may be a promising direction for disentangling the mechanisms behind pathogen-specific immune priming.

Finally, it should be noted that although priming with bacteria has variable effects, RNAi protection against viruses is highly specific and can reproducibly generate a sustained effect that protects against secondary infection, reminiscent of immune memory in vertebrates. siRNA can be amplified by RNA-dependent polymerase, which generates secondary siRNA and propagates the protection (Bonning and Saleh, 2021; Pradeu et al., 2024).

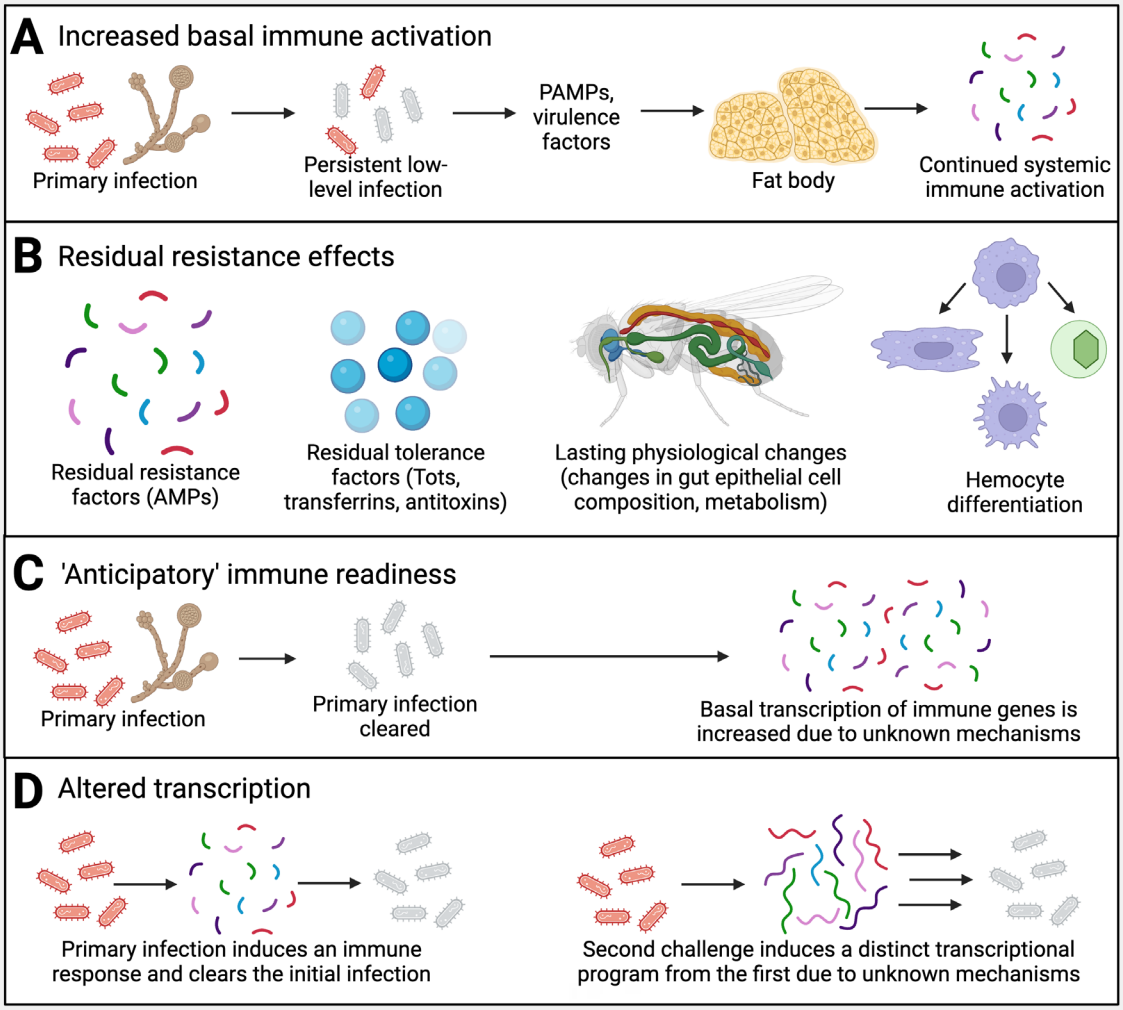


Figure Box 8 Immune priming in Drosophila
Drosophila lacks the adaptive immune mechanisms known in vertebrates but regardless shows improved survival upon re-infection with certain pathogens. Depending on the physiological response elicited by the initial pathogen and dose, these effects are attributable to some combination of **A** increased basal immune activation due to persistent low-level infection, **B** residual effectors or cellular changes, **C** increased basal immunity due to unknown mechanisms, or **D** a change in physiology resulting in induction of a distinctly different transcriptional response upon secondary infection. For discussion on innate immune memory, see (Pradeu et al., 2024; Pradeu and Du Pasquier, 2018; Tang et al., 2023). The sustained RNAi response is not shown. Figure created with BioRender.com, CC-BY-NC-ND.

10
Gut and epithelial immunity

Barrier epithelia, which are constantly exposed to microorganisms, require robust systems for recognizing and managing pathogens while protecting symbionts. This is particularly crucial for insects like *Drosophila*, which primarily feed on decaying material. Various factors including physical and chemical barriers, peristalsis, and inducible defense mechanisms, all work together to provide protection in the gut (Buchon et al., 2013a, 2013b; Miguel-Aliaga et al., 2018; Tafesh-Edwards and Eleftherianos, 2023b). The gut is a compartmentalized organ with functional diversification of the immune system along the length of the gut, highlighting the importance of compartment-specific defense mechanisms (Buchon et al., 2013b; Marianes and Spradling, 2013) (**Figure 28**). While most studies have focused on the midgut, it is likely that regions of the foregut (the cardia/proventriculus in adults and larvae, and the crop in adults) play important roles in host defense (Stoffolano and Haselton, 2013; Zhu et al., 2024).

A. Physical and chemical barriers to infection

Increased intestinal permeability is a direct precursor of mortality in flies, revealing the importance of the gut barrier in health (Rera et al., 2012; Sekihara et al., 2016). The digestive tract is lined with a protective protein-chitin barrier, shielding it from abrasive food particles and enteric pathogens (Hegedus et al., 2009). While the foregut and hindgut feature an impermeable cuticle, the midgut relies on the more permeable peritrophic matrix. The peritrophic matrix is continuously produced in the proventriculus of larvae and cardia of adults by specific cells, and is modified as it travels along the midgut (Hegedus et al., 2009; King, 1988; Miguel-Aliaga et al., 2018; Rizki, 1956). A subset of enteric neurons innervating the anterior midgut regulate the proventricular structure and the permeability of the peritrophic matrix (Kenmoku et al., 2016). Elimination of these neurons or loss of function of the *Crystallin* gene (*Crys*), which encodes a structural component, causes a leaky peritrophic matrix phenotype. *Crystallin* deficient flies show higher susceptibility to oral bacterial and viral infection and ingestion of toxin, confirming its protective role (Bonnay et al., 2013; Kuraishi et al., 2011; Nehme et al., 2007; Shibata et al., 2015; Villegas-Ospina et al., 2021). The peritrophic matrix also influences Imd pathway immune reactivity, likely by limiting the diffusion of peptidoglycan from the gut lumen into gut epithelial cells (Kuraishi et al., 2011). Interestingly, elimination of the peritrophic matrix at the adult stage through knockdown of the *drop-dead* gene is not lethal (Conway et al., 2018).

Most entomopathogenic bacteria, such as *Pseudomonas entomophila*, *Serratia marcescens* and *Bacillus thuringiensis* infect their host by producing pore-forming toxins, which cross the peritrophic matrix and target the midgut epithelium (Hertle, 2002;

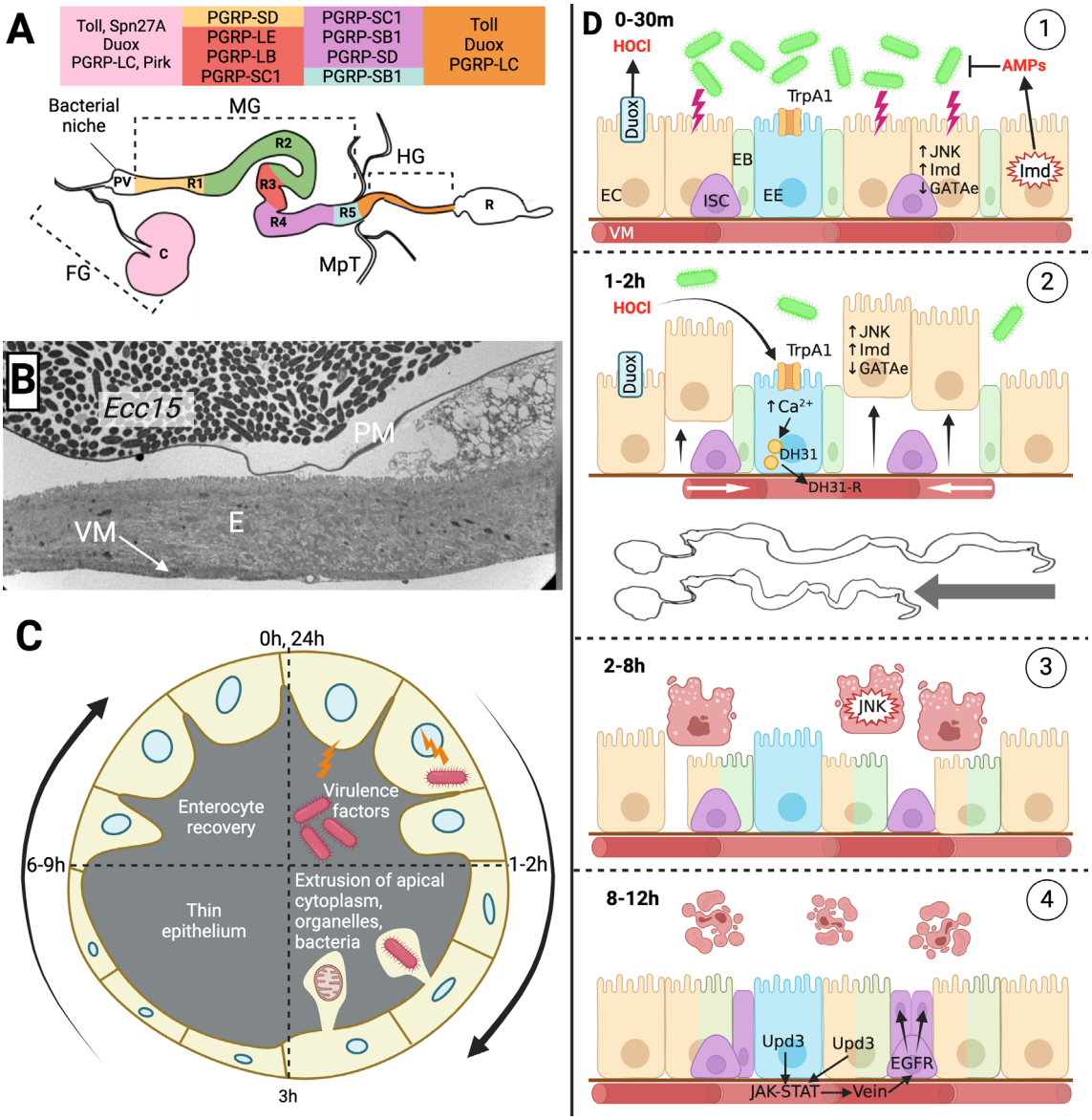


Figure 28 The gut immune response

A Schematic showing regions of the *Drosophila* adult gut. PV, proventriculus; FG, foregut; C, crop; MG, midgut; MpT, Malpighian tubules; HG, hindgut; R, rectum. The foregut and hindgut are of ectodermal origin and are lined with cuticle, while the midgut is derived from the endoderm and is lined with peritrophic matrix. Colored regions show differential expression of positive and negative regulators of immune pathways in the major gut regions, which tunes the immune response in the gut to accommodate commensal bacteria and eliminate pathogens. Toll pathway genes are highly expressed in the fore- and hindgut regions but absent in the midgut. The Imd receptor PGRP-LC and ROS-producing oxidase Duox are also more strongly expressed in the fore- and hindgut than the midgut. The Imd negative regulator Pirk is strongly expressed in the crop. The adult midgut can be further subdivided based on differences in morphology, cell composition

and gene expression into regions R1-R5. R3 contains the copper cell region, which produces high acidity and aids in bacterial elimination (Buchon et al., 2013b; Dutta et al., 2015). The larval midgut is similarly divided based on morphology and expression, but regional gene expression differs from the adult fly (Bosco-Drayon et al., 2012). **B** Electron microscopy cross section of *Drosophila* gut orally infected with *P. carotovorum Ecc15* bacteria. PM, peritrophic matrix; E, epithelium; VM, visceral muscles. **C** Schematic of gut cross-section showing epithelial thinning response following oral infection with bacteria producing pore-forming toxins (such as *Serratia*). Apical extrusion of cytoplasm reduces transit of bacteria that have entered the epithelium to the hemolymph, reducing opportunity for systemic infection. Enterocytes rapidly recover following this response. Inspired by (Lee et al., 2016). **D** Gut responses following oral infection with *Ecc15*. EC, enterocyte (tan); ISC, intestinal stem cell (purple); EB, enteroblast (green); EE, enteroendocrine cell (blue); VM, visceral muscles (red). **D1**, Pathogenic bacteria in the lumen activate JNK and Relish in a subset of enterocytes, suppressing GATAe activity. Imd activation triggers AMP expression in a subset of gut cells to suppress pathogens. The reactive oxygen species HOCl is produced in the gut lumen by Duox in enterocytes (see **Box 9**). **D2**, Enterocytes with increased JNK and Relish activity and reduced GATAe delaminate and become disordered. Trp1A channels in enteroendocrine cells bind HOCl and initiate calcium flux leading to export of DH31 neuropeptide, which likely binds the receptor DH31-R in the longitudinal visceral muscles and causes them to spasm, shortening the gut and promoting expulsion of bacteria through increased defecation. **D3**, Enterocytes strongly activate JNK and undergo anoikis (apoptosis as a result of delamination). Differentiation of enteroblasts to enterocytes rapidly restores gut epithelium. Gut length rapidly recovers. **D4**, Proliferation of intestinal stem cells mediated by EGF-R restores enteroblasts, and differentiation completes restoration of the gut epithelium. JAK-STAT-mediated production of the EGF-R ligand Vein in the visceral muscles in response to Upd3 secreted by enteroblast and enterocytes promotes EGF-R activity. Compiled with data from: (Benguettat et al., 2018; Buchon et al., 2010; Zhou et al., 2013). Figure created with BioRender.com, CC-BY-NC-ND.

