REVIEWS

Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology

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Abstract | Since the discovery of antimicrobial peptide responses 40 years ago, the fruit fly *Drosophila melanogaster* has proven to be a powerful model for the study of innate immunity. Early work focused on innate immune mechanisms of microbial recognition and subsequent nuclear factor-κB signal transduction. More recently, *D. melanogaster* has been used to understand how the immune response is regulated and coordinated at the level of the whole organism. For example, researchers have used this model in studies investigating interactions between the microbiota and the immune system at barrier epithelial surfaces that ensure proper nutritional and immune homeostasis both locally and systemically. In addition, studies in *D. melanogaster* have been pivotal in uncovering how the immune response is regulated by both endocrine and metabolic signalling systems, and how the immune response modifies these systems as part of a homeostatic circuit. In this Review, we briefly summarize microbial recognition and antiviral immunity in *D. melanogaster*, and we highlight recent studies that have explored the effects of organism-wide regulation of the immune response and, conversely, the effects of the immune response on organism physiology.

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The fruit fly *Drosophila melanogaster* is a whole-animal model system that has been used to study how physiological responsiveness is integrated with immunity and how dysregulation of this integration leads to pathology. The study of *D. melanogaster* is highly relevant to mammalian biology given the evolutionary conservation of many of the key signal transduction pathways and transcriptional regulators that control development, metabolism and immunity[1–3]. Indeed, many of the organ systems of flies are homologous to those in vertebrates (FIG. 1). Flies can be used to study infectious diseases as they are infected naturally by bacteria, fungi and viruses, and can also be experimentally infected with human pathogens[4–6]. *D. melanogaster* is also a useful model in which to study the response of arthropods and insect vectors to human pathogens. Most important to experimental immunologists, there is a wide array of genetic tools for studying flies that allow for the fine manipulation of cells and tissues both spatially and temporally[7,8]. In the past few years, these genomic and molecular genetic technologies have provided many insights into immunity in this model system.

In this Review, we discuss key observations that demonstrate that *D. melanogaster* has much to offer to our understanding of innate immunity and how immune regulation fits into the broader context of organism biology. We begin by highlighting some of the key mechanisms and pathways involved in innate immune recognition of infection in *D. melanogaster*. We then discuss intestinal immunity, as the gut is at the intersection between immune defence and metabolic control. Next, we discuss the interplay between metabolism and immunity, in which *D. melanogaster* research has also begun to provide important mechanistic insights. Finally, we highlight recent discoveries in *D. melanogaster* that have advanced our understanding of the modulation of immunity — both locally and systemically — by hormones and endocrine signalling molecules produced by cells in the brain.

Microbial recognition

In 1972, Hans Boman and colleagues[9] discovered an inducible antibacterial immune response in *D. melanogaster,* thereby introducing a new model system in which to study innate immunity. This led to the
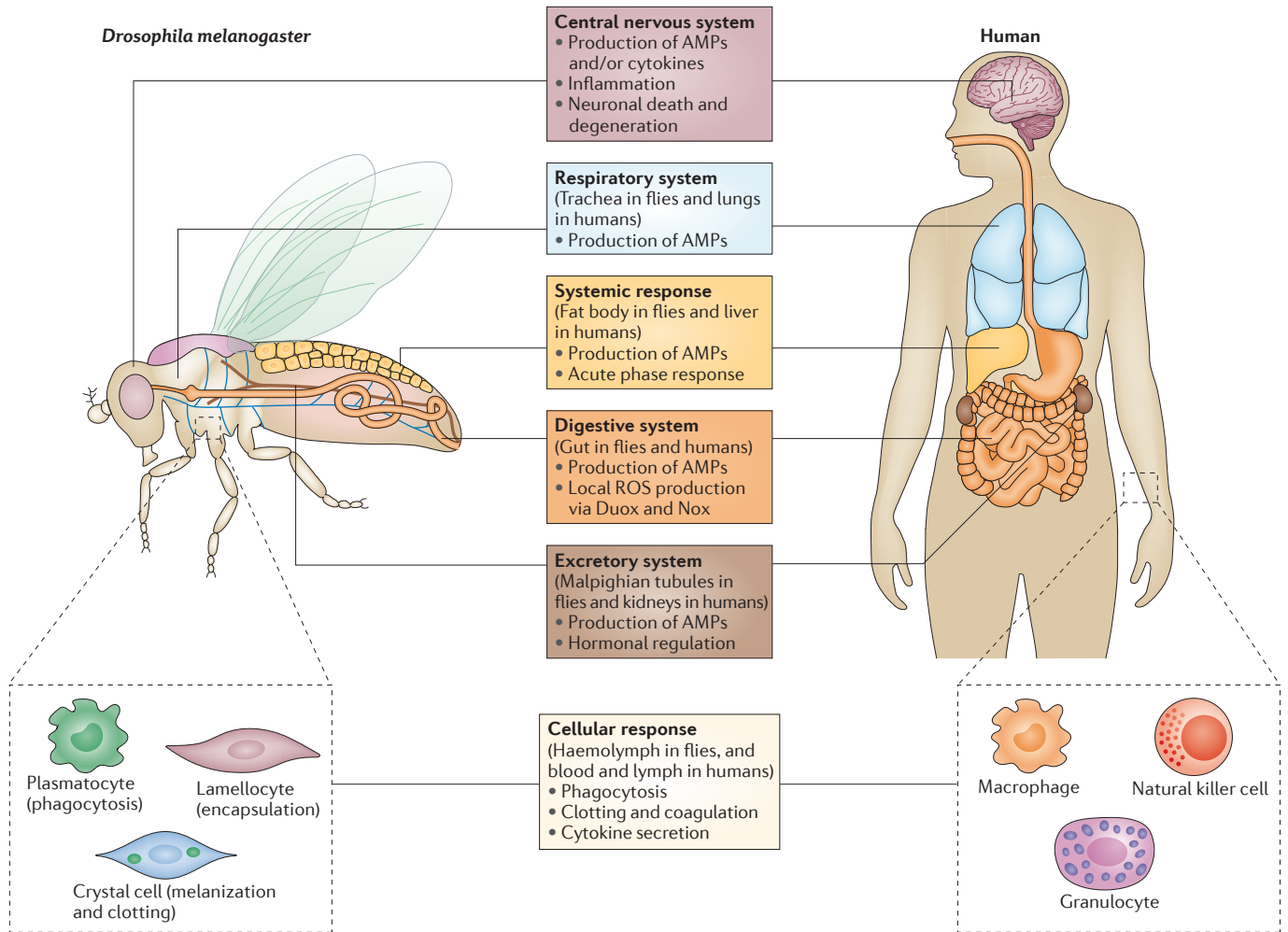
Figure 1 | **Innate immunity in *Drosophila melanogaster*.** The organ systems of *Drosophila melanogaster* are analogous to those in vertebrates; the gut absorbs nutrients, whereas the fat body stores nutrients and functions as a nutrient sensor, similar to the mammalian liver and adipose tissue. The Malpighian tubules in flies carry out the same basic functions as the kidneys in vertebrates. The *D. melanogaster* heart is essential for the circulation of nutrients and immune cells; however, flies have an open circulatory system, rather than a vasculature, and oxygen is delivered by an independent tracheal system. Several *D. melanogaster* organ systems contribute to innate immune defence. Similar to mammals, who have various blood cell types, flies have several types of circulating cells, collectively known as haemocytes. Immediately upon infection, macrophage-like plasmatocytes begin to phagocytose microbial invaders. Other circulating cells, such as the crystal cells in larvae, activate melanization, which generates bactericidal reactive oxygen species (ROS) at infection sites and promotes coagulation. Large pathogens are encapsulated by large haemocytes known as lamellocytes. The hallmark of the *D. melanogaster* humoral response is the inducible synthesis and secretion of antimicrobial peptides (AMPs), which are released into the haemolymph as a systemic response. The fat body is a primary systemic source of AMPs. Barrier epithelial cells in *D. melanogaster* are also capable of generating AMPs, similar to mammals. The trachea, Malpighian tubules and gut produce tissue-specific AMPs in response to local microbial infection. In the gut, the inducible generation of ROS by NADPH oxidases — such as dual oxidase (Duox) and NADPH oxidase (Nox) — has a role in both pathogen infection and regulation of the gut microbiota. The *D. melanogaster* central nervous system coordinates both organism physiology and immunity through the secretion of hormones.