Kurz et al., 2003; Lee et al., 2016; Nehme et al., 2007; Opota et al., 2011). Some bacteria such as *P. entomophila* also secrete proteases that degrade the peritrophic matrix, facilitating the action of pore-forming toxins (Shibata et al., 2015). Cross-linking of the matrix by enzymes like transglutaminase creates a balance between resistance to pore-forming toxins and sufficient permeability for nutrient absorption (Hachfi et al., 2023; Kuraishi et al., 2011; Lee et al., 2016; Shibata et al., 2015). In addition to the peritrophic matrix, tight septate junctions of the epithelium prevent entry of most pathogens from the gut to the hemolymph compartment (Bonnay et al., 2013; Kuraishi et al., 2011; Nehme et al., 2007; Shibata et al., 2015; Villegas-Ospina et al., 2021) (but see Systemic immune activation in response to oral infection, page 118). We still know little about the structure of *Drosophila* peritrophic matrix, the role of the numerous *Drosophila* peritrophin genes, or how this matrix is generated in the cardia/proventriculus and modified along the digestive tract. However, an extensive single cell characterization of the foregut, including the proventriculus, has recently provided new insight on this complex structure, paving the way for a genetic dissection of this important immune and digestive barrier (Zhu et al., 2024).

Although the *Drosophila* genome encodes a large set of mucin and mucin-related proteins that are enriched in prolines and potentially glycosylated threonines and serines (Buchon et al., 2013b; Syed et al., 2008), we know almost nothing about mucins in the *Drosophila* digestive tract and their possible role in host defense. The gut of *Drosophila melanogaster* includes an acidic region (~pH 2) called the copper cell region or

R3 region (Buchon et al., 2013b; Overend et al., 2016). The maintenance of the low pH in this region is dependent on H^+ V-ATPase, together with K^+/Cl^- and $Na^{2+}/\text{-}HCO3^-$ transporters (Buchon et al., 2013b; Overend et al., 2016). Suppression of the acidic region by silencing the V-ATPase gene increases susceptibility to *Pseudomonas* and results in a higher abundance of key members of the gut microbiota (*Acetobacter*, *Lactobacillus,* and *Lactiplantibacillus*), pointing to its role in host defense and homeostasis.

Interestingly, the pH of the acidic region is reduced in germ-free *Drosophila*, indicating of a role of the gut bacteria in shaping the pH conditions of the gut (Barron et al., 2024; Overend et al., 2016). Compartmentalization of the gut tends to decline with age, leading to reduced acidity in the R3 region (Buchon et al., 2013a; Li et al., 2016). This leads to a concomitant increase in microbiota load and dysbiosis, with a change in the *Acetobacter*/*Lactobacillus* ratio, contributing to gut dysplasia and aging (Li et al., 2016).

B. Inducible antimicrobial responses in epithelia

Transcriptomic studies reveal that the gut inducible immune response is complex and compartmentalized (Buchon et al., 2013b, 2009). Imd-deficient flies are susceptible to oral bacterial infection, highlighting its role as a key regulator of the gut immune response (Buchon et al., 2009; Liehl et al., 2006; Marra et al., 2021a; Ryu et al., 2006). Ingestion of Gram-negative bacteria triggers specific regional expression of AMP genes (Buchon et al., 2009b) through the Imd transmembrane receptor PGRP-LC in the ectodermal parts of the gut, and the intracellular receptor PGRP-LE in the midgut (Bosco-Drayon et al., 2012; Joshi et al., 2023; Neyen et al., 2012) (**Figure 28A**).

Negative regulators of the Imd pathway, including enzymatic PGRPs (PGRP-LB, PGRP-SC1A/1B/2) that scavenge peptidoglycan and gut-specific regulators (Trabid, LUBEL), balance the antibacterial response and immune tolerance (Aalto et al., 2019; Bosco-Drayon et al., 2012; Costechareyre et al., 2016; Fernando et al., 2014; Lhocine et al., 2008; Paredes et al., 2011). Flies lacking these negative regulators exhibit excessive and harmful immune activation to innocuous infection (Paredes et al., 2011). Peptidoglycan fragments (notably TCT) can also traverse the gut and remotely induce a systemic immune response through signal transduction involving the hemocytes (Basset et al., 2000; Charroux et al., 2018; Neyen et al., 2012) (see Systemic immune activation in response to oral infection, page 118). Regional transcription factors like Nubbin and Caudal further shape the Imd pathway response along the gut (Dantoft et al., 2016; Lindberg et al., 2018; Ryu et al., 2004; Ryu et al., 2008). Both the JNK and Imd pathways contribute to enterocyte delamination, a cell shedding process that might promote bacterial elimination (Loudhaief et al., 2017; Zhai et al., 2018a) (**Figure 28C, D**). To mitigate excessive damage, it has been proposed that Diedel, a cytokine that inhibits the Imd pathway (Lamiable et al., 2016b), is produced by the fat body and binds to integrins of gut epithelial cells to oppose their delamination and apoptosis (Mlih and Karpac, 2022).

The Toll and melanization pathways are functional in the foregut and hindgut, which are of ectodermal origin, but not in the midgut (**Figure 28A**). Interestingly, some putative antifungal peptides with homology to Drosomycin, Drsl2 and Drsl3, are induced in the gut via the JAK-STAT pathway (Buchon et al., 2009b; Osman et al., 2012). Several lysozymes are also constitutively expressed in the midgut at high levels. These likely play a digestive role but could shape the immune response by cleaving immunogenic peptidoglycan (Hultmark, 1996; Marra et al., 2021a). Many aspects of gut immunity act

to control systemic immune activation in response to bacteria within the gut lumen (see Systemic immune activation in response to oral infection, page 118).

C. Pathogen expulsion via gut peristalsis

Ingestion of pathogenic bacteria triggers strong contractions of longitudinal visceral muscles which shorten the gut, facilitate rapid expulsion of bacteria, and limit opportunities to colonize the midgut (Benguettat et al., 2018; Du et al., 2016) (**Figure 28**). HOCl is produced by Duox in enterocytes upon oral infection (**Box 9**), which is sensed by the TrpA1 receptor in enteroendocrine cells. This receptor produces the neuropeptide DH31, which activates spasms in nearby visceral muscles. Ongoing work suggests that in larvae, oral infections trigger contractions of a gut sphincter that traps bacteria in the anterior midgut and exposes them to antimicrobial peptides produced by Imd signaling (Tleiss et al., 2024). Interestingly, only pathogens such as *P. carotovorum Ecc15* are trapped in the anterior midgut, while symbiotic bacteria such as *L. plantarum* can pass through and reach the posterior midgut. This mechanism is specific to larvae; in adults, pathogenic bacteria are rapidly expelled by peristalsis (Tleiss et al., 2024).

D. Epithelial thinning and renewal

Oral bacterial infection triggers increased epithelial renewal through stem cell proliferation. This process, which is crucial to maintaining gut integrity, involves many pathways including EGFR and JAK-STAT (Biteau and Jasper, 2011; Buchon et al., 2010, 2009b; Jiang et al., 2011, 2009). Epithelial renewal in response to the symbiotic bacteria *Lactobacillus plantarum* or the opportunistic pathogen *Pectobacterium carotovorum Ecc15* involves the NADPH oxidase Nox (Iatsenko et al., 2018; Jones et al., 2013; Patel et al., 2019). Lactate produced by *L. plantarum* is metabolized by the host to produce the Nox substrate NADPH, which increases ROS production and stimulates epithelial turnover (Iatsenko et al., 2018) (**Figure 28**, **Box 9**).

Study of the roles of Duox in the gut have produced some contradictory results. An initial study using RNAi suggested Duox was required to produce microbicidal ROS (Ha et al., 2005; Westlake et al., 2024), but ROS has highly variable effects on different species of gut microbes, promoting the growth of some while mildly inhibiting others (Sekihara et al., 2016). Recent results suggest that Duox may contribute to bacterial elimination primarily by playing a signaling role in gut peristalsis (Benguettat et al., 2018) (see Pathogen expulsion via peristalsis, page 113). Notably, Duox also promotes tracheal branching, facilitating gut oxygenation needed to sustain epithelial renewal (Perochon et al., 2021; Tamamouna et al., 2021). Oxygenation levels also likely influence composition of gut microbiota. A recent study shows that ROS produced by Duox in the Malpighian tubules triggers Upd3 production in response to oral infection. Upd3 is then flushed forward from the Malpighian tubules to the anterior midgut by a countercurrent flow initiated by infection, where it stimulates epithelial renewal (Liu et al., 2023). Countercurrent flow has been described in the digestive tract of several insects (Terra, 1988) and may reveal further mechanisms through which Malpighian tubules contribute to gut immunity. Several studies also find a role for Uracil produced by pathogenic but not symbiotic bacteria in stimulating Duox activity and ROS production (Du et al., 2016;

Box 9 ROS production from NADPH and ROS detoxification

Duox and Nox transmembrane oxidases can produce extracellular superoxide (O_2^-, very unstable) from oxygen while oxidizing NADPH to $NADP^+ + H^+$ (Lambeth and Neish, 2014). Duox has an additional extracellular peroxidase domain that can produce hydrogen peroxide (H_2O_2, comparatively stable) from O_2^- (**Figure Box 9**). The peroxidase domain of Duox may also produce hypochlorous acid (HOCl), which is highly unstable and may be microbicidal (Ha et al., 2005, but see Westlake et al., 2024) or fulfill a signaling role in expulsion of pathogenic bacteria (Benguettat et al., 2018; Du et al., 2016). Because they are highly reactive, ROS typically act locally in the region that they are produced. Iatsenko and colleagues proposed that the Nox substrate NADPH is generated by oxidation of microbiota-derived lactate by the intestinal lactate dehydrogenase (Iatsenko et al., 2018). Both Duox and Nox are activated by increased calcium concentration through an EF hand domain that binds Ca^{2+}. Duox activity is regulated by the Gaq-Phospholipase Cß-Ca^{2+} pathway (Ha et al., 2009a) while the *Duox* gene can be transcriptionally upregulated by the MEKK1-P38c-ATF2 pathway (Chakrabarti et al., 2014; Ha et al., 2009b).

In the gut, Nox has a signaling role in stimulating stem cell proliferation in response to stress (Iatsenko et al., 2018; Jones et al., 2013; Patel et al., 2019). Duox has been implicated in multiple processes including (**i**) sclerotization of the peritrophic membrane in mosquitoes (Kumar et al., 2010); (**ii**) production of signaling ROS involved in visceral muscle contraction (Benguettat et al., 2018; Tleiss et al., 2024), wound healing (Chakrabarti and Visweswariah, 2020; Razzell et al., 2013), and Upd3 production in Malpighian tubules (Liu et al., 2023); (**iii**) production of microbicidal ROS (Ha et al., 2005) (but see Westlake et al., 2024); and (**iv**) tracheal development (Jang et al., 2021; Kizhedathu et al., 2021). Null mutations in *Duox* are lethal; the dominant lethal recessive mutation $Duox^{Cy}$ causes the curly wing phenotype commonly used as a genetic marker (Hurd et al., 2015).

Consistent with a need to control ROS to prevent damage to the host through processes such as lipid peroxidation, the *Drosophila* genome encodes a range of enzymes involved in ROS detoxification: (**i**) three catalases that convert hydrogen peroxide to water (the cytoplasmic Cat, the extracellular Immune-Regulated Catalase (IRC), and CatB which is likely localized to the peroxisome and mitochondria); (**ii**) three superoxide dismutases that convert superoxide radicals to water and hydrogen peroxide (cytoplasmic SOD1, mitochondrial SOD2, extracellular SOD3); and (**iii**) several peroxidases (e.g., Pxd, Gtpx, Gpxl) which like catalases can convert hydrogen peroxide to water but also organic hydroperoxides such as peroxidated lipids (LOOH or LOOR) to L-OH (Lennicke and Cochemé, 2020). ROS can also be produced by the mitochondrial respiratory chain (mainly by Complex I, where O_2^- which is transformed into H_2O_2 by SOD2) and during the melanization reaction (see **Figure 15**).

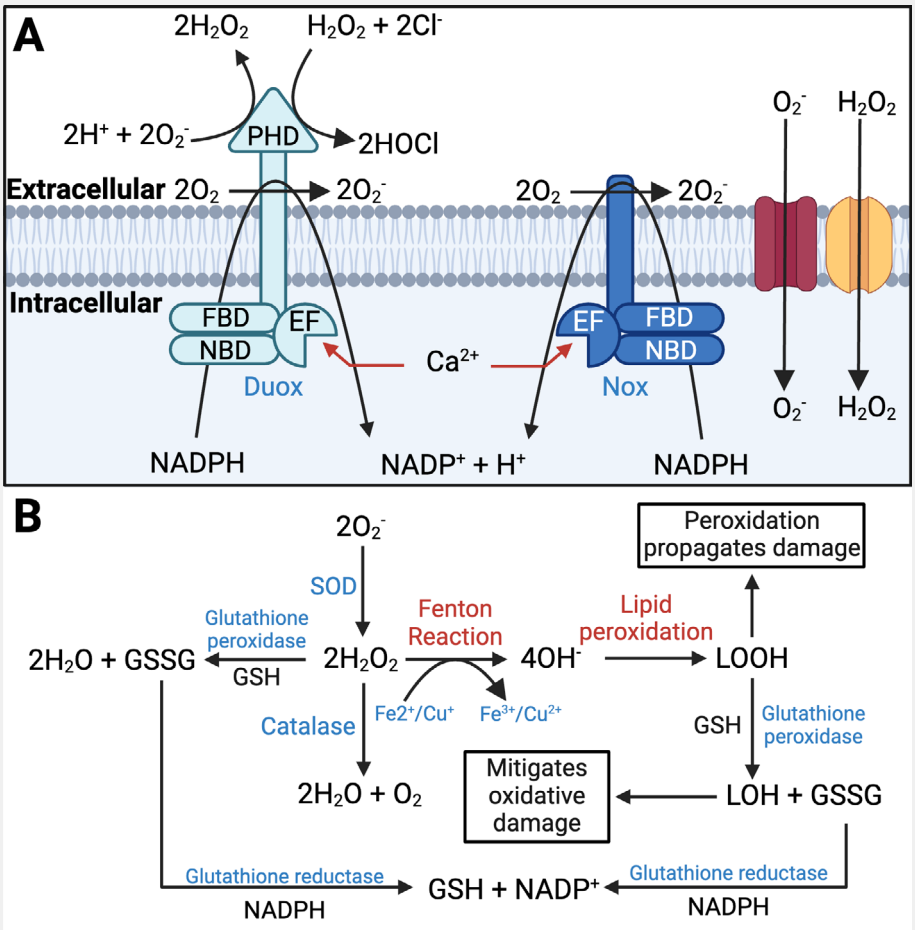


Figure Box 9 ROS production by NADPH

A Production of reactive oxygen species (ROS) by Nox (NADPH oxidase) and Duox (dual oxidase). Both proteins have an intracellular NADPH-binding domain (NBD) and FAD-binding domain (FBD). Increased intracellular Ca^{2+} activates Nox and Duox through their EF hand domains. Upon activation, Nox and Duox bind intracellular NADPH and transfer electrons to extracellular O_2, generating superoxide (O_2^-). The extracellular peroxidase homology domain (PHD) of Duox promotes dismutation of O_2^- to less reactive H_2O_2, and in the presence of chloride ions (Cl^-), production of highly reactive hypochlorous acid (HOCl). Superoxide and hydrogen peroxide generated by NADPH oxidases can act extracellularly or cross cell membranes via chloride channels (red) and aquaporin channels (orange), respectively, and act intracellularly. **B** Major oxidation and reduction reactions of ROS. To detoxify ROS and mitigate damage to host proteins, superoxide dismutases (SOD) convert superoxide to hydrogen peroxide, while catalases convert hydrogen peroxide to water and oxygen. Damage to host proteins is promoted by Fenton reactions: In the presence of proteins that contain copper or iron ions (e.g., cytochrome c oxidases, prophenoloxidases), H_2O_2 is further oxidized to produce highly reactive hydroxyl radicals (OH^-) which can produce peroxidated lipids (LOOH) and damage host tissues. Glutathione peroxidases can reduce peroxidated lipids (LOH) and mitigate damage through oxidation of glutathione substrate (GSH to GSSG), which is then reduced in the presence of glutathione reductase and NADPH. Compiled with data from: (Fisher, 2009; Fukai and Ushio-Fukai, 2011; Kim and Lee, 2014). Figure created with BioRender.com, CC-BY-NC-ND.

Kim et al., 2020; Lee et al., 2015, 2013). Overall, the role of Duox in the gut and other tissues requires clarification, but recent studies indicate that Duox-dependent ROS plays a signaling role rather than a direct microbicidal role.

Interestingly, symbiotic or pathogenic microbes not only impact intestinal stem cell proliferation but also differentiation, thus changing epithelial composition. All microbes stimulate the Imd/Relish pathway (NF-κB), but pathogens additionally generate stress and damage that stimulate the JAK-STAT pathway, leading to accumulation of enteroendocrine cells (Broderick et al., 2014; Jneid et al., 2023; Liu et al., 2022b). Higher numbers of enteroendocrine cells could contribute to microbe elimination by increasing peristalsis (Benguettat et al., 2018; Ye et al., 2021).

In addition to epithelial renewal, *Drosophila* intestinal epithelia undergo an evolutionarily conserved thinning response when exposed to hemolysin, a pore-forming toxin secreted by *Serratia marcescens*. During this process, epithelial cells extrude most of their apical cytoplasm without lysing, then recover their initial thickness within a few hours (**Figure 28C**). This is a rapid and efficient response that may promote tolerance by expelling damaged organelles and preventing transcellular bacterial transit to the hemolymph, with pore-forming toxins serving as alarm signals (Lee et al., 2016; Socha et al., 2023).

E. Systemic immune activation in response to oral infection

In mammals, invasive bacterial pathogens such as *Shigella*, *Salmonella* or *Listeria* penetrate the intestinal epithelium and spread systemically (Ribet and Cossart, 2015). In *Drosophila,* the cuticle and peritrophic matrix that line the digestive tract are thought to provide an efficient physical barrier that restrict contact between pathogens and the gut epithelium. Although there is currently no direct demonstration of entomopathogenic bacteria gaining access to the cytoplasm of the *Drosophila* intestinal epithelium, the *Serratia marcescens* strain *Db11* (Flyg et al., 1980) can efficiently cross the *Drosophila* intestinal barrier to reach the hemolymph (Lee et al., 2016; Nehme et al., 2007). Currently, we have little histological information on how and where peptidoglycan and *S. marcescens* cross the digestive tract barrier. While *S. marcescens* is highly pathogenic upon systemic injection and triggers an antibacterial response, infection with this same bacterium via an oral route has reduced pathogenicity and fails to trigger an immune response, despite bacteria entering the hemolymph (Kocks et al., 2005; Nehme et al., 2007). The observation that *S. marcescens* infection is less pathogenic upon oral infection is consistent with the recent observation that this bacterium can switch from a pathogenic to commensal strategy upon ingestion by the fly (Wang et al., 2024). Circulating plasmatocytes play an important role in control of *S. marcescens* that enter the hemolymph via the gut. Bacteria infiltrate the hemolymph in *domino* mutant larvae lacking hemocytes, indicating that the cellular response is required to eliminate bacteria that opportunistically cross the gut barrier (Braun et al., 1998).

Interestingly, bacteria such as *P. carotovorum Ecc15* and *P. entomophila* can trigger a strong systemic immune response in *Drosophila* after oral infection, although they appear to be confined to the intestine (Basset et al., 2000; Vodovar et al., 2005). This is likely mediated by the translocation of small peptidoglycan fragments from the gut

lumen to the hemolymph. This notion is supported by the observation that (**i**) ingestion of monomeric peptidoglycan can stimulate a strong systemic immune response in *PGRP-LB* deficient flies that lack the ability to degrade and reduce immunogenicity of peptidoglycan (Charroux et al., 2018; Charroux and Royet, 2022; Paredes et al., 2011; Zaidman-Rémy et al., 2006), and (**ii**) that this response dependent on the PGRP-LCx/PGRP-LCa heterodimer that senses monomeric peptidoglycan (Neyen et al., 2012). One study alternatively points to a key role of nitric oxide (NO) as a signaling molecule in gut to fat body signaling (Foley and O'Farrell, 2003), but the effect of NO might be indirect (Westlake et al., 2024). Another study proposed that activation of Imd signaling in the gut, either genetically or by pathogenic infection, promotes the hemocyte-mediated conversion of hemolymph sugars to polyols. Accumulated polyols in the hemolymph then activate fat body Imd signaling through upregulation of Mmp2, which cleaves the PGRP-LC ectodomain at the surface of fat body cells (Yang et al., 2019) (see **Box 4**). Hemocytes are also essential for gut-to-fat body signaling in larvae following *Ecc15* ingestion (Basset et al., 2000; Charroux and Royet, 2009). Gut regions are associated with adherent hemocyte populations, which may be resident, such as those adhering to the larval proventriculus (Zaidman-Rémy et al., 2012) or induced to attach upon oral infection (Ayyaz et al., 2015). These may play a signaling role and help co-ordinate a systemic response to gut infection. Oral infection with *Ecc15* also activates the Imd pathway in Malpighian tubule cells through PGRP-LE sensing, which impairs their filtration function and leads to a bloating phenotype caused by fluid accumulation and fat body wasting (Zugasti et al., 2020).

F. Regulation of microbiota load in the gut

The microbiota promote gut homeostasis by stimulating basal epithelial renewal, promoting differentiation of stem cells to enterocytes rather than enteroendocrine cells, and by inducing a low level of Imd pathway activity (Broderick et al., 2014; Buchon et al., 2009; Liu et al., 2022b). Microbiota load is regulated by the same defense mechanisms that combat pathogens. Copper cell region acidity and AMPs limit microbiota load, which is critical in old flies that have higher bacterial counts (Buchon et al., 2009; Li et al., 2016; Marra et al., 2021a; Overend et al., 2016). Peristalsis also gradually eliminates most gut microbes, except for bacteria resident in the proventriculus niche of adults (Dodge et al., 2023; Pais et al., 2018).

Several additional mechanisms help to maintain the microbiota while preventing immune activation (Bosco-Drayon et al., 2012; Charroux et al., 2018; Paredes et al., 2011). First, regional expression of PGRP-LC and PGRP-LE receptors likely contributes to differential expression of negative regulators of Imd, compartmentalizing immune activation. Activation of the Imd pathway via PGRP-LC mediates the microbicidal response in the anterior gut, while activation via PGRP-LE creates a protective zone for bacteria in the posterior midgut[18] (Bosco-Drayon et al., 2012; Charroux et al., 2018; Guo et al., 2014; Neyen et al., 2012) (**Figure 28A**). Second, regional transcription factors such as Caudal limit expression of AMPs and favor expression of negative regulators in the

18 Similarly, in the plant pest fruit fly *Bactrocera*, expression of antimicrobial peptides through PGRP-LC in the anterior gut blocks pathogen entry, while expression of negative regulators in distal parts of the gut define a zone that favors establishment of symbiotic bacteria (Yao et al., 2022).

posterior part of the gut creating an environment more favorable to the microbiota (Choi et al., 2008; Ryu et al., 2004; Ryu et al., 2008). Third, symbiotic bacteria tend to stimulate low Imd pathway activity due to their growth characteristics. Peptidoglycan fragments that activate the Imd pathway are released upon bacterial cell division, and are produced more quickly by fast-growing pathogens than established microbiota members (Arias-Rojas et al., 2023; Attieh et al., 2020; Zaidman-Rémy et al., 2006). Fourth, some microbiota members such as *L. plantarum* have cell walls with a thick peptidoglycan layer and teichoic acid modifications that protect them from antimicrobial peptide activity and increase persistence in the gut (Arias-Rojas et al., 2023; Attieh et al., 2019; Zaidman-Rémy et al., 2006).

Interestingly, chronic Imd pathway activation tends to select for AMP-resistant pathobionts, leading to dysbiosis and further immune activation (Aalto et al., 2019; Kosakamoto et al., 2020; Ryu et al., 2008). In contrast, suppression of the Imd pathway tends to increase microbiota load upon aging, leading to higher rates of epithelial renewal and reduced lifespan (Buchon et al., 2013a, 2009). Thus, multiple mechanisms balance the level of Imd pathway activation in the gut.

An open question is whether *Drosophila* can shape its microbial environment by seeding antimicrobial peptides into its surroundings through salivary gland secretions or defecation of AMPs produced in the gut. External digestion through the release of amylases has been proposed in other arthropods and *Drosophila* (Miguel-Aliaga et al., 2018), and antimicrobials or lysozymes could be similarly expelled in fly species that feed on bacteria, either to shape the external microbiome, or to predigest food bacteria.

G. Local immune responses in other tissues

Similar to its role in the gut, the Imd pathway is the primary regulator of antimicrobial defense in other epithelia (Tzou et al., 2000). For example, the tracheae have an intact Imd pathway that responds to natural infection by expressing AMPs and Tsf1 (Gendrin et al., 2013; Wagner et al., 2008). The immune transcriptome of the trachea is otherwise less complex than that of the gut, as it is composed of a simple epithelium and does not undergo epithelial renewal (Bossen et al., 2023; Gendrin et al., 2013; Wagner et al., 2009). Tracheal infection instead induces genes involved in the stress response and oxidoreduction and suppresses a set of chitin binding proteins (e.g., Twdl), suggesting that the chitinous tracheal intima is remodeled following infection. These results show that Imd activation in various epithelia induces sets of core and tissue-specific transcriptional responses.

In contrast, the Toll pathway is usually not involved in local epithelial immunity, likely because its intricate extracellular signaling cascade cannot function in lumenal fluids as it does in hemolymph (but see (Bahuguna et al., 2022)). Antimicrobial peptide genes such as Drosomycin are also expressed constitutively in some tissues (e.g., female spermathecae, salivary gland) independent of the Toll and Imd pathways but under the control of developmental transcription factors such as the POU transcription factor Drifter for reproductive organs and Caudal for salivary glands (Ferrandon et al., 1998; Junell et al., 2010; Ryu et al., 2004; Tzou et al., 2000) (see The genitalia as an immune tissue and infection route, page 121). Melanization is also operational in some epithelia, such as the gut and tracheae. Two serpins, Spn28D and Spn77B, specifically regulate melanization in the trachea, although the source of PPO for these reactions is

unclear (Scherfer et al., 2008; Tang et al., 2008). Interestingly, Spn77B deficient larvae with melanized tracheae also induce the Toll pathway at a low level in the fat body, likely through Psh.