discovery of antimicrobial peptides (AMPs), which not only have conserved sequences from insects to mammals[10], but are also regulated by conserved nuclear factor-κB (NF-κB) signalling cascades[11]. The discovery of *D. melanogaster* Toll (also known as Toll-1) as a key component of the antifungal immune response was a defining moment in the study of innate immunity, as this signalling pathway is similar to the myeloid differentiation primary response protein 88

(MYD88)-dependent Toll-like receptor (TLR) pathway in mammals[12,13] (FIG. 2). *D. melanogaster* encodes nine Toll proteins, and although Toll is indirectly involved in pathogen recognition and functions as a receptor for the secreted cytokine Spätzle, Toll-7 directly recognizes viral glycoproteins, which is similar to TLRs in mammals[14–16]. Toll-8, which is expressed by the respiratory epithelium, negatively regulates NF-κB signalling[7].
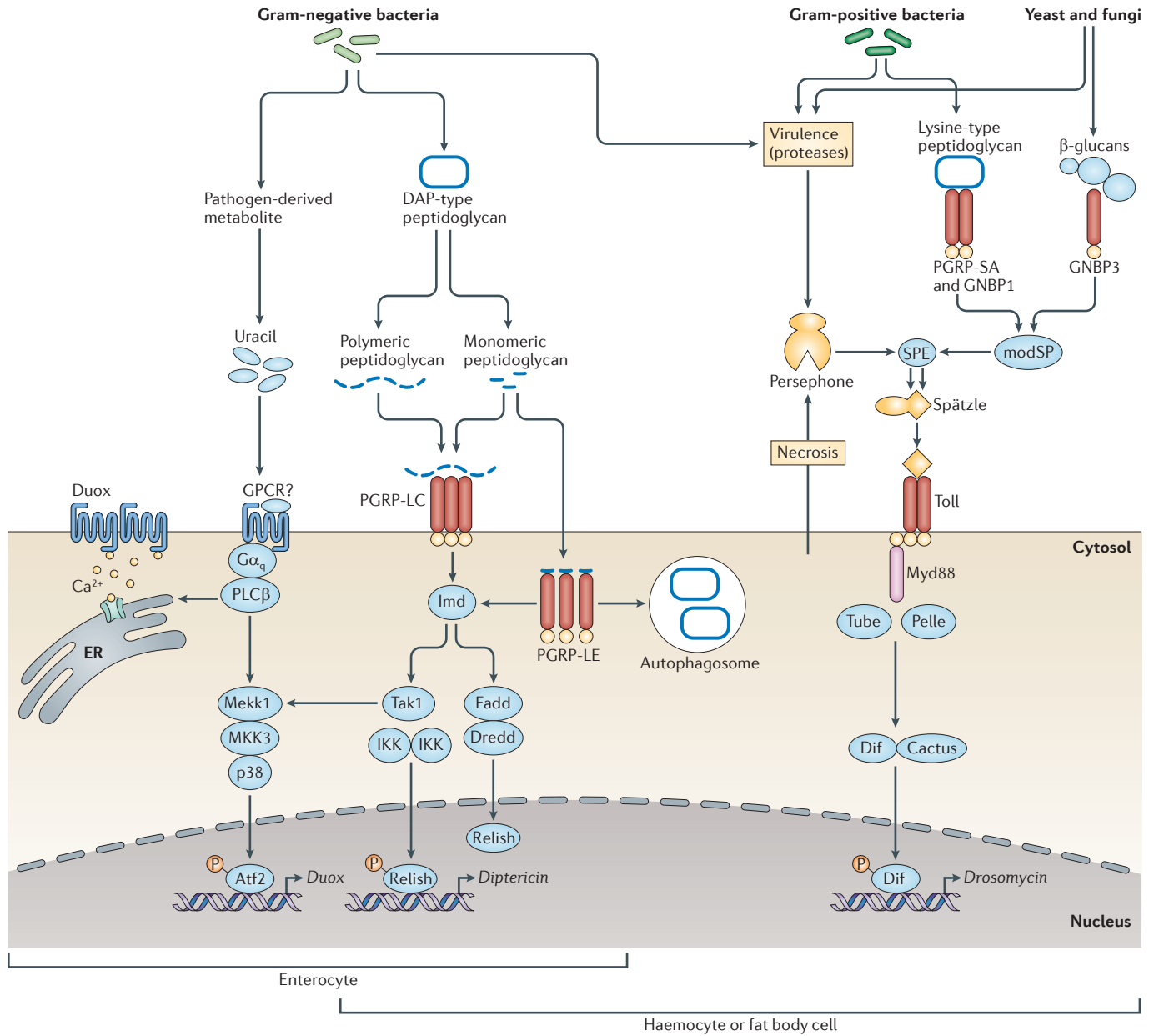
Figure 2 | Immune recognition of microbial agents in *Drosophila melanogaster*. Two classical signalling pathways control inducible immune responses to bacteria and fungi in *D. melanogaster*: the Toll pathway and the immune deficiency (Imd) pathway. The Toll pathway is active in the fat body and, together with the Imd pathway, controls the systemic production of antimicrobial peptides (AMPs). The Imd pathway is also active in barrier epithelial surfaces including the gut, and functions in antimicrobial responses together with reactive oxygen species (ROS)-generating enzymes, such as dual oxidase (Duox). These pathways are activated in response to the detection of microbial cell wall components. Peptidoglycan recognition protein LC (PGRP-LC) and PGRP-LE recognize the diaminopimelic acid (DAP)-type peptidoglycan from Gram-negative bacteria and certain Gram-positive bacteria, and activate the Imd pathway. PGRP-SA and Gram-negative bacteria-binding protein 1 (GNBP1) recognize the lysine-type peptidoglycan of Gram-positive bacteria, and GNBP3 recognizes the β-glucans of yeasts and fungi to activate Toll signalling. In addition, the Toll pathway can be activated through the sensing of danger signals — including microbial proteases — or abnormal cell death, triggering the maturation of the protease Persephone. In all variations of the Toll pathway, immune recognition activates a proteolytic cascade that culminates in the maturation of the cytokine Spätzle, which is mediated by the protease Spätzle-processing enzyme (SPE). Toll activation ultimately leads to the nuclear translocation of the nuclear factor-κB (NF-κB) transcription factor Dif, to induce the expression of AMP genes such as *Drosomycin*, as well as other target genes. Activation of the Imd pathway leads to the nuclear translocation of the NF-κB transcription factor Relish to activate the expression of AMP genes such as *Diptericin*. ROS also have important roles in antimicrobial defence and Duox activity is triggered by the recognition of uracil, which is a pathogen-derived small molecule that activates an unidentified G protein-coupled receptor (GPCR) and promotes the release of calcium from the endoplasmic reticulum (ER). In addition, both the Imd pathway and GPCR signalling, through a phospholipase Cβ (PLCβ)-dependent pathway, lead to the activation of a Mekk1–p38 mitogen-activated protein kinase (p38 MAPK) axis that promotes the sustained expression of Duox upon infection. Atf2, activating transcription factor 2; Dredd, death-related ced-3/Nedd2-like caspase; Fadd, FAS-associated death domain orthologue; Gα$_q$, G protein α$_q$-subunit; IKK, inhibitor of NF-κB kinase (also known as Ird5); MKK3, MAPK kinase 3 (also known as Licorne); modSP, modular serine protease; Tak1, TGFβ-activated kinase 1.

Spätzle is a cystine-knot cytokine that is structurally similar to mammalian interleukin-17 (IL-17)[17], and is produced as a pro-form that is activated by proteolytic cleavage in response to both infection and cellular damage through serine protease cascades that converge on the activation of Spätzle-processing enzyme (SPE). For example, some virulent fungi and bacteria release proteases during infection, and the circulating host serine protease Persephone senses these virulence-associated proteases and triggers SPE activation; this process is referred to as effector-triggered immunity[18,19]. Endogenous signals released by necrotic cells also trigger a Persephone-dependent response[20].

In addition, Spätzle cleavage is triggered by microbial cell wall components. For example, fungal cell walls are recognized by the circulating β-glucan receptor Gram-negative bacteria-binding protein 3 (GNBP3)[19], whereas lysine-type peptidoglycan — which is common to many Gram-positive bacteria — is recognized by a complex of GNBP1 and peptidoglycan recognition protein SA (PGRP-SA)[21]. Both ligand–receptor complexes trigger activation of the same protease, modular serine protease (modSP). In turn, modSP initiates a protease cascade that culminates in SPE activation and Spätzle cleavage[19,22].