Strikingly, many of the proteins contributing to the first line of defense during metamorphosis are provisioned by massive apocrine secretion by the larval salivary glands, which express complete immune pathways (Beňová-Liszeková et al., 2021; Nandy et al., 2018). Many organs, including the salivary gland and Malpighian tubules, express antimicrobial peptides and future research may reveal important roles for these organs in host defense.

11
Sex and immunity

Sexual intercourse is a major source of infection. Thus, potent and specialized host defense mechanisms exist in sexual organs of male and female flies. Mating also has important consequences on the defense response in females by diverting resources to reproduction. Moreover, male and female flies face different evolutionary pressures, resulting in sexual dimorphisms in immunity. Thus, sex and mating status are important parameters to take into consideration when considering the fly immune system (Belmonte et al., 2020; Schwenke et al., 2016).

A. The genitalia as an immune tissue and infection route

Like other epithelia, reproductive tissues can be an entry point for infection. Copulation in *D. melanogaster* invariably results in wounding of the intima of the female genitals by the male aedeagus (Kamimura, 2010; Mattei et al., 2015), providing a direct route through which systemic infection and mortality may occur (Miest and Bloch-Qazi, 2008; Zhong et al., 2013). Melanization and wound healing programs are activated in the genital epithelium immediately following mating, likely to repair copulatory wounds (Delbare et al., 2023; Kamimura, 2010). In male flies, Gram-negative peptidoglycan or bacteria externally applied to the male genitals can activate local and systemic immune responses, and can establish infections through this route in immunocompromised flies (Gendrin et al., 2009). Thus the genitals, like other *Drosophila* epithelia, express a variety of AMPs to protect tissues in contact with the external environment and limit pathogen entry (Ferrandon et al., 1998; Tzou et al., 2000; Wagner et al., 2008). Much of the constitutive expression of AMPs in the reproductive tract is independent of Imd, and instead controlled by other systems including the POU/Oct factor Dfr/Vvl, and the transcription factor Caudal (Junell et al., 2010; Ryu et al., 2004; Tzou et al., 2000).

B. The local immune response to mating

Mating induces a host of immediate transcriptional and translational responses in the female reproductive tract, including upregulation of proteins involved in cytoskeletal organization, cell migration, and tissue morphogenesis. This drives the extensive morphological and physiological remodeling of the reproductive tract to prepare for egg production that occurs post-mating, and may also contribute to healing of wounds inflicted during mating (Delbare et al., 2023; Mattei et al., 2015) (**Figure 29**). Mating also initiates mild transient upregulation of immune genes including AMPs and serine proteases

both locally in the genital epithelia and reproductive organs, and in the abdominal fat body of female flies (Fricke et al., 2020; Innocenti and Morrow, 2009; Mack et al., 2006; McDonough-Goldstein et al., 2021; McGraw et al., 2004). Transient immune activation in the genital epithelia and fat body following mating are dependent on Sex Peptide (Domanitskaya et al., 2007; Kapelnikov et al., 2008; Peng et al., 2005). Indeed, ectopic expression of Sex Peptide in the female fat body is sufficient to induce a significant expression of AMP genes via the Toll and Imd pathways (Peng et al., 2005), however the precise mechanism by which Sex Peptide activates immune pathways has not been fully characterized. As the hydroxyproline motif of Sex Peptide is required for this activity, it may activate immunity through chemical mimicry of sugar components of the bacterial cell wall (Domanitskaya et al., 2007). Introduced microbes also appear to play a role in mating-induced immune activation, as mating with axenic males leads to lower immune gene induction in females (Delbare et al., 2020). Males undergo very different and comparatively mild transcriptional changes in response to mating (Fowler et al., 2019; McKean and Nunney, 2001; Rai et al., 2023; Winterhalter and Fedorka, 2009).

Sex Peptide is one of many male accessory gland proteins (Acps) incorporated into the seminal fluid of males that have a variety of effects on female physiology, such as temporarily decreasing female receptivity and attractiveness to other males in addition to altering metabolism and immunity (Avila et al., 2010; McGraw et al., 2008; Newell et al., 2020; Ram and Wolfner, 2007). Seminal fluid has antimicrobial properties thought to combat infection in the female following mating (Lung et al., 2001). One candidate for this activity is the ejaculatory duct specific protein Andropin. However, while Andropin has similarities to antibacterial peptides, it lacks comparable *in vitro* activity to Cecropin A (Samakovlis et al., 1991), and the peptides responsible for antimicrobial activity have not been conclusively identified. Male courtship song prior to mating may pre-emptively upregulate stress proteins in females such as Turandot M, which could improve female survival against sexually transmitted infections (Zhong et al., 2013).

C. Consequences of mating on host defense

In addition to transient immune activation and remodeling of the reproductive tract, mating induces a permanent change in female fly metabolism linked to reproduction and biogenesis (Gioti et al., 2012; Gordon et al., 2022; Innocenti and Morrow, 2009; Kapelnikov et al., 2008), which results in persistent immune suppression and decreased resistance to a variety of infections (Fedorka et al., 2007; Gordon et al., 2022; Short and Lazzaro, 2010). Sex Peptide is retained in the female genitals and has persistent activity, increasing production of Juvenile Hormone (JH) which suppresses ecdysone-mediated potentiation of immunity (Flatt et al., 2008; Schwenke and Lazzaro, 2017; Zhang and Palli, 2009). Although antimicrobial peptide (AMP) gene transcription in mated females in response to infection is only mildly suppressed or delayed if at all (Flatt and Kawecki, 2007; Gordon et al., 2022; Wigby et al., 2008), transcription may not accurately reflect immune protein production due to post-transcriptional regulation (Lauwers et al., 2009; Vasudevan et al., 2017; Wei et al., 2009) and metabolic limitations (see Metabolic adaptation associated with systemic antimicrobial responses, page 69). A recent paper showed that immune activation in mated females overloads translational demand on the fat body, which is responsible for both reproductive and immune protein production.

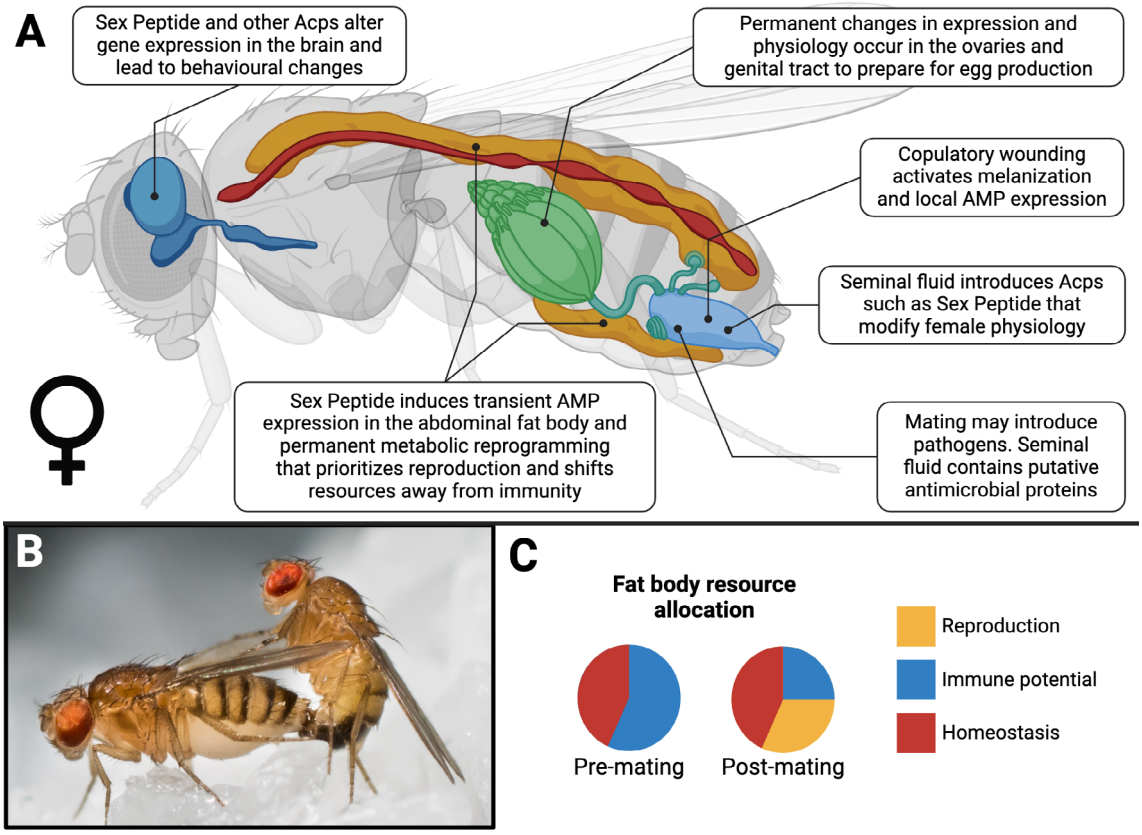


Figure 29 The post-mating response in female flies
A Summary of changes in female flies following first mating. Many post-mating changes are induced by male accessory gland proteins (Acps) such as Sex Peptide introduced during mating (Avila et al., 2011). **B** Mating flies, photo credit Francisco Romero Ferrero, via Wikimedia Commons. **C** Following mating, more fat body resources are allocated to egg production, leaving fewer resources for production of immune proteins such as AMPs. Figure created with BioRender.com, CC-BY-NC-ND.

This causes endoplasmic reticulum (ER) stress that reduces effector translation and efficiency of the immune response (Gupta et al., 2022). Reciprocally, fecundity is reduced with increasing immune activation through detection of peptidoglycan by octopaminergic neurons in the brain (Kurz et al., 2017), indicating that synthesis of AMPs incurs a reproductive cost (Nystrand and Dowling, 2020; Schwenke et al., 2016). These results show that there is a physiological trade-off in females between egg production and immune defense that may affect survival in a pathogen-dependent manner, depending on the metabolic and immune resources needed to resist and tolerate specific infections. Due to the interaction with metabolism, female survival to certain pathogens following mating may be highly dependent on diet (Rai et al., 2023).

D. Sexual dimorphism and immunity in *Drosophila*

Sexual dimorphisms arise from differences in natural selection imposed on males and females, notably in regard to reproduction. The nature and significance of sex differences in *Drosophila* immunity has not yet been fully explored, though many observations point to their existence. Differences in the immune response and susceptibility to pathogens based on sex are, however, widely present across animals. Recent studies in *Drosophila* have highlighted that biological sex influences *Drosophila* host defense in different ways, although only a few broad generalizations can be made given the current state of research *(e.g., "males are more susceptible than females to pathogen X")*, which is reviewed extensively by (Belmonte et al., 2020). Importantly, this is not just a question of sex, as the mating status is an important parameter (see Consequences of mating, page 122). The female fat body provisions energy both for oogenesis and for induced immune responses. As a consequence, mating status has a marked effect on survival in females (Camus et al., 2018; Fedorka et al., 2007). Experimental inhibition of translation in the fat body prior to mating improves female survival upon infection relative to uninhibited controls when translation is restored. This suggests that the metabolic needs of female homeostasis and immunity are at odds (Gupta et al., 2022). Sexual dimorphism is also expected in the gut immune response as the female gut is more plastic with higher basal and induced levels of stem cell activity (Hudry et al., 2016; Regan et al., 2016). Other tissues, most obviously the ovaries and testes, also contribute to differential gene expression of immune genes between male and female flies. Furthermore, adult females harbor higher hemocyte numbers (Duneau et al., 2017b; Kleinhesselink et al., 2011). A major cause of sexual dimorphism results from diet*sex interactions, which could also partially explain some of the inter-lab variation in the field. For instance, dietary protein content has a greater effect on fitness in females compared to males (Camus et al., 2019; Regan et al., 2016), and protein in lab diets is derived from brewer's yeast, purchased from regional suppliers that likely have yeast strain differences (Sannino and Dobson, 2023). Triglyceride metabolism further relies on genes with sexually dimorphic expression (Wat et al., 2020), which could explain some dimorphisms in baseline immune state.

It is common to observe mild differences in susceptibility between males and females, but the multiple influences of sex on various parameters makes the mechanistic interpretation of these differences difficult. Moreover, few studies have been systematically designed to test sexual dimorphisms in infection susceptibility, such that many inferences are made based on post-hoc observations, and inter-study methodological differences are not controlled for (see Belmonte et al., 2020). In some cases, opposite trends have been reported even using the same pathogen (e.g., (Vincent and Sharp, 2014) and (Chowdhury et al., 2019), which differed in mating status). Females are reported to suffer increased mortality against *P. rettgeri*, due to activation of the Toll-SP pathway (Duneau et al., 2017b), which is triggered by cleavage of the serine protease Persephone. Flies with mutations affecting Toll at or downstream of Persephone lack this sexually dimorphic response to *P. rettgeri*. Persephone itself may be activated by bacterial proteases secreted by *P. rettgeri*, which is consistent with recent findings on this protein (Issa et al., 2018; Nakano et al., 2023). Other studies using different genetic backgrounds have found mixed results for a sexual dimorphism following *P. rettgeri* infection (Mullinax et al., 2023; Shit et al., 2022), collectively suggesting this result relies partly on lab effects, and partly on genetics. Genetic background itself interacts with mating to impact

post-infection bacterial load (Short and Lazzaro, 2010). A male-specific erect wing response was also observed in *Baramicin A*-deficient males, but less so in females (Hanson et al., 2021), although the mechanism behind this remains unclear. The genetic tools available in *Drosophila*, notably the ability to change the phenotypic sex of tissues (Cline and Meyer, 1996; Hudry et al., 2016; Regan et al., 2022) make *Drosophila* a suitable model to analyze sexual dimorphism in immunity. However, these studies require careful consideration of the route of infection, nature of pathogens, genetic background, and nutritional conditions, which have complex interactions with biological sex. Systematic study will further elucidate the underlying causes of sexually dimorphic responses after infection.

12

Immunity in non-infectious disease

Immune system function is not limited to the control of infectious agents. It also plays an important role in maintaining tissue homeostasis, removing dead cells and limiting tumor growth (Eberl and Pradeu, 2018). Dysregulation of the immune system upon aging or in other contexts is now linked to many diseases in both *Drosophila* and humans. Thus, current research is trending towards analyzing the roles of immune system components in processes beyond traditional host defense such as autoimmunity, cancer, and neurodegeneration, with parallels to human diseases (Yamaguchi, 2018). We briefly highlight some of these findings here.

A. Autoimmunity

Constitutive activation of immune programs is deleterious, causing lethality or shortened life span (De Gregorio et al., 2002a; Garschall and Flatt, 2018; Hanratty and Dearolf, 1993; Lemaitre et al., 1995a; Levashina, 1999; Paredes et al., 2011). The exact causes of these immunopathologies are not known, although toxic apoptosis, ROS exposure, metabolic wasting and dysbiosis have been invoked (Wang et al., 2023).

One of the largest phenotypic classes of mutations in *Drosophila* are 'melanotic tumor' mutants, which have black melanized bodies free-floating within the larval body cavity or attached to internal organs (Minakhina and Steward, 2006; Rizki, 1960; Watson et al., 1992) (**Figure 30**). Melanotic tumors are not necessarily cancerous; rather, they represent an auto-immune reaction that typically involves encapsulation of self-tissues by lamellocytes at the larval stage (see Encapsulation, page 100). Genotypes causing melanotic tumors can be divided into two classes (Avet-Rochex et al., 2010; Watson et al., 1991): Class I have normal cellular immune systems but abnormal self-recognition (often due to mutations in endocytic pathways or extracellular matrix components), while Class II have an overactive immune system (mutations activating Toll, JAK-STAT) defined by ectopic activation of hemocytes and an abnormal response to normal tissue (Minakhina and Steward, 2006). Like autoimmune diseases, the frequency of these non-invasive pseudo-tumors is influenced by genetic background and environmental conditions (e.g., Kim and Choe, 2014; Mortimer et al., 2021). Melanotic tumors can be induced by simultaneous disruption of the basement membrane and cell integrity or apicobasal polarity (Kim and Choe, 2014; Rizki and Rizki, 1983), or by necrotic cell death (Park et al., 2020). Constitutive activation of Toll in the fat body (e.g., using gain of function alleles such as Tl^{10b}) is also sufficient to induce lamellocyte differentiation and a melanotic tumor phenotype (Schmid et al., 2014). As Tl^{10b} larvae have abnormal fat body cells (Gerttula et al.,

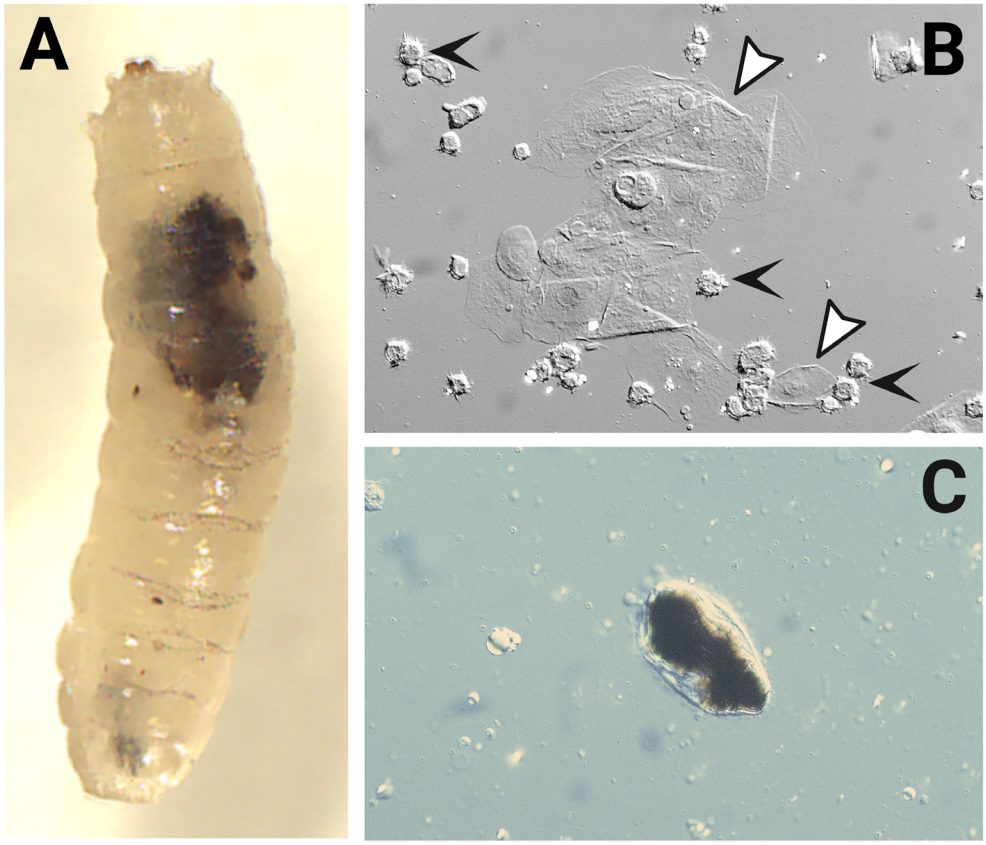


Figure 30 Autoimmunity in Drosophila: Melanotic tumors
A Melanotic tumor around the larval fat body in a Tu^{Sz1} mutant background. This line has (**i**) a mutation in the gene encoding the JAK-STAT kinase Hop causing precocious hemocyte differentiation and (**ii**) a mutation in the GCS1 gene which disrupts N-glycosylation of extracellular matrix proteins covering the fat body that identify it as self-tissue (Mortimer et al., 2021) (Photo Nathan Mortimer and Todd Schlenke). **B**, **C** The mutation Tl^{10b} causing the constitutive activation of the Toll pathway leads to melanotic tumor formation (Gerttula et al., 1988; Lemaitre et al., 1995a). **B** Lamellocytes (white arrowheads) are flat cells much larger than plasmatocytes (black arrowheads) that adhere in layers to non-self material, forming a melanized capsule (Photo credit, B. Lemaitre CC). **C** Melanotic tumor mutants may have melanized lamellocyte-encapsulated bodies free-floating in the larval body cavity; these are usually pieces of loosely adherent tissue that have detached from organs such as the fat body (Photo credit, B. Lemaitre CC).

1988; Lemaitre et al., 1995a), this suggests that simultaneous activation of Toll and the presence of abnormal tissues is sufficient to induce encapsulation of host tissues. These studies suggest that patrolling hemocytes identify basement membrane as self and only react to certain cues perceived as absence of self or danger signals, such as necrosis or basement membrane breach. Loss of membrane N-glycosylation leads to melanotic tumor formation only in the presence of activated hemocytes (Mortimer et al., 2021), indicating that both aberrant tissues and overactive hemocytes contribute to this phenotype.

Increased topology of the surface of the fat body due to either inhibition of endocytosis or greatly increased exocytosis (overactivation of protein production) can also trap extracellular matrix components and other proteins at the surface of the fat body, causing fibrotic accumulations that chronically activate the humoral and cellular immune responses (Csordás et al., 2020; Zang et al., 2015).

B. Immunity in tumor control

Several neoplastic tumor models are available in *Drosophila*, combining mutations disrupting epithelial polarity (*Dlg*, *Scrib*) and over activating proto-oncogenes (*Src*, *Ras*). These models provide a platform to study cancer progression and interactions between tumors and their environment (Bilder et al., 2021; Enomoto et al., 2018). Similar to wounds, many tumors induce ROS through Duox, secrete Upd3, attract hemocytes and stimulate the Toll pathway. Both cellular and humoral immune responses as well as the microbiota affect tumor growth (Bangi et al., 2012; Parisi et al., 2014; Zhou and Boutros, 2020).

Hemocytes and JNK signaling may either promote or restrict tumor growth, depending on tumor characteristics. Hemocytes bind to disrupted basement membrane of tumors and express TNF/Eiger, activating JNK in tumor cells and causing apoptosis (e.g., Chen et al., 2012; Pérez et al., 2017). In an allograft Notch-induced neural stem cell model, hemocytes bind and engulf tumor cells to restrict growth in a process that requires NimC1, Draper, and Croquemort but not Eater. In addition to their protective role, it was suggested that hemocytes may also increase host morbidity by producing damaging extracellular reactive oxygen species (Voutyraki et al., 2023). In tumors where apoptosis is inhibited (e.g., $>Ras^{V12};scrib^{-/-}$), the high levels of JNK signaling induced by hemocyte-derived Eiger through the TNF receptor Grindelwald increase metastatic growth through overexpression of the matrix metalloproteinase *Mmp1*. High levels of ROS in these tumors attract more hemocytes, leading to amplification of the signal and exacerbating tumor growth (Andersen et al., 2015; Diwanji and Bergmann, 2020; Fogarty et al., 2016). Similarly, recruitment of hemocytes by senescent cells in the larval hindgut can promote tumorigenesis through non-autonomous activation of JNK signaling (Datta and Bangi, 2024). Recent studies indicate that Grindelwald mediates the systemic and apoptotic functions of Eiger, whereas the alternate TNF receptor Wengen has roles mainly in the central nervous system (Palmerini et al., 2021).

In vitro studies have shown that some antimicrobial peptides (AMPs) have antitumoral activity, making them the current focus of translational studies aiming to combine AMP treatment with cellular antitumor therapy (Jafari et al., 2022). It is not yet clear whether endogenous AMPs can have similar activity *in vivo*, and which mechanisms allow these molecules to target and attack aberrant host cells. Recent studies have highlighted the antitumoral effect of *Drosophila* AMPs in two cancer models caused by mutations disrupting the hematopoietic organ (e.g., *Mxc*) and imaginal discs (e.g., *dlg*) (Araki et al., 2019; Kinoshita et al., 2022; Parvy et al., 2019). These studies revealed that *in vivo,* some AMPs have cytotoxic effects that selectively enhance apoptosis of tumor cells. Parvy et al. showed that the cationic AMP Defensin is secreted from the trachea and fat body and binds *dlg* imaginal disc tumor cells due to their increased exposure of phosphatidylserine, a negatively charged phospholipid (Parvy et al., 2019). Phosphatidylserine exposure is increased in tumor cells by hemocytes, which bind to tumors and

secrete TNF/Eiger, activating JNK signaling. These studies provided the first *in vivo* examples of endogenous AMPs acting as anti-cancer agents. AMPs can also have a protective effect: in a $>Ras^{v12}$ salivary gland tumor model, expression of the AMP *Drosomycin* prevents tissue damage by suppressing JNK pathway activity (Krautz et al., 2020). In a non-AMP example of immune interaction with tumors, overproduction of clotting factors (e.g., Fondue) in a fly ovarian tumor model causes lethal hypercoagulation (Hsi et al., 2023). Thus, various aspects of the immune system are engaged depending on the tumor model, which can have either pro- or anti-tumoral effects.

C. Immunity in neurodegeneration

In recent years, *Drosophila* has emerged as a powerful model to study neurodegeneration, and several models mimicking human diseases have been developed, including Alzheimer's, Parkinson's, and Huntington's diseases (Dabool et al., 2019; Nainu et al., 2019). Both humoral and cellular immune programs are critical for nervous system function and maintenance. Phagocytic glia play a major role in brain health, but must be tightly regulated (Kurant, 2011). Defective phagocytosis in the brain (e.g., Draper mutants) leads to neurodegeneration through accumulation of debris (Draper et al., 2014; Elguero et al., 2023), while excessive phagocytosis can lead to abnormal neuronal cell death by phagoptosis[19] (Hakim-Mishnaevski et al., 2019). Impaired autophagy leads to age-dependent neuronal loss, associated with overactivation of immunity (Shukla and Giniger, 2019).

The Imd pathway is induced in many neurodegenerative contexts and is suspected to play an active role in disease progression (Cao et al., 2013; Kounatidis et al., 2017; Li et al., 2018; Petersen et al., 2013, 2012). Mutations of the gene encoding the Imd transcription factor Relish can rescue neurodegeneration in several genetic contexts (*Dnr1, ATM*, *Cdk5α*, *Draper*, etc.). AMPs may exhibit pro-neurodegenerative activities, possibly by targeting negatively charged neurons, which may have naturally high phosphatidylserine exposure similar to the trachea (Cao et al., 2013; Hanson and Lemaitre, 2020) (**Figure 21**). Strikingly, loss of the AMP *Metchnikowin* protects against traumatic brain injury and amyotrophic lateral sclerosis-mediated neuronal loss (Lee et al., 2023; Swanson et al., 2020). The determinants of cause and effect, and their mechanisms of action, remain to be elucidated.

Neurodegeneration and brain dysfunctions have been associated with infection and inflammation in humans (Amor et al., 2014; Leblanc and Vorberg, 2022). As in mammals, the *Drosophila* brain is protected from systemic infection by the blood-brain barrier, composed of the perineural and subperineural glial layers (Benmimoun et al., 2020; Desalvo et al., 2011). There is however a window at the pupal stage where activation of the Imd pathway in the glia or brain infection by group B *Streptococcus* can recruit plasmatocytes into the central nervous system, across the blood brain barrier (Winkler et al., 2021). Interestingly, infection with *Enterococcus faecalis* induces permeabilization of the blood brain barrier in adult flies, associated with higher JAK-STAT reporter activation and expansion of septate junction markers in subperineural glial cells (Kim et al., 2021b).

19 Phagocytes play an important role in removal of apoptotic cells, a process called efferocytosis. However, phagocytes can also play an active role in the process by killing live target cells (Brown and Neher, 2012; Zohar-Fux et al., 2022). This phagocytosis-induced cell death is called phagoptosis.

Bidirectional interactions between the brain and the immune system are complex and poorly understood. There is no doubt that similar to human disease contexts, cellular and humoral immune programs contribute to neurodegeneration in *Drosophila*. However, it is unclear whether immune programs are initiators of neurodegenerative diseases, or primarily respond to pre-existing disease states and exacerbate neurodegenerative phenotypes by causing collateral damage.