The immune deficiency (Imd) pathway is a second important microbial-sensing system in *D. melanogaster* and is similar to the tumour necrosis factor (TNF) pathway and TIR domain-containing adaptor protein inducing IFNβ (TRIF)-dependent TLR pathways (FIG. 2). The Imd pathway is triggered by the recognition of diaminopimelic acid (DAP)-type peptidoglycan (which is produced by Gram-negative bacteria and *Bacillus* species) by surface-bound PGRP-LC and cytosolic PGRP-LE. This initiates an NF-κB signalling pathway that functions in parallel with the Toll-triggered NF-κB pathway and induces the expression of an overlapping but distinct set of effector proteins, including AMPs[23–25]. Both PGRP-LC and PGRP-LE activate strong NF-κB signalling, whereas the cytosolic receptor PGRP-LE also triggers an antibacterial autophagic response that is essential for the control of intracellular pathogens such as *Listeria* species[23,26]. Although the Toll and Imd signalling pathways have been well studied, there is still much to be learnt about the molecular mechanisms underlying these NF-κB-activating systems, and future studies will continue to contribute to our understanding of these ancient and highly conserved pathways. Integrating these pathways into whole-organism biology will provide a better understanding of the homeostatic mechanisms that result in pathology when dysregulated.

Nucleic acid and virus recognition

Nucleic acid recognition is central to host detection of viruses and to antiviral immunity in diverse organisms, from plants to mammals[27,28]. Although in some cases NF-κB-dependent pathways are triggered in response to viruses, how viruses activate this pathway and the effector proteins induced by this sensing are less clear[29,30]. The antiviral Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling pathway that is activated during viral infection

in mammals is also involved in the response to some viruses in flies. However, evidence suggests that in flies, this pathway is induced by damage-associated molecules that are released during cytolytic infection, rather than by direct sensing of viral ligands[31]. Furthermore, studies in flies have revealed that small noncoding RNAs have fundamental roles in the defence against diverse viruses in the soma and selfish genetic elements in the germ line[32].

RNA interference and exogenous viruses. Studies that were first carried out in plants and later in *D. melanogaster* demonstrated that RNA interference (RNAi) has a major role in defence against viral pathogens[28,33] (FIG. 3). During viral replication, viral RNAs are processed into small interfering RNAs (siRNAs), which are then used to direct antiviral RNAi[34–36]. Thus, loss-of-function mutations in the host genes required for this RNAi pathway lead to an increased susceptibility to viral infection[35,37–39]. DNA viruses are also subject to RNAi-mediated restriction, as their genomes encode foreign double-stranded RNA (dsRNA) structures (such as repetitive hairpins and convergent transcripts)[40,41]. However, the mechanisms by which particular RNA structures or intermediates are targeted during infection remain unclear[42–44]. Intriguingly, RNA fragments from various different RNA viruses can be reverse-transcribed early during infection to generate DNA of viral origin. These are potentially transcribed to generate additional substrates for RNAi to amplify the signal and provide a form of long-term inhibition of viral replication[45]. Many viral pathogens of insects encode suppressors of RNAi, highlighting the central role of RNAi in antiviral responses. Moreover, viral suppressors that bind dsRNAs also attenuate interferon (IFN) signalling in vertebrates by blocking the recognition of these potent pathogen-associated molecular patterns by other classes of pattern recognition receptors[46–50].

Insects have two somatic small RNA pathways — the antiviral siRNA pathway (which is Dicer-2 dependent) and the modulatory microRNA (miRNA) pathway (which is Dicer-1 dependent). Mammals only have one *DICER* gene (also known as *DICER1*) that carries out the functions of *D. melanogaster* Dicer-1 and Dicer-2. As the miRNA pathway components are essential for life in all organisms, the antiviral activity of DICER in mammals has been more difficult to study and its importance is debated (reviewed in REF. 51). Indeed, it has been suggested that in mammals, the RNA-based antiviral RNAi pathway has been replaced by the protein-based antiviral IFN response. However, two recent studies found that RNAi could have an antiviral role in some contexts; for example, in undifferentiated cells, which do not have a robust IFN pathway[52,53].

Other factors involved in nucleic acid recognition and antiviral defence. In addition to the small RNA pathways, other RNA-binding proteins act as pattern recognition receptors to induce antiviral responses. In mammals, the DEAD-box helicases retinoic acid-inducible gene I (RIG-I) and melanoma

Selfish genetic elements
DNA sequences that enhance their own transmission relative to other elements in the genome, and that are thought to be either neutral or detrimental to the fitness of the organism. These elements, which include transposons, constitute a large proportion of eukaryotic genomes.

RNA interference
(RNAi). RNA-directed inhibition of gene expression that is typically achieved by causing the degradation of specific mRNA molecules.
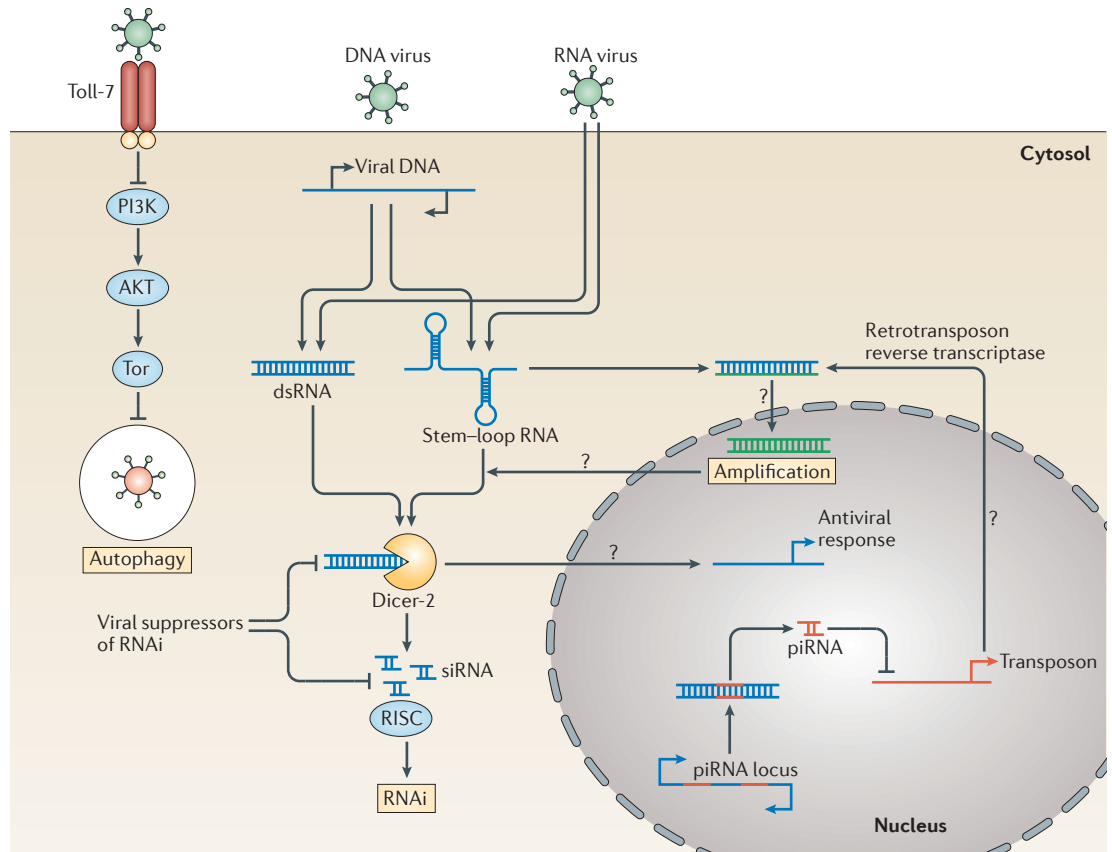
Figure 3 | **Nucleic acid recognition and antiviral defences in *Drosophila melanogaster*.** RNA viruses often encode structured RNAs or produce double-stranded RNA (dsRNA) intermediates. Some DNA viruses produce convergent transcripts that form dsRNAs and also transcribe structured RNAs. These RNAs are recognized and cleaved by Dicer-2 to form virus-derived small interfering RNAs (siRNAs), which are loaded into an Argonaute 2-containing RNA-induced silencing complex (RISC) to silence viral expression. Dicer-2 also initiates the transcription of antiviral genes through an as-yet-unidentified pathway. Endogenous retrotransposon-encoded reverse transcriptase can generate viral-derived cDNA, which can be further transcribed and amplify the RNA interference (RNAi) response. Another RNAi pathway, the PIWI-interacting RNA (piRNA) pathway, protects the cell from endogenous mobile genetic elements, especially those in the germ line. In addition to these nucleic-acid-triggered responses, some viruses can be directly sensed by Toll-7 to induce antiviral autophagy dependent on the conserved AKT pathway involving phosphoinositide 3-kinase (PI3K) and target of rapamycin (Tor).

differentiation-associated protein 5 (MDA5; also known as IFIH1) — which are collectively known as RIG-I-like receptors (RLRs) — are canonical cytosolic receptors that bind to viral RNAs, activate NF-κB and induce IFN production[54,55]. Recent studies in flies have linked the small RNA pathway to signalling, suggesting greater similarities. The closest *D. melanogaster* homologue of mammalian RLRs is Dicer-2, which not only cleaves viral RNAs into siRNAs but also induces the transcription of antiviral effector proteins[56]. Recent genetic screens in *D. melanogaster* have found antiviral roles for additional DEAD-box helicases[57,58], such as DDX17 (known as Rm62 in *D. melanogaster*), which has antiviral activity against arthropod-borne bunyaviruses in a range of species from insects to humans[57]. Many open questions remain about how diverse RNAs are recognized and how these activities are integrated to restrict infection. Even less is known about DNA virus recognition and future studies are required to unveil the mechanisms involved.

D. melanogaster as a model for gut immunity

The intestine is a major entry point for pathogens and is therefore central to the defence against infection. The insect intestine is structurally similar to that in vertebrates, and also contains a complex microbiota (FIG. 4). In both insects and vertebrates, the intestine is a highly compartmentalized tubular organ[59] with an epithelial monolayer that is physically separated from the lumen by barriers. One such barrier is the peritrophic membrane that lines the epithelium — analogous to mammalian mucus — and acts as a first line of defence against microbial invaders[60]. In addition, *D. melanogaster* encodes multiple mucin-like proteins that produce layers of mucus that are maintained between the peritrophic membrane and the gut epithelium; many of these mucin-like proteins are regulated by infection, which suggests an important but understudied role in immunity[61,62]. *D. melanogaster* has emerged as a powerful model in which to investigate interactions between pathogens and gut-associated microorganisms in the intestinal tract[59].
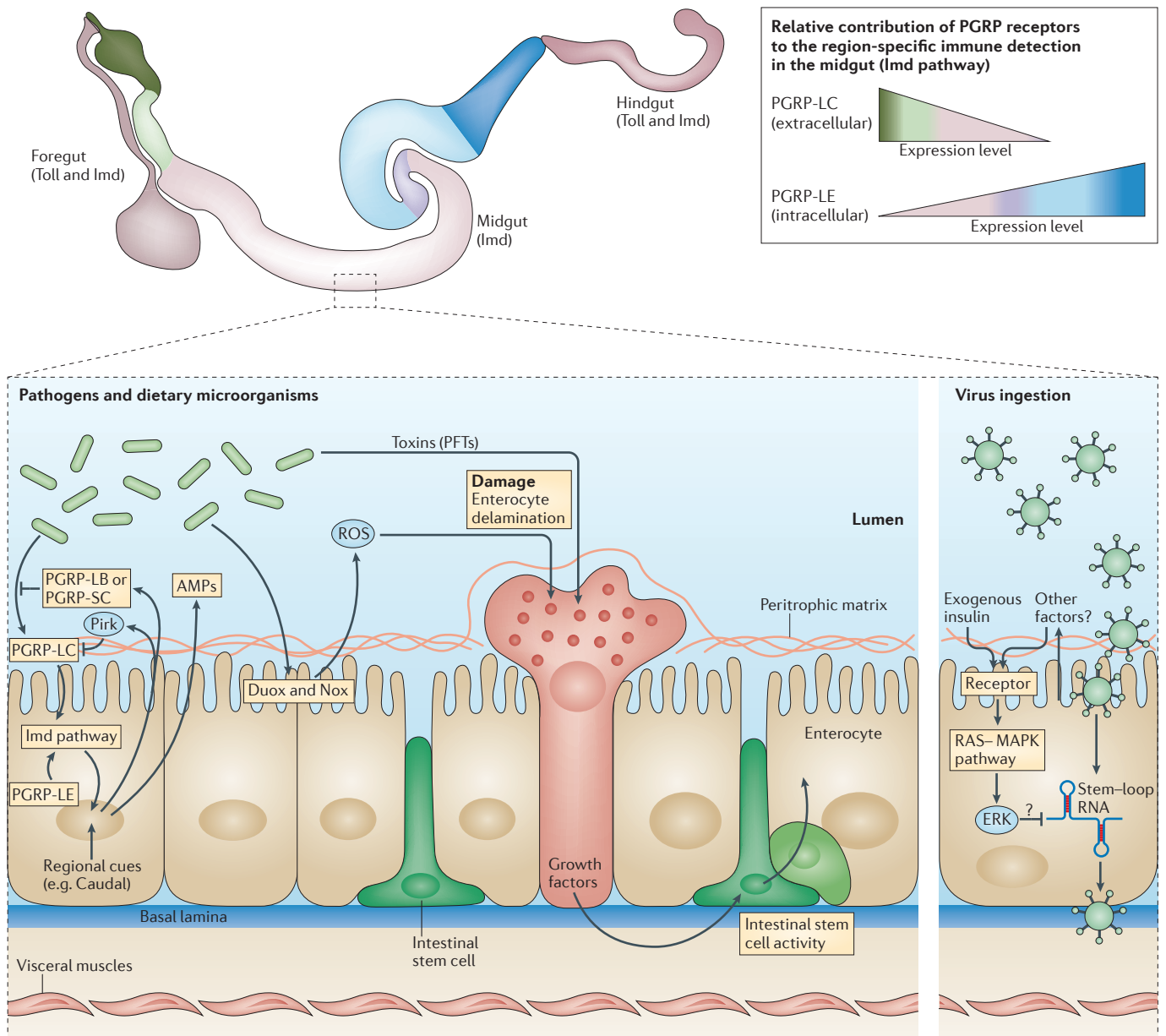
Figure 4 | ***Drosophila melanogaster* intestinal immune response to infection.** The digestive tract of *Drosophila melanogaster* is highly regionalized, and is composed of the ectodermal foregut, the endodermal midgut and the ectodermal hindgut. The midgut is the primary site of digestion and absorption, and is protected by a chitinous membrane known as the peritrophic matrix. The midgut is subdivided into domains that have distinct physiological properties. The gut immune response is highly regionalized; the Toll pathway is active only in the foregut and hindgut, whereas the immune deficiency (Imd) pathway mostly regulates the immune response in the midgut. In response to Imd activation, different regions of the gut express different antimicrobial peptides (AMPs); this regional regulation depends on local, developmentally programmed cues (such as local expression of the transcription factor Caudal). The recognition of extracellular and intracellular bacterial components takes place in different gut regions; immune recognition in the midgut occurs by either membrane-bound peptidoglycan recognition protein LC (PGRP-LC) in the anterior midgut or by intracellular PGRP-LE in the middle and posterior midgut. The gut epithelium also produces reactive oxygen species (ROS) in response to both the natural microbiota and more virulent microorganisms by activating the NADPH oxidases dual oxidase (Duox) and Nox. Together with toxins, such as pore-forming toxins (PFTs), excessive gut immune responses can cause tissue damage and enterocyte delamination, which is rapidly repaired through the proliferation and differentiation of intestinal stem cells. To decrease damage and maintain tissue homeostasis, negative regulators of the Imd pathway, such as poor Imd response upon knock-in (Pirk) and the amidases PGRP-LB and PGRP-SC, prevent excessive activation of the immune response. Sensing of viral infection leads to the induction of antiviral extracellular signal-regulated kinase (ERK) activation via a RAS–mitogen-activated protein kinase (MAPK) pathway in the gut epithelium.