13
Behavioral immunity

Like other insects, *Drosophila* uses its sensory system to detect pathogens and trigger various behaviors that prevent infection. These mechanisms include avoidance of pathogen-associated odors, spore removal by grooming, food uptake blockage, and sickness behaviors required for efficient health recovery. Thus, the *Drosophila* immune and nervous systems cooperate to increase fitness and protect the next generation (Davis and Schlenke, 2022; Montanari and Royet, 2021). Neuronally controlled behaviors are complex and likely of greater importance in survival to pathogens than is currently appreciated, as this is a nascent branch of study.

A. Avoidance and food uptake blockage in response to pathogenic microbes

Drosophila detect and adapt behavior in response to an array of volatile olfactory cues related to the microbial environment. *Drosophila* are attracted to odors from *Saccharomyces cerevisiae* and *Lactobacillus plantarum* symbionts, but are repelled by *Acetobacter malorum* (Venu et al., 2014). Metabolites produced by lactobacilli such as propionic and butyric acid are sensed by specific odorant receptors to stimulate appetite (Depetris-Chauvin et al., 2017). In contrast Geosmin, a volatile associated with harmful fungi or bacteria, is a repellent sensed by odorant receptor Or56A (Stensmyr et al., 2012). Similarly, a feces-derived phenol sensed by Or46A prevents *Drosophila* feeding or egg laying on potentially pathogenic bacteria (Mansourian et al., 2016). Beyond avoidance, infection by *P. entomophila* and *P. carotovorum Ecc15* induces a food uptake blockage that likely limits infection (Chakrabarti et al., 2012; Keita et al., 2017). Surprisingly, infection by pathogens such as *P. entomophila* alters the odors emitted by flies, greatly increasing production of pheromones associated with courtship and aggregation. This response is expected to benefit the pathogen by attracting healthy flies and enhancing pathogen dispersal (Keesey et al., 2017).

Feeding and egg laying assays show that *Drosophila* have a strong aversive response to the bacterial cell wall component LPS, dependent on the chemosensory cation channel TrpA1 in gustatory neurons (Keita et al., 2017; Soldano et al., 2016). In many studies using LPS, it is not clear whether LPS itself is the elicitor, or whether other contaminating microbial molecules induce the neuronal response. Of note, it is now well established that LPS provided by SIGMA™ is contaminated with lipopeptides and DAP-type peptidoglycans, which produced confusing findings in earlier innate immunity research (Kaneko et al., 2004). Toxic food consumption elicits complex

long-term post-ingestion behaviors that evoke disgust memory (Charroux et al., 2020; Kobler et al., 2020), while feeding on beneficial microbiota also modifies fly behaviors and sensory capacity (Fischer et al., 2017; Wong et al., 2017a).

B. Grooming as hygienic behavior

Grooming involves brushing the body and wings with the legs and cleaning the legs and the antenna with the mouthparts. Grooming is a very important hygienic behavior in removing spores of entomopathogenic fungi (Yanagawa et al., 2014; Zhang et al., 2020b; Zhukovskaya et al., 2013). In addition to spores, grooming behavior can be triggered by various chemicals such as LPS from SIGMA™ and quinine (Yanagawa et al., 2018, 2017, 2014). Multiple sensing modalities have been implicated in grooming including contact chemoreceptors, the olfactory system, and the Imd receptor PGRP-LC in the case of Gram-negative bacteria (Yanagawa et al., 2018, 2017, 2014). A recent study has shown that the *D. melanogaster* chemosensory protein CheA75a recognizes the *Metarhizium* Mcdc9 CFEM membrane protein, a group of proteins that mediate spore-host attachment. Fungi that have lost Mcdc9 fail to stimulate grooming behavior and kill flies more quickly (Shang et al., 2023). In this example that echoes effector-triggered immune mechanisms, a virulence factor that promotes spore attachment is now highjacked by the host to sense pathogens (Pradeu et al., 2024; Remick et al., 2023; Stuart et al., 2013).

C. Reduction of egg laying upon infection

Infected insects display post-infection behaviors such as feeding on specific diets that stimulate host defense, thermoregulatory behaviors that promote resistance, reduction of egg laying (Babin et al., 2023; Kurz et al., 2017), and modulation of sleep and activity (Lee and Edery, 2008; Mallon et al., 2014; Shirasu-Hiza et al., 2007; Surendran et al., 2017; Vale and Jardine, 2017; Vincent et al., 2022). Most of these disease-induced behaviors still require mechanistic characterization in *Drosophila*. Reduced egg laying following infection has been well characterized in *Drosophila*; this mechanism may transiently shift host resources from reproduction to immunity (see Consequences of mating on immunity, page 122). This behavior is triggered by the sensing of peptidoglycan by PGRP-LE in a subset of octopaminergic neurons in the central brain. This then prevents follicular cell rupture, a step required for egg-laying (Kurz et al., 2017; Masuzzo et al., 2019). Strikingly, this study revealed that in neurons, immune pattern recognition receptors can directly sense infection and modify behavior. Thus, the nervous system can directly react to microbial cues. This finding echoes the recent observation that recognition of peptidoglycan by the pattern recognition receptor NOD2 in mouse neurons affects body temperature and appetite (Gabanyi et al., 2022). Of note, several short peptides (IM33, Nemuri, Diptericin B, GNPB3-like), some of which have demonstrated antimicrobial activity, have been linked to brain function, although their immune and/or neurological roles in the brain are not yet clear (Barajas-Azpeleta et al., 2018; Toda et al., 2019; Xu et al., 2023b)

D. Behavioral immunity against parasitoid wasps

Parasitoids lay their eggs inside *Drosophila* larvae or pupae, which kill the host by consuming its tissues. *Drosophila* have an arsenal of behaviors to escape infestation by parasitoid wasps (Davis and Schlenke, 2022). Larvae roll to dislodge wasp ovipositors, a behavior that involves class IV nociceptive neurons (Hwang et al., 2007). *Drosophila* larvae and adults avoid sites smelling of *Leptopilina* wasps through specific olfactory sensory neurons: larvae sense the wasp odor iridomyrmecin, while adults detect actinidine and nepetalactol through the olfactory receptors Or49a and Or85f. Wasp odors can also prime progenitor hemocytes of the lymph gland to differentiate into lamellocytes. This involves the Or42a olfactory receptor, leading to production of extracellular GABA by projection neurons and the activation of the HIF/SIMA transcription factor in the lymph gland (Madhwal et al., 2020; Shim et al., 2013). Upregulation of the immune-associated peptide IBIN (Induced By INfection) is also observed in the optic lobes upon wasp sighting. IBIN itself plays a role in the mating response triggered by wasp sighting, suggesting that immune-regulated genes may have a role in behavior (Ebrahim et al., 2021).

Strikingly, *Drosophila* can also exhibit parental behaviors to protect their progeny from wasp infection (**Figure 31**). Sight and olfaction of wasps reduce *Drosophila* female oviposition rate by inducing apoptosis in the ovaries, and promote egg deposition on

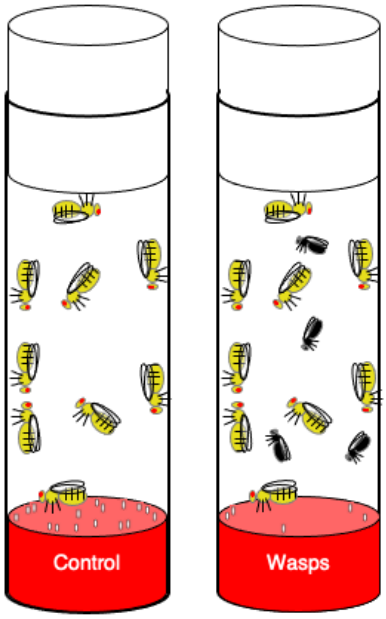


Figure 31 Behavioral immunity: reduced egg laying upon wasp infestation
Drosophila females reduce egg laying in the presence of parasitoid wasps. This parental behavior driven by olfactory and visual cues is expected to reduce wasp infestation. Schema kindly provided by Todd Schlencke.

ethanol rich substrates that are aversive to wasps (Kacsoh et al., 2013). Multimodal sensory integration regulates these behaviors, which are ultimately mediated by NPF neuropeptide signaling (Bozler et al., 2019). Anti-parasitoid behaviors display memory and can take place to a certain extent even after the wasp is removed (Kacsoh et al., 2015a, 2015b). Thus, anti-wasp behavioral immunity involves higher order neuronal function, such as memory that involves the mushroom body. These studies collectively reveal that a significant number of *Drosophila* behaviors are devoted to deterring infections.

14

Evolution of the immune system

With hundreds of related species sequenced, access to population genetic and genomic resources, and genetic tools in *D. melanogaster*, *Drosophila* is a powerful model to study how the immune system evolves in response to selection by natural challenges (Kim et al., 2021a; Mackay et al., 2012; Sackton et al., 2007). The short life cycle of fruit flies further enables experimental evolution approaches, with relatively easy tractability of both host and pathogen genetics.

A. Immune trade-offs with reproduction and other physiological functions

The purpose of an immune response is to preserve host fitness. Fitness is often defined in terms of reproductive success, which can encompass host survival for the purpose of later reproductive output, and can be extended to competitive capacity of offspring. This places a limit on immune readiness to instead prioritize growth, development, and reproductive output. Landmark studies identified a trade-off where increased resistance to wasps through higher investment in energetically-costly hemocytes slowed larval growth (Kraaijeveld et al., 2002; Kraaijeveld and Godfray, 1997). Indeed, excessive hemocyte numbers have been shown to limit starvation resistance in larvae due to their high metabolic demands (Ramond et al., 2020b) (see The hemocytes are a central metabolic hub, page 104). Single cell RNA sequencing has also shown that evolved resistance against wasps is associated with constitutive upregulation of immune genes associated with increased differentiation of lamellocyte precursors, the cell type that encapsulates wasp eggs (Leitão et al., 2020). Such energy trade-offs become more readily apparent in nutrient-poor conditions. For instance, fecundity shows a negative correlation with resistance to infection specifically in food-limited conditions (McKean et al., 2008). Trade-offs in immune readiness also take place in immune-competent tissues that perform multiple physiological roles. As previously discussed (see Consequences of mating on immunity, page 122), the fat body is not only involved in the production of immune effectors, but also provisions yolk during oogenesis. The transcriptional programs of reproduction and homeostasis are at odds with the metabolic needs of immunity (Gupta et al., 2022; Uttenweiler-Joseph et al., 1998). Therefore, female flies deploy resources towards fecundity that could otherwise be spent on improving immune readiness.

B. Variation within species

There is striking variability in resistance of wild-type flies to different pathogenic infections (Bangham et al., 2008; Bou Sleiman et al., 2015; Lazzaro et al., 2004; Orr and Irving, 1997). It therefore stands to reason that loci underlying resistance to infection are not fixed in the wild. The evolutionary processes that generate this variation include geographic (Hanson et al., 2019a) and seasonal selection (Behrman et al., 2018). Seasonal selection may rely on dynamic processes such as fluctuating pathogen presence, which drives frequency-dependent selection (balancing selection) (Chapman et al., 2019). This balancing effect maintains polymorphic alleles (Unckless and Lazzaro, 2016), which may have unique competence against certain pathogens. An alternate hypothesis is that alleles with unique competence against one pathogen might come at a cost to host fitness, and so are selected against in times when pathogen presence is low (Perlmutter et al., 2024).

These studies highlight variation in genes evolving under natural selection. The consequences of that variation are readily seen, as many encode polymorphisms at loci with major effects. For instance, genetic variation in the *edl* gene determines resistance against the parasitoid wasp *Leptopilina boulardi* (Hita et al., 2006, 1999), and recurrent loss of *lectin-24a* expression leads to increased susceptibility to wasp parasitization (Arunkumar et al., 2023). Multiple loci have large impacts on resistance against different viral infections (see Restriction factors, page 31). More recently, polymorphisms and gain/loss of AMPs have been described that can explain variability in resistance of wild flies to fungal or bacterial infections. A segregating duplication in the antifungal gene *Baramicin A* is observed in a notable proportion of the *Drosophila* Genomic Resource Panel (DGRP) fly stocks, which increases gene expression and presumably provides a protective effect (Hanson et al., 2021; Hanson and Lemaitre, 2022). Natural polymorphisms in the Buletin and Metchnikowin peptides are similarly associated with differences in survival upon infection (Hanson et al., 2022; Perlmutter et al., 2024). The retention or loss of *Diptericin* genes throughout the Drosophilidae lineage is closely associated with geography and host ecology. Naturally-occurring variation in these genes greatly affects defense against ecologically relevant *Providencia* and *Acetobacter* bacteria (Hanson et al., 2023; Unckless et al., 2016). In these studies, a *DptA* S69R allele provides protection against *P. rettgeri*, while naturally-occurring presence or absence of *DptB* determines susceptibility to *Acetobacter* systemic infection both within and across species. Pressures maintaining the alternate allele are not known, though it has been suggested that *DptA* S69R could interact with the host microbiome (Mullinax et al., 2023). For now, the selective forces maintaining polymorphisms remain poorly understood, but recent studies identify many examples that await characterization.

C. Variation between species

The *Drosophila* innate immune system is built on ancient and broadly-conserved signaling pathways (e.g., JAK-STAT, Toll/Imd NF-κB, cGAS-STING, JNK, MAPK) and immune effector mechanisms (e.g., AMPs, phagocytosis). These signaling pathways, and processes such as phagocytosis, are conserved in mammals (Buchmann, 2014; Flajnik and Du Pasquier, 2004; Leulier and Lemaitre, 2008; Magor and Magor, 2001), and the core genes of these pathways are very well-conserved across *Drosophila* species. However, some

mechanisms of realized immunity are more lineage-restricted, such as the melanization of arthropods (Palmer and Jiggins, 2015) or antiviral defense mechanisms of *Drosophila* (Hédelin et al., 2024; Imler et al., 2024).

This variation stems from gene duplication and loss events (e.g. (Palmer and Jiggins, 2015; Ruzzante et al., 2022; Salazar-Jaramillo et al., 2014)), and from positive selection (elevated rate of non-synonymous mutations) shaping the immune response. Indeed, immune genes evolve more rapidly than other genes in the genome (Kosiol et al., 2008; Sackton et al., 2007; Shultz and Sackton, 2019). In fruit flies, RNAi, receptor, and signaling genes are often seen as "hotspots" of evolution (Hill et al., 2019; Van Mierlo et al., 2014). For example, multiple approaches have found selection on the Relish cleavage complex and the Imd receptor PGRP-LC (Begun and Whitley, 2000; Jiggins and Kim, 2007; Obbard et al., 2009; Sackton et al., 2007). Contrary to vertebrates (Hollox and Armour, 2008; Lynn et al., 2004; Semple et al., 2003; Tennessen, 2005), early studies failed to recover signals of positive selection in *Drosophila* effectors (Lazzaro, 2008), despite evidence of AMP polymorphisms in natural populations (Lazzaro, 2003; Unckless and Lazzaro, 2016). Perhaps owing to advances in genomic resources and analytical techniques, many examples of positive selection in *Drosophila* AMPs are now well-described (Chapman et al., 2019; Early et al., 2017; Hanson et al., 2016; Hill et al., 2019; Unckless and Lazzaro, 2016).

D. Immune novelty through gene duplication and loss

Immune genes experience frequent gene duplication (copy number variation), which gives rise to extant multi-gene families that are often arranged in tandem in the genome. Immune genes are frequently found on chromosome 2R (especially AMP families). Strikingly, unrelated genes with functional relationships are often clustered together at a locus (e.g., *Toll*, *spz, grass*, and *pelle* together on the tip of chromosome 3R, *Cactus* and *Dorsal/Dif* on the 2nd chromosome) (**Figure 32**). This clustering could reflect an evolutionarily-favored state, for instance by allowing efficient chromatin unpacking for transcriptional co-regulation. Indeed, immune inducible genes that are not related are often clustered (De Gregorio et al., 2002b; Spellman and Rubin, 2002).

Gene copy number variation is particularly common among immune receptors and effectors (Clemmons et al., 2015; Ekengren and Hultmark, 2001; Gao Band Zhu, 2016; Hedengren et al., 2000; Quesada et al., 2005). Such duplications can allow partitioning of ancestral functions to daughter genes (i.e. subfunctionalization) (**Figure 33**). For instance, the transcription factor Dorsal regulates dorsoventral embryonic patterning, but a duplication of *dorsal* in the *Drosophila* ancestor gave rise to Dorsal-related immunity factor (Dif), which is the primary Toll NF-κB transcription factor in adult flies during the systemic immune response (Mayo, 2008; Zhou et al., 2015). Duplication of immune effectors may alternately provide the genome with raw material to perform novel roles. *Drosophila PPO3* stems from a duplication of *PPO2* and is expressed only in lamellocytes that defend against parasites (Binggeli et al., 2014; Dudzic et al., 2015). Both the *PPO3* gene and lamellocytes have been secondarily lost in *Drosophila sechellia*, a species adapted to feed on morinda fruit that is toxic to would-be parasites (Salazar-Jaramillo et al., 2014; Salazar-Jaramillo and Wertheim, 2021). Although lamellocytes are unique to the Melanogaster group, other species have their own specialized cells that perform a similar function (nematocytes, multinucleated giant hemocytes) (Kacsoh et al., 2014;

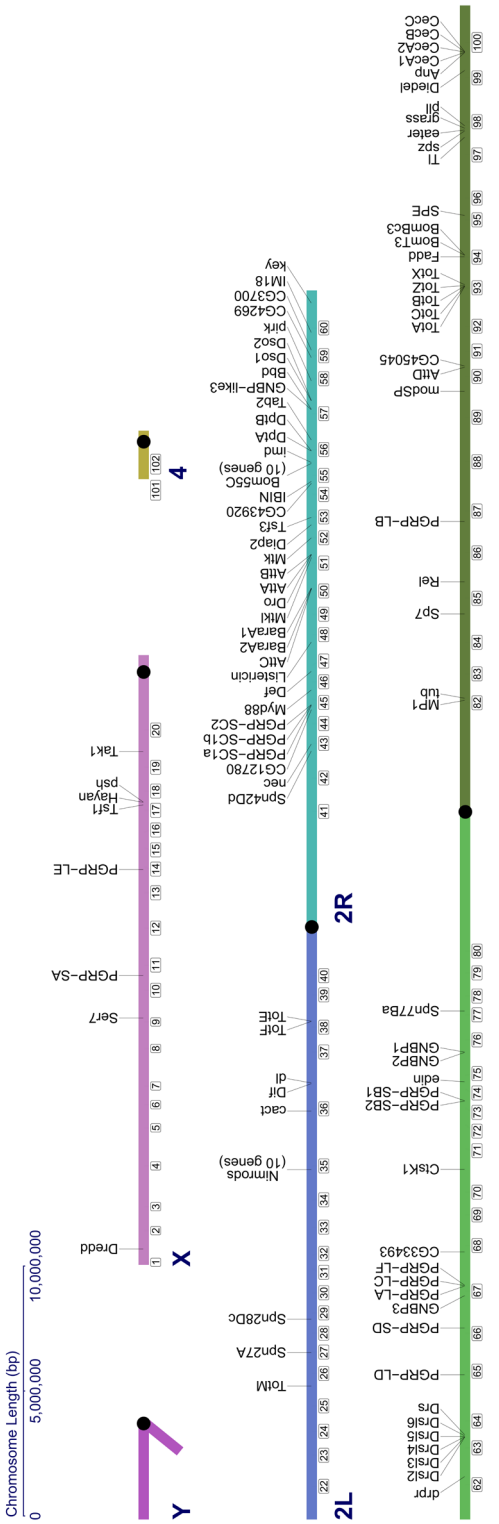


Figure 32 Cytological location of genes involved in the antimicrobial response
Genes belonging to one protein family are often clustered at a genomic locus following tandem duplication events (examples include Bomanins, Turandots, Cecropins, Drosomycins, and Nimrods). Some genes that are related in regulation or function, but not sequence, are also grouped together: *GNBP-like3/Bbd* in 57A; *Tl/spz/pll/grass* in 97D; and *dl/Dif/cact* in 35F-36C (Khush and Lemaitre, 2000). Chromosome 2R is of special note, bearing a high density of genes related to Toll/Imd signaling, including many host defense peptides. Genome-wide transcriptome studies have revealed that immune genes with similar expression patterns are also often clustered together on the genome (De Gregorio et al., 2002b; Spellman and Rubin, 2002). The cytological positions of immune genes were provided by FlyBase.

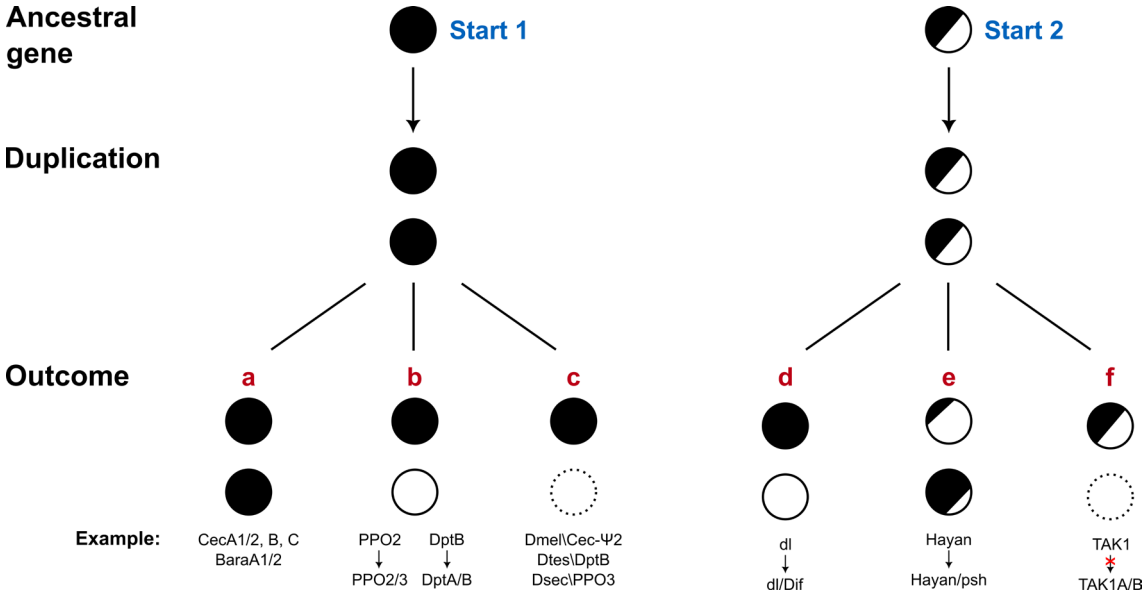


Figure 33 Routes for evolution of immune novelty by gene duplication
Two start points are given. In **Start 1**, a gene with relatively singular function undergoes duplication. Following duplication, three outcomes are possible. **a** No important additional change (Hanson and Lemaitre, 2022; Ramos-Onsins and Aguadé, 1998): this outcome likely results in increased transcriptional potential of the gene family, but does not generate novel function for the gene family itself. **b** Neofunctionalization, where one gene copy takes on a novel function, can arise if the gene provides a good scaffold to build on for addressing a pre-existing evolutionary pressure (Dudzic et al., 2015; Hanson et al., 2023; Salazar-Jaramillo et al., 2014). **c** Pseudogenization can occur through genetic drift or evolutionary selection, wherein the extra daughter gene either offers no selective advantage, or is dead-on-arrival, or the host niche shifts, creating a context where the net effect of the gene becomes deleterious (Hanson et al., 2023; Ramos-Onsins and Aguadé, 1998). In **Start 2**, a gene with multiple roles undergoes duplication, which can lead to: **d** Subfunctionalization, wherein the two genes specialize near-completely for alternate roles (Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000a); or **e** partial subfunctionalization, where both daughter genes evolve to become specialized for alternate roles of the parent gene, but retain somewhat overlapping function (Dudzic et al., 2019; Nakano et al., 2023; Shan et al., 2023). **f** Pseudogenization can also occur under purifying selection, where gene copy number is tightly regulated and genomic duplications are quickly purged. Such instances of gene duplication likely occur but are unlikely to be retained in the genomes of extant species.

Márkus et al., 2015), emphasizing how a common need can be addressed by parallels of evolution. The antimicrobial peptide *Diptericin A* (*DptA*) is similarly a duplication of an ancestral *DptB*-like gene, which diverged rapidly in the ancestors of the subgenera *Sophophora* and *Drosophila* (Hanson et al., 2023). The two extant *Diptericin* genes show specific importance in defense against different microbes across *Drosophila* species.

In contrast to effectors, duplication or loss of signaling cascade intermediates is rare, which is thought to reflect the need to precisely control dosage of positive and negative regulators (Sackton et al., 2007) (**Figure 33**). Perhaps the reason signaling cascade intermediates experience high rates of positive selection (Begun and Whitley, 2000; Hill

et al., 2019; Jiggins and Kim, 2007; Obbard et al., 2009) is because they do not readily duplicate. This makes them vulnerable to disruption, as they provide an evolutionarily stable cascade of proteins for suppressors of immunity to target. In response, hosts must prevent disruption either by evolving suppressor-blockers, or by evolving minor changes in the targeted proteins themselves that would allow them to escape pathogen suppressors. The low copy number variation that is common in these genes may therefore make them focal points in "red queen" host-pathogen arms races[20] (e.g., see Bitra et al., 2012; Hamilton et al., 1990), although the framing we propose here would benefit from robust empirical investigations.

An exception to low copy number variation in signaling genes may be those genes that have multiple isoforms or physiological roles. *Persephone* is an ancestral duplication of the serine protease Hayan, which has dual roles in propagating extracellular Toll signaling and cleaving PPO in the melanization response (Dudzic et al., 2019; Nakano et al., 2023). *Persephone* resembles only one of two *Hayan* isoforms, and is essential in Toll activation in response to pathogen proteases but has a very minor role in melanization (Dudzic et al., 2019; Ligoxygakis et al., 2002b). Strikingly, a parallel truncation of the *Hayan/persephone* daughter genes has occurred in *D. ananassae* to produce *persephone*-like genes at vice versa loci, suggesting this partitioning of specialized isoform functions to daughter genes was favored more than once (Dudzic et al., 2019).

Despite inducibility of the immune response, which is thought to limit immune costs to host fitness, immune effectors are lost when pathogen pressures shift due to changes in ecology. Determining whether these losses are driven by passive drift or active selection against as-yet unidentified costs of these genes is a burgeoning avenue of research. Many studies have now identified model infection systems with promising evolutionary relationships (Arunkumar et al., 2023; Hanson et al., 2023; Salazar-Jaramillo and Wertheim, 2021; Unckless et al., 2016). Such systems will inform on the environmental and internal pressures that contribute to evolutionary maintenance of immune modules.

E. Experimental evolution

The short life cycle of *Drosophila melanogaster* combined with modern capacity for high-throughput sequencing now allows analysis of the evolution of *Drosophila* under selective pressures in the laboratory. "Evolve and resequence" studies use experimental evolution to adapt populations to a novel environment, followed by next-generation sequencing to analyze genetic changes. By placing a polymorphic population under selection for several generations, we can detect variants that increase in frequency or become fixed, enabling monitoring of molecular evolution in real time on a genome-wide scale (Long et al., 2015). This type of experimental setting can not only identify traits that are susceptible to selection, but also reveal new immune mechanisms. Consistent with our mechanistic understanding of the *Drosophila* immune system, the route of infection is an important parameter in the selection process, and both resistance and disease tolerance mechanisms can undergo selection (Martins et al., 2013; Paulo et al., 2023).