Gut microorganisms of D. melanogaster. Recent experiments with wild and laboratory-reared flies indicate that the gut microbiome of *D. melanogaster* consists of 1–30 bacterial species, which are mostly of the *Lactobacillus* and *Acetobacter* genera[63–69] (*Lactobacillus* species are also part of the normal human microbiome). This simple microbiome composition and the fact that these bacterial species are all culturable allow for efficient functional analyses through the use of germ-free and gnotobiotic flies[70].

As in mammals, the intestinal microbiota influences the metabolic and immune status of flies. Although flies that are reared under germ-free conditions are viable when provided with a rich food source, the presence of the gut microbiota is essential under limiting conditions, and promotes key aspects of development and intestinal homeostasis. First, gut microorganisms promote *D. melanogaster* growth through the induction of the insulin pathway, which in turn accelerates larval development[71–73]. This process is controlled by *Acetobacter pomorum* (through the generation of acetic acid) and *Lactobacillus plantarum* (potentially through alterations in circulating amino acid levels). Given these relationships, it is clear that dysbiosis of the microbiota can affect systemic growth, and that there may be a link between dysbiosis and metabolic disorders in flies, as there is in humans[74]. Second, germ-free flies have broad alterations in intestinal physiology. The microbiota positively regulates the renewal of the midgut epithelium through stem cell proliferation[69,71,75]. Intestinal stem cells (ISCs) differentiate into two essential lineages — absorptive epithelial cells and secretory enteroendocrine cells. Moreover, microorganisms skew cell lineage commitment, as germ-free flies have an increased ratio of enteroendocrine cells to absorptive epithelial cells[69]. Indeed, a common feature of both pathogenic and commensal microorganisms is their ability to promote the production of unpaired 3 (Upd3) — a cytokine analogue of mammalian IL-6 — by the gut epithelium[62,69,75]. In turn, Upd3 promotes homeostatic responses by altering the ISC niche function, and by increasing ISC proliferation and differentiation[69,71,75]. The microbiota triggers this homeostatic programme during steady-state conditions, whereas intestinal pathogens induce a stronger homeostatic response to repair infectious damage to the epithelium[71,75–77]. Flies deficient in this compensatory proliferation succumb rapidly to infection, which provides strong evidence for the central role of active tissue repair in survival. However, it is unclear whether repair mechanisms alter bacterial elimination (resistance), the ability to withstand infection (tolerance) or both. The relationship between the microbiota and epithelial cell proliferation has direct parallels with colorectal cancer, in which the microbiota has been suggested to be an aetiological factor[78]. A similar relationship occurs in mammalian lungs, where IL-6 is produced in response to chemical injury, and coordinates both immune and homeostatic responses[79].

In a range of organisms from flies to humans, the microbiota also has protective roles. Germ-free larvae are more susceptible to pathogenic infection by *Candida albicans* than larvae reared in normal conditions[80]. This protective effect is probably mediated by direct competition for a survival niche, as the conventional microbiota remained protective in immune-deficient animals. This type of niche competition is important in humans and is best exemplified by *Clostridium difficile*, which can take hold when the normal niche is depopulated of healthy commensal microorganisms during antibiotic treatment[81]. Germ-free mice also show increased susceptibility to pathogens, reduced cytokine production and altered stem cell activity. Together, these findings suggest that *D. melanogaster* is a useful model in which to identify both host pathways and microbial metabolites that regulate immunity and intestinal physiology.

Reactive oxygen species in gut immunity. In response to bacterial ingestion, organisms from flies to humans induce two key responses to fight infection — the production of reactive oxygen species (ROS) and the expression of AMPs[59]. ROS are induced by two conserved NADPH enzymes; dual oxidase (Duox) generates microbicidal hydrogen peroxide (H_2O_2) and hypochlorous acid[82–84], whereas NADPH oxidase (Nox) generates H_2O_2. In addition to its microbicidal activity, ROS have instructive roles in tissue repair, wound healing and haematopoiesis in both flies and mammals, during which they act as a second messengers or signalling modulators[85–87]. How particular bacteria are sensed to induce these responses is unclear. In *D. melanogaster*, commensal and pathogenic microorganisms stimulate ROS production through Duox and Nox; commensals, such as *L. plantarum,* stimulate Nox, whereas pathogenic microorganisms, such as *Erwinia carotovora* subsp. *carotovora* 15 (also known as *Pectobacterium carotovorum*) or *Pseudomonas entomophila,* activate Duox[82–84]. Flies that lack Duox activity are more susceptible to infection with enteric pathogens and have increased mortality in the presence of dietary bacteria and yeasts[82]. This suggests that ROS are central to both resistance to infection and regulation of commensals. In addition, flies that are deficient in either Duox or Nox, or that lack the ROS-detoxifying enzyme immune-regulated catalase, have a shortened lifespan, which demonstrates that healthy ageing can only be achieved when there is tight regulation of the activity of NADPH oxidases[84,88,89].

As excessive ROS production is ultimately toxic to the host, a sophisticated regulatory network controls Duox expression and activity in *D. melanogaster*[90]. The transcriptional induction of Duox depends on the conserved p38 mitogen-activated protein kinase (p38 MAPK)–activating transcription factor 2 (Atf2) pathway[90]. This pathway can be induced by the metabolite uracil and the bacterial cell wall component peptidoglycan[90,91]. Uracil is selectively secreted by two distinct pathogenic bacteria and sensed by an as-yet-unidentified G protein-coupled receptor that activates phospholipase Cβ (PLCβ; also known as Plc21C in *D. melanogaster*) in a pathway that depends on the G protein $α_q$-subunit ($Gα_q$), which in turn drives p38 MAPK signalling[88,90]. Peptidoglycan also activates p38 MAPK through the activation of the Imd pathway and TGFβ-activated kinase 1 (Tak1)[88,90]. Uracil stimulates Duox activity through the PLCβ pathway, which promotes the release of Ca^{2+} from endoplasmic reticulum stores to bind and activate Duox directly[88,90]. However,

it remains unclear whether uracil is the sole agonist of Duox activity. Nevertheless, when levels of uracil are low, concentrations of cytosolic Ca^{2+} are reduced and Duox activity is attenuated[88]. This attenuation is further enforced because basal levels of PLCβ induce MAPK phosphatase 3 (Mkp3), which reduces the expression of Duox in a calcineurin B-dependent manner[90]. Loss of Mkp3 in the fly gut increases the levels of ROS, triggering increased epithelial cell death and early ageing[90]. This suggests that multiple layers of control are required to ensure that Duox activity is kept proportional to the microbial threat and is maintained at levels that are beneficial to the host. The recent identification of Duox as a key regulator of innate immunity in species ranging from worms to humans suggests that Duox function and, in turn, the role of ROS in infection, are an ancient and central immune defence mechanism[92,93]. Whether microbiota-derived metabolites such as uracil are involved in this regulation in mammals remains unclear.

Local regulation of gut immunity and links with ageing. Throughout the animal kingdom, AMPs are produced by the gut to control infection. Indeed, ROS and AMPs are complementary, as AMPs are crucial to control ROS-resistant bacteria[94]. In both flies and mammals, the induction of AMP production is compartmentalized, and distinct AMPs are expressed regionally throughout the gut[62,95–97]. This suggests that, as is found in mammals, local cues modulate the immune response throughout the gut in flies. Spatial control of AMP expression is not well understood, but probably involves regulation by locally expressed transcription factors. For example, expression of the homeobox gene *Caudal* is restricted to the *D. melanogaster* posterior midgut, where it represses the expression of AMPs[63]. Mutations in *Caudal* are associated with increased AMP expression in this gut region, resulting in dysbiosis, cell death and early lethality[63].

It is known that systemic control of AMPs is NF-κB dependent, whereas the regulation of AMPs in the gut is more complex. The Toll pathway, which is a key systemic activator of NF-κB, is non-functional in the midgut. In addition, the expression of microbial-sensing receptors that activate the Imd pathway is also regionalized in the gut; PGRP-LC is mainly expressed in the anterior midgut, whereas PGRP-LE is expressed in the middle and posterior midgut[96,98] (FIG. 4). This suggests that bacterial sensing and the production of specific AMPs are regionally controlled within the gut to fine-tune the immune response. For example, in the posterior midgut, the transit of food and bacteria slows down, and the enrichment for cytosol-expressed PGRP-LE might prioritize immune responses to invasive species that gain access to the cytosol in this region over other types of responses[98]. Furthermore, the JAK–STAT pathway regulates three AMPs of the Drosomycin class in the midgut but not elsewhere in the fly gut[62,99]. Importantly, the JAK–STAT pathway is induced not by microbial products but by the cytokine Upd3, which is activated locally by stress or damage[75,77,100]. So, the intestinal immune response in *D. melanogaster* is poised to recognize not only microorganisms but also damage caused by infection or toxins, similar to how virus-induced JAK–STAT activation is thought to occur.