20 The 'red queen' hypothesis states that species must continuously adapt and evolve to hold their own against pathogens and predators, which are also continuously evolving to better exploit their host or prey.

Resistance to infection often rapidly increases with selection, indicating the presence of standing genetic variation in the population. Surprisingly, selection for increased survival to a pathogen does not always lead to increased costs, as shown by the maintenance of immunity under pathogen free relaxed conditions over several generations (Faria et al., 2015). Experimental evolution studies have linked resistance to parasitoid wasps with increased hemocyte numbers, differentiation of hemocytes into a pre-lamellocyte state poised for deployment, and increased constitutive and inducible humoral (Toll, Imd, and JAK/STAT pathways) responses (Kraaijeveld et al., 2002; Kraaijeveld and Godfray, 1997; Leitão et al., 2020; Zhou et al., 2024). Adaptation to one pathogen can also lead to cross-resistance of the host against several parasites (Martins et al., 2014; Singh et al., 2021). In some cases, increased resistance is not linked to a change in the *Drosophila* genome, but to changes in symbionts such as *Wolbachia* (Faria et al., 2016).

In a reverse approach, pathogen evolution can be studied in wild-type and immune-deficient fly lines over several rounds of infection to identify how pathogens adapt to the immune system. In one study, *Drosophila*-adapted *E. faecalis* strains resistant to *Drosophila* immunity are characterized by mutations that increase resistance to various antibiotics and alter properties of the bacterial cell surface (Wadhawan et al., 2022). Experimental evolution of host and pathogen reveals another perspective on the immune system that complements mechanistic approaches and allows testing of hypotheses on the evolution of the immune system. These studies also emphasize that *Drosophila* strains kept in pathogen-free laboratory environments for many decades may have undergone erosion of immune defenses, as illustrated by the presence of cryptic immune deficient mutations in some lab stocks (e.g., *NimC1, Imd, PPO3*) (Dudzic et al., 2015; Honti et al., 2013; Lemaitre et al., 1995b).

15
Conclusions

Our knowledge of the *Drosophila* immune system is a vast body of slowly accumulated concepts and information. Following initial characterization of recognition and signaling factors in the early 2000s, the later advent of CRISPR-Cas9 allowed us to study effector genes such as antimicrobial peptides, which were used as immune readouts for many years but were not amenable to classical genetic techniques. Functional studies have improved our knowledge of how effectors contribute individually or collectively to immunity, and we can now confidently attribute many Toll and Imd pathway contributions directly to effector activity (Hanson et al., 2019b). At the same time, the crucial discovery of Bomanins in 2015 (Clemmons et al., 2015) revealed that we are still ignorant of many important mechanisms of defense. Future studies should investigate mechanisms behind the broad range of protection provided by the Bomanins. Although the notion that some of these peptides may block the impact of bacterial toxins rather than having direct microbicidal activity is appealing (Huang et al., 2023; Xu et al., 2023a), a direct role in fungus-killing has also been suggested (Lin et al., 2019; Lindsay et al., 2018). In either case, explaining how a peptide family can block such a wide array of challenges is a tough puzzle. New ways of thinking about and measuring resistance and tolerance (e.g. SPBL, BLUD (Duneau et al., 2017a)) are part of an ever-improving ability of drosophilists to dissect causes of mortality. The function or mechanism of many other inducible proteins such as TEPs ore IDGFs are also poorly understood. Thus, we are still far from understanding how the humoral response transforms the hemolymph into a compartment hostile to pathogens while protecting host tissues. The recent identification of factors that protect the host from autotoxic immune responses (e.g., Turandots, Materazzi, catalases), reveals the complexity of host tolerance mechanisms that maintain vital functions such as oxygenation, osmoregulation, and removal of damaged proteins during the immune response. Recent studies have also revealed how 'non-immune' tissues such as the muscles play a key role in host defense (Kierdorf et al., 2020; Yang and Hultmark, 2017). Future studies may reveal how various organs co-ordinate to adapt host physiology and metabolism in the immune response.

Although sensing and signaling has been the topic of studies for decades, it would be naïve to think that this process is fully understood in *Drosophila*. Important classes of receptors, such as CD36 homologs, scavenger receptors, Nimrods, cGLRs and even some PGRPs (e.g., PGRP-LA, PGRP-LCy) have not been functionally characterized. Alternative modes of Toll and Imd activation by ROS and cGLRs, and how hemocytes contribute to pathway activation, are still unclear. Multiple pathways downstream of ROS have been shown to trigger Upd production that activates the JAK-STAT systemic wound response, but it is unclear how this can be reconciled with JAK-STAT activation by the cytoskeletal component actinin (Gordon et al., 2018).

Following identification and ordering of canonical Toll and Imd pathway components, many studies have identified additional factors modifying pathway activity through interactions with universal cellular processes such as ubiquitination, sumoylation, endocytosis, and glycosylation. It is not yet clear if these factors modify pathway activity in general or tissue-specific ways, and this requires more study. For example, it has been shown that the JNK pathway is activated by TAK1 downstream of the Imd pathway (Boutros et al., 2002; Silverman et al., 2003). However, it is unclear whether this mode of JNK activation is operational in all tissues. Thus, how pathway activation and inhibition differ in the fat body, in various hemocyte types, or in epithelia is an important question. Characterizing immune responses in tissues such as the salivary gland, Malpighian tubules, digestive tract, reproductive tissues, and tracheae might reveal new mechanisms of defense that remain undiscovered. A blossoming understanding of *Drosophila* physiology has made it possible to gain a better whole-body understanding of immunity, including inter-organ communication during the immune response.

Most studies in *Drosophila* immunity have focused on specific stages, namely third instar larvae and adults, for reasons of convenience. Future studies should explore the role of the immune system throughout the *Drosophila* life cycle, notably during metamorphosis, which remains largely unknown. Both humoral and cellular mechanisms are likely important during pupariation, as revealed by the high expression of antimicrobial peptide genes and the critical role of hemocytes in metamorphosis (Stephenson et al., 2022). Aging is associated with chronic activation of the immune system and a decline in hemocyte number and immune reactivity (Arias-Rojas et al., 2023; Arias-Rojas and Iatsenko, 2022; Clark et al., 2014; Corbally and Regan, 2022; Garschall and Flatt, 2018; Hanson and Lemaitre, 2023; Horn et al., 2014; Khan and Prasad, 2013; Rera et al., 2012; Zerofsky et al., 2005). Although it is currently a subject of intense study, there is still a lot to learn concerning the complex interplay between immunity, microbiota, and aging. A major goal in the aging field - as it is more generally when analyzing the complex relationships between immunity, microbiota and diseases - is to understand if deregulation of the immune system or dysbiosis are causal factors that precipitate aging or simply bystander reactions accompanying this process. A third more holistic view is that immunity and the microbiota mutually influence each other in complex ways that, when disrupted, lead to a 'vicious cycle' that promotes and maintains a disease state. According to this idea (van de Guchte et al., 2018), homeostasis represents a stable state of equilibrium that serves health, but perturbation of this equilibrium beyond the limits of resilience can induce a shift to a stable pre-disease state that is generally healthy, but more likely to be triggered towards the development of chronic diseases. The powerful genetic tools available in *Drosophila* allow study of these complex relationships *in vivo*.

Many key immune processes including melanization, phagocytosis, and encapsulation remain poorly characterized. Studies of hemocytes have now revealed central roles in metabolism and repair in addition to immunity. CRISPR-Cas9 methodology offers the opportunity to study gene family members collectively or individually and clarify their roles in immune programs. The remarkable work of Katja Bruckner on connections between neurons and sessile hemocyte populations, and of Ulrich Theopold on pyroptosis-like behavior of crystal cells and tumor defense, offer fascinating new options for researchers. Hotly debated topics in vertebrate immunity such as contributions of

lysosomal enzymes, ROS, and acidity to microbe killing in the phagosome, may be receptive to genetic dissection in *Drosophila*. Similarly, beyond RNAi, we still know little of the effector mechanisms that restrict viral infection (e.g., *pastrel*, *Vago*, STING-regulated genes).

How the nervous and immune systems interact to shape both behavior and host defense is a developing area of research that can take advantage of many new insights into dual roles for immune genes in neurology and defense, alongside advances in mapping the *Drosophila* brain. For instance, we still know little of how the nervous system reacts upon brain or systemic infection, which offers an area for future studies. Furthermore, *Drosophila* presents a unique model for population genetics and ecology that can lead to dissection of general principles behind ecological and evolutionary factors that shape immune systems in general. New and well-assembled genomes across species, as well as RNA sequencing advances, now allow studies in a phylogenetic or experimental evolutionary framework. These studies can analyze how *Drosophila* co-evolves with mutualist and pathogenic microbes, and be used as a strategy to identify new immune genes or functions (Lezcano et al., 2023; Paulo et al., 2023; Wadhawan et al., 2022). These represent only a few of the many exciting research possibilities offered by the fly immune system.

The genetic approaches we are able to apply in *Drosophila* are powerful because they produce results reliable enough to be built upon in a cumulative manner. An informed community that is able to contextualize, correct, or build upon the findings of others is crucial in shaping a solid dataset. Being open to non-immune research conducted in the *Drosophila* model system leads to new discoveries and avoids viewing the immune system in artificial isolation. The accumulated work that has illuminated the complexities of the immune system paves the way for new discoveries that will continue to refine our understanding of host defense and innate immunity.

"Immunity" and beyond

Although we highlight many promising areas of research in *Drosophila* immunity, the most exciting discoveries cannot be predicted, and likely require broad exploration of the many facets of the *Drosophila* immune system. In the last fifteen years, our view of the *Drosophila* immune system has been greatly extended by major conceptual changes occurring in the field of immunology at large (Pradeu et al., 2024). These include increased awareness of symbiotic interactions (endosymbionts, microbiota, symbiont mediated immunity), interest in the pathogen side of immunity, the critical role and specificity of barrier epithelia such as the gut, the complexity and plasticity of hemocyte functions, immunometabolism, increased appreciation for the importance of disease tolerance and behavioral defense, the non-immune functions of the immune system, and the necessity of considering the interrelated evolutionary and ecological framing of *Drosophila* as an organism. In some respects, we must accept that our initial view of the immune system was naïve, and gradually explore new topics that open new horizons of research.

This expansion of our concept of innate immunity is not without issue; in particular, it has become more difficult to draw lines around what actually constitutes an 'immune system'. The term 'immune' could be applied to all factors that contribute to survival to infection. However, this opens the door to nearly unlimited extension of the

concept of 'immunity' to any factor that influences health, fitness, or resilience of the host, including developmental factors such as ecdysone and basic cellular mechanisms like mitochondrial function or autophagy. A major challenge in studying the immune system is establishing causal links and distinguishing direct and indirect effects, although both may be of value. Alternatively, the term 'immune' could be applied only to mechanisms of resistance that target microbes and parasites. The Toll and Imd pathways are confidently called 'immune' pathways because they regulate host defense peptides with direct effects on pathogens. Complications quickly arise when considering pathways such as JAK-STAT, which plays a role in the systemic wound response[21] or the JNK pathway, which regulates cytoskeletal changes and antioxidant responses, but yet have critical roles in host survival to infection.

Many terms used in describing innate immunity are ambiguous because of strong connotations with adaptive immunity (specificity, memory), or because they represent archaic holdovers from early immune studies that were less precise in their understanding of molecular processes or mechanisms. The terms 'cellular immunity' and 'humoral immunity' can easily be applied to distinguish phagocytosis by hemocytes from the production of antimicrobial peptides by the fat body, but the situation is less clear for melanization, which has both cellular and humoral facets. Terms are more precise when they refer to specific molecular processes, but this restricts usage and may prevent useful generalizations. The adoption of scientific terms is strongly influenced by their selling value, as evidenced by an explosion in the use of the term 'inflammation' in recent decades. Early fly immunologists were reluctant to use this term, which was originally associated with migration of blood cells from vessels that are absent in insects. Over time however, we have gradually transitioned from 'mechanisms of *Drosophila* bear similarities to the inflammatory response of mammals' to 'inflammatory mechanisms of *Drosophila*'. The term 'inflammation' is now broadly used in *Drosophila* to describe NF-κB pathway activation, migration of hemocytes to wound sites in embryos, or anti-wasp responses.

The tendency to describe and conceptualize the fly immune system in terms of mammalian immunology is worrisome, as it can create bias and blind us to important mechanisms of defense unknown in mammals. Some key discoveries in *Drosophila* immunity were driven by the simple desire to understand its function, independent of mammalian immunity. Surprisingly, these breakthroughs are now used to justify using *Drosophila* exclusively as a model to approach human biology. Research does not occur in isolation and is strongly influenced by grant agencies, politics, and journal editors that may overvalue '*Drosophila* as a model' to the detriment of '*Drosophila* as itself'. This is not to suggest that we reject the comparative approach, but rather encourage recognition that insect immunity has its own importance in our understanding of the world, particularly in light of climate change effects on agricultural pests, insect vectors, and pollinators. The recently renewed relationships between drosophilists and entomologists is heartening and gives hope that immune research can move forward with increased dialogue between these communities and others with interest in invertebrates.

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21 The JAK-STAT pathway also regulates some putative antifungal peptides (Drs-like peptides) in the gut (Buchon et al., 2009a; Osman et al., 2012; Buchon et al., 2009b)

We have attempted here to encompass some of the intriguing progress made in *Drosophila* immunity that has accompanied major conceptual and methodological advances in recent years. It is difficult to predict what the next big findings will be, as we are far from exploring the full extent of a field that has only offered new intrigue with each discovery. This has and will continue to depend on the unique passion of drosophilists, with the support of granting agencies, to push forward and share new knowledge on the fascinating immune system of this little fly. Exciting discoveries await us.

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