Similar to ROS production, excessive or prolonged activation of NF-κB signalling is deleterious to the host and causes developmental defects and advanced ageing. To prevent these effects in the intestine, the gut secretes enzymatic PGRPs, such as PGRP-SC or PGRP-LB, that digest peptidoglycan and thus decrease the local levels of this immunostimulant[101–103]. In addition, several negative regulators of the Imd pathway have been identified: poor Imd response upon knock-in (Pirk) modulates PGRP-LC and PGRP-LE signalling[104–106]; PGRP-LF is a PGRP receptor that dimerizes with PGRP-LC to decrease its ability to activate Imd signalling[107]; and SkpA is proposed to ubiquitylate and degrade the active NF-κB homologue Relish[108]. Finally, transcription factors such as Caudal function as locally expressed negative regulators of target genes activated by Relish, enabling fine-tuning of their expression[63]. Flies deficient for PGRP-LB or Caudal have shortened lifespans that can be rescued by the elimination of commensal bacteria[63,103], which highlights the important role of these negative regulators in maintaining immune homeostasis in the complex milieu of the gut. Accordingly, mammals encode four PGRPs (*PGLYRP1* to *PGLYRP4*) that are required for healthy management of the microbiota, and deficiencies in these PGRPs are associated with dysbiosis and increased susceptibility to colitis[109]. However, the sequence of events that underlies the loss of intestinal homeostasis remains unclear in both flies and mammals.

These studies demonstrate the complexity of the regulation and compartmentalization of the *D. melanogaster* gut immune response. The constant exposure to microorganisms throughout life makes it challenging to maintain this delicate balance, and recent studies have shown that gut immunity affects healthy ageing in *D. melanogaster*[75,110–112]. The microbial load and diversity increases with age[64,75,112], despite an increased production of ROS and AMPs in the intestine[69,75,90,112]. This hyperactivation is likely to have multiple effects on the gut. First, ROS cause damage to the epithelium. Second, the increased levels of ROS and AMPs will affect the microbiota species differently and cause dysbiosis. Third, this dysbiosis induces chronic epithelial stress that stimulates high levels of ISC proliferation, which alters both the differentiation and the renewal of the gut tissue[75,111,112]. These age-related changes in gut homeostasis are correlated with barrier failure and the induction of systemic immune responses, which are potentially caused by the translocation of bacteria or bacterial products across the failed epithelium of the gut[110]. Recent investigations into the mechanisms underlying this age-related dysbiosis have shown that the transcription factor forkhead box subgroup O (Foxo) is chronically activated in the gut of ageing flies, potentially due to oxidative stress, and that Foxo dampens the expression of PGRP-SC2 — a negative regulator of the Imd pathway — resulting in hyperactivation of the immune response[112]. These results shed light on the links between intestinal immune homeostasis, dysbiosis and barrier dysfunction. Given the parallel findings of age-related dysbiosis in humans[113], these studies point to mechanisms that are likely to occur in ageing humans.

Systemic control of the immune system

Hormones have central roles in regulating whole-organism physiology, including direct effects on immunity[114]. In addition, circadian circuits, which are known as homeostatic oscillators of physiology, also affect immunity in flies and humans[115–117]. Indeed, an increasing number of connections between immunity and metabolic homeostatic control have been made in recent years. Acute and chronic infections can trigger metabolic dysregulation, and many metabolic syndromes are associated with inflammation, which has led to the hypothesis that inflammation drives metabolic dysfunction[118]. Dissecting this relationship has provided insights into both immunity and the aetiology of metabolic syndromes. In this section, we aim to integrate recent studies that have explored the interplay between physiology, metabolism and immune defence in flies.

Nutrient sensing and immune defence. Insulin signalling is central to metabolic regulation, and studies have implicated inflammatory signalling in the regulation of insulin responsiveness[118] (FIG. 5). Similar to the mammalian liver and white adipose tissue, the *D. melanogaster* fat body stores excess fat as triglycerides and responds to metabolic shifts[119,120]. The fat body responds to dietary signals by releasing factors that affect insulin secretion, organism growth and metabolism[121,122]. A delicate balance between fat storage and breakdown is crucial to maintain energy homeostasis. Peptide hormones released by specialized cells are key regulators of metabolism in both flies and mammals; in flies, these include glucagon-like peptide[123] and insulin-like peptides[124,125], which are mostly produced by neurosecretory cells in the brain. *D. melanogaster* insulin-like peptides bind a single insulin receptor that is orthologous to insulin receptors in vertebrates. Insulin receptor activation in both insects and mammals induces the same two conserved downstream pathways — those mediated by AKT (also known as Akt1 in *D. melanogaster*) and extracellular signal-regulated kinase (ERK; known as Rolled in *D. melanogaster*). On binding of insulin to its receptor, AKT negatively regulates the transcription factor Foxo, which otherwise suppresses the storage of energy as fat and glycogen, thereby promoting long-term energy storage. AKT also positively regulates target of rapamycin (Tor; known as mTOR in mammals), which is central to growth factor signalling, as it cell-autonomously responds to amino acids, ATP and oxygen; induces anabolic processes including translation; and inhibits catabolic pathways including autophagy[126]. In turn, ERK activation affects organism growth, and modulates insulin sensitivity and metabolic flux[127,128]. Importantly, adult flies fed a high-fat or high-sugar diet develop excess adiposity and insulin resistance, suggesting that the molecular mechanisms of insulin resistance are conserved[126]. In adult flies, insulin functions in metabolic homeostasis, resistance to stress, fecundity, lifespan and, as shown more recently, has direct links to immunity[125,129].

In flies, as in humans, infections result in metabolic disruption. The molecular mechanisms that drive shifts in metabolic homeostasis, including cachexia caused by infection, are incompletely understood and probably have diverse aetiologies, including inflammatory signalling pathways[130,131]. Redistribution of resources from metabolism to immunity during infection is thought to be central to these shifts. Indeed, prolonged or excessive immune activation can drive metabolic dysregulation and cause wasting in mammals and flies[132]. In flies, systemic infection with *Mycobacteria* species, *Listeria* species or fungi leads to metabolic shifts such as decreases in total triglyceride levels[130,131,133]. Infection of fly haemocytes leads to a systemic reduction in AKT activation, which increases Foxo activity and triggers the loss of metabolic stores[131]. Foxo can also directly affect inflammatory signalling by binding to and activating the promoters of a subset of NF-κB-dependent target genes, which suggests direct coordination of NF-κB and Foxo pathways[134]. In this way, genes encoding AMPs can be activated under normal physiological conditions in response to the oscillating energy status of cells and tissues; whether this is beneficial or can lead to pathologies under some conditions remains unclear. Furthermore, in the fed state, the fat body secretes the leptin family hormone Upd2, which is a ligand for the JAK–STAT pathway[135]. This affects brain insulin secretion and directly communicates metabolic information between the fat body and the brain, influencing organism growth and energy metabolism. As the JAK–STAT pathway also has fundamental roles in immunity, this is another clear example of integrating metabolic activities with immune defence.

Mechanistic links between metabolism and immunity are probably integrated in the *D. melanogaster* fat body. Not only does the fat body have roles that are similar to the mammalian liver and adipose tissue, but it is also immune responsive and can produce large quantities of AMPs during infections. Although mammals have separated these functions into distinct adipose tissue, liver and haematopoietic systems, it is likely that overlapping pathways regulate metabolic and immune functions through conserved signalling systems. Indeed, pathogen-sensing and nutrient-sensing pathways directly regulate each another in flies[130,136].

However, immune defence is energetically costly[137], and this robust response must draw resources from other physiological processes[130,138]. Activation of Toll signalling in the fat body of larvae either genetically or by systemic infection leads to decreased AKT signalling and increased Foxo-dependent transcription, leading to the depletion of nutrient stores and reduced organism growth[130]. Shifting the metabolic state of the host affects fecundity, which suggests that there are trade-offs between immunity and host fitness. Furthermore, tissue necrosis at distal sites activates the Toll–AKT crosstalk in the fat body, promoting an energy-wasting phenotype and decreased levels of circulating *S*-adenosylmethionine (SAM), which is a major building block for organism growth[139]. Tissue-derived DNA damage, which is another classic danger signal, also leads to the systemic induction of pro-inflammatory signalling and alters insulin signalling in the fat body[140]. Together, these data suggest that crosstalk between the microbial recognition system and AKT signalling evolved to

Autophagy

An evolutionarily conserved process in which an acidic double-membrane-bound vesicle, known as an autophagosome, sequesters intracellular contents (such as damaged organelles and macromolecules, or pathogens) and targets them for degradation through fusion to lysosomes. This process is essential for the response to starvation because it facilitates the recycling of cellular components, and it can also be targeted to intracellular bacteria or viruses to restrict their growth.

Haemocytes

Cells found within the haemolymph of an insect that are equivalent to the blood cells in vertebrates. Different types of haemocyte are plasmatocytes, crystal cells and lamellocytes. These cells have important roles in immunity through the secretion of cytokines and phagocytic clearance of invaders.
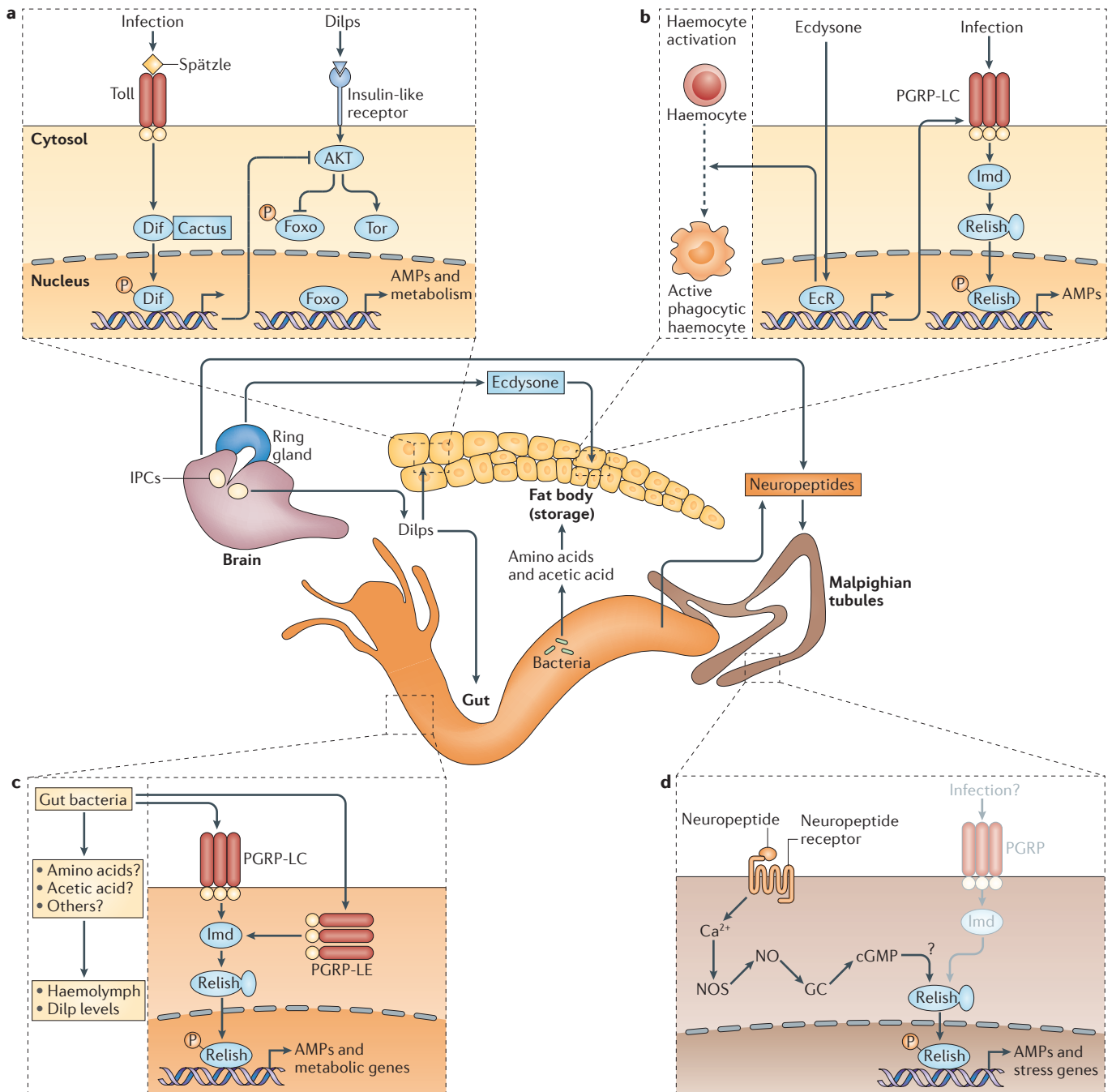
Figure 5 | **Systemic regulation of *Drosophila melanogaster* immune responses.** The *Drosophila melanogaster* immune response is integrated with multiple inter-organ regulatory circuits. **a** | The fat body is a central regulator of metabolic control. Here, Toll signalling through the nuclear factor-κB (NF-κB) transcription factor Dif inhibits insulin signalling and retards growth, whereas forkhead box subgroup O (Foxo) — the downstream target of the insulin pathway — regulates the production of antimicrobial peptides (AMPs) and anabolic genes. **b** | In larvae, the steroid hormone Ecdysone is produced in a secretory organ associated with the brain (known as the ring gland), whereas the source of Ecdysone is less well characterized in adult flies. Ecdysone is a major regulator of the *D. melanogaster* lifecycle, as well as the immune response, through controlling the expression of peptidoglycan recognition protein LC (PGRP-LC) in the fat body and the phagocytic activity of pupal haemocytes. **c** | The gut microbiota further influences the insulin signalling pathway by regulating the production of *Drosophila* insulin-like peptides (Dilps) in the brain. Dilp production and robust growth are triggered by acetic acid that is produced by *Acetobacter* species in the gut, and potentially triggered by optimization of amino acid levels provided by other components of microbiota. **d** | The central nervous system further regulates immune responses in the Malpighian tubules through the production of neuropeptides that are thought to trigger a nitric oxide (NO)-dependent NF-κB response in this organ. The faded part of this panel indicates a hypothetical role for the Imd pathway. cGMP, cyclic guanosine monophosphate; EcR, Ecdysone receptor; GC, guanylyl cyclase; Imd, immune deficiency; IPC, insulin-producing cell; Nos, nitric oxide synthase; Tor, target of rapamycin.

balance energy expenditure between organism growth and the acute demand of combating infection. This has an impact on the induction of protective mechanisms locally (such as autophagy) and systemically (such as AMPs and SAM). Thus, as in humans, chronic inflammation can directly affect metabolic state and insulin resistance.

Metabolic pathways and inflammatory responses are integrated at the level of transcription by the transcription factor myocyte enhancer factor 2 (Mef2), which activates distinct classes of genes depending on the state of the organism[136]. Under normal conditions, Mef2 is phosphorylated, potentially by kinases downstream of AKT signalling, and promotes the expression of anabolic enzymes. However, immune stimulation reduces its phosphorylation levels and alters its target genes to those encoding immune effectors, which results in decreased anabolism and increased immunity[136]. Mef2 has also been linked to the regulation of Eiger, which is the *D. melanogaster* homologue of TNF[141]. TNF signalling is involved in cell death pathways and immunity in a range of organisms from flies to humans, which suggests another link between metabolism and innate responses. Furthermore, many infections attenuate AKT signalling and thus Mef2 may emerge as a central control point.

Attenuation of AKT signalling upon microbial recognition can also activate autophagy. Sensing of cytosolic *Listeria* species by PGRP-LE and of viruses by Toll-7 induces autophagy in infected cells to restrict infection[15,26]. In addition, the ubiquitin ligase Parkin promotes a specialized form of autophagy known as mitophagy to target intracellular pathogens such as mycobacteria in both flies and humans[142].

In addition to the effects of inflammatory signalling on growth that are discussed above, growth signals can affect inflammatory signalling. Insulin and growth factor signalling both induce the MAPK–ERK pathway, which is involved in growth and development, and also lead to the activation of Pirk, which is a negative regulator of inflammatory NF-κB signalling[104–106,143]. This negative feedback is probably protective, as hyperactive inflammatory signalling is detrimental to all species and is a hallmark of metabolic disorders[144]. ERK signalling also promotes antiviral defence in the intestine[145]. Oral challenge of vector insects by blood-acquired viral pathogens occurs during a nutrient-rich blood meal, and this triggers antiviral ERK signalling in the absorptive epithelial cells. This couples antiviral defences with nutrient acquisition and might be an ancient prophylactic defence mechanism in the gut. Furthermore, nutrient signals can influence systemic immunity, as diet restriction leads to decreased resistance to infection with *Listeria* species but increased tolerance to infection with *Salmonella* species[146].

Hormonal control of immunity. In addition to insulin, the steroid hormone Ecdysone modulates immune responses. Ecdysone is the major regulator of the insect life cycle[147] and signals through a classical nuclear hormone receptor heterodimer consisting of the Ecdysone receptor (EcR) and Ultraspiracle (USP), which are orthologous to mammalian farnesoid X receptor and liver X receptor, and retinoid X receptor, respectively[148]. Early fly embryos, which are devoid of Ecdysone, cannot mount an antibacterial response unless exogenous Ecdysone is provided[149]. Later during embryogenesis, Ecdysone pulses released from extra-embryonic tissues provide the priming needed for immune responsiveness[149]. During the third and final larval stage, a large Ecdysone pulse initiates pupariation, which regulates many aspects of physiology, including the death of many larval tissues, haemocyte-mediated phagocytosis of dead cells and full induction of genes encoding AMPs upon infection[150–153]. Hormone-triggered haemocyte activation involves several actin-dependent processes — including haemocyte motility and phagocytic activity — that are also required for resistance to infection and for wound healing at the pupal stage[154]. During development, the embryo is covered in a shell that protects it from external challenge, and thus the embryo may not require AMP production until hatching. By contrast, larvae are constantly exposed to pathogens, possibly even more so during moulting, when the cuticle must be rebuilt. During pupariation, when the entire organism is remodelled, it has been suggested that microorganisms are released from the gut into the haemolymph[154]. Thus, the coupling of immune priming with these major developmental transitions could confer a survival advantage, as it may tailor the immune response to deal with relevant threats.

In adult flies, the induction of AMP gene expression and host survival following bacterial infection requires Ecdysone signalling, as it is crucial for activation of the Imd pathway and directs the expression of the upstream receptor PGRP-LC[152]. It remains unclear which Ecdysone-triggered transcription factors directly regulate PGRP-LC expression but its upregulation during infection suggests that Ecdysone itself may be induced. Ecdysone is known to be regulated by multiple stressors, including heat shock and sleep deprivation[155]. Nutrient deprivation increases Ecdysone levels in the ovaries to block oogenesis, thereby serving a checkpoint function[156]. So, Ecdysone-dependent induction of PGRP-LC expression may integrate stress signals with immune function. Like stress, ageing is associated with a dysregulated immune system, elevated AMP expression, defects in phagocytosis, elevated levels of cytokines (such as IL-6 in vertebrates) and immunosenescence. Reduced Ecdysone signalling increases lifespan[157], which suggests that it has a fundamental role in age-dependent changes in immunity. Given that infection is inherently stressful, stress hormone priming of both the humoral Imd pathway and the cellular phagocytic immune response may protect the animal from infection, but also lead to the pathology that is found in aged animals.

Other hormones, including neuropeptides, respond to physiological stressors and modulate the immune response in *D. melanogaster* (FIG. 5). Excretory functions are partially achieved by tubular epithelial structures known as the Malpighian tubules, which are analogous to vertebrate kidneys. The function of Malpighian

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Mitophagy
A specialized form of autophagy that selectively targets and degrades mitochondria.

tubules is regulated by neuropeptides that respond to stressors; Malpighian tubules also sense systemic infection through tissue-autonomous sensing of peptidoglycan[23,158]. Neuropeptides — such as *D. melanogaster* Capability (also known as Capa-1)[159], which enhances tolerance to desiccation, and neuropeptide receptors, such as guanylyl cyclase at 76C (Gyc76C)[160] — elicit an immune response in the Malpighian tubules and parallels were observed in human embryonic kidney cells, suggesting that physiological stressors, such as desiccation, interact with immune pathways through neurohormonal signalling across hosts.

Neuronal–inflammatory connections

The brain coordinates many systemic responses, including the production of hormones. The brain is also relatively immune privileged, so as to protect non-dividing yet essential neuronal cells. Recently, neuronal inflammation has been linked to age-related pathological processes, including neurodegeneration[161]. Indeed, neurodegenerative triggers — such as amyloid-β peptide — can activate classical microbial-sensing receptors such as TLRs and the NOD-, LRR- and pyrin domain-containing 3 (NRLP3; also known as NALP3) inflammasome[161,162]. Mechanistically, it is unclear how these pathways intersect. Interestingly, recent studies in *D. melanogaster* models of neurodegeneration have linked activation of the insect innate immune response to disease progression. In a *D. melanogaster* model for Alzheimer's disease that is based on the expression of the amyloid-β42 peptide in the developing eye[163], loss of any intracellular component of the Toll pathway, but not of the Imd pathway or the canonical ligand Spätzle, ameliorated the amyloid-β42-induced eye phenotype[163]. These findings suggest a conserved connection between innate immune signalling and amyloid-β-dependent pathologies in the central nervous system.

The Imd pathway is linked to neurodegeneration in other models. In one study, spontaneous vacuolar lesions in the *D. melanogaster* brain neuropile — a hallmark of neurodegeneration — were found to occur at a markedly increased rate in flies that lack defence repressor 1 (Dnr1)[164], which is a negative regulator of the Imd pathway[165]. Increased neurodegeneration in the *Dnr1* mutant was found to be NF-κB dependent, and enforced expression of AMPs in either the neurons or glia was sufficient to phenocopy neurodegeneration[164]. In another system, mutation of *ATM* (which encodes ataxia telangiectasia mutated kinase; also known as Tefu) in glia induced NF-κB-dependent neurodegeneration, suggesting a previously unknown connection between the ATM-dependent DNA damage response pathway and Imd immune signalling. Moreover, NF-κB activation in the glia alone was sufficient to drive neuronal cell death and vacuolar lesions in the fly central nervous system[166,167]. However, it remains unclear how the loss of ATM affects Relish and NF-κB signalling, and whether this involves any of the known components of the ATM-dependent DNA damage response pathway. In a third study, a *D. melanogaster* model of retinal degeneration also revealed a dependence on NF-κB

signalling[168]. Light-dependent *NorpA*-deficient retinal degeneration was strongly suppressed by mutations in the Imd signalling components Relish, Kenny (also known as IKKγ) or death-related ced-3/Nedd2-like caspase (Dredd; also known as caspase 8). However, this phenotype was not affected by the loss of either *Imd* or *Fadd* (which encodes FAS-associated death domain orthologue), which are upstream components in the classical Imd pathway. Together, these four studies support a previously unknown role for components of the Imd pathway in neuronal cell death, with their effects being partially mediated by NF-κB-dependent AMP expression. Clearly, inappropriate activation can lead to serious pathology[169]. Although these papers describe sterile inflammatory conditions contributing to neurodegeneration, the theme here is similar to what is observed in the gut — restoring homeostasis after inflammatory responses is key to maintaining healthy organs.

Future perspectives

In this Review, we have described the diverse areas of immunity that have been studied in *D. melanogaster* in recent years. Early studies focused on molecules involved in sensing and signalling, whereas more recent studies have started to investigate immunity using a more holistic approach. Studies in flies can reveal how immunity and its dysregulation can affect whole-body pathophysiology. Such studies have also shown that innate immune genes are under strong positive selection[170,171], which suggests that parasites impose strong evolutionary pressures on their hosts. Animals from fruit flies to humans are colonized by a range of microorganisms that must be tolerated by the ever-evolving and adapting immune response, and how these bacteria have co-evolved with other organisms remains to be elucidated. Dissecting the complex relationships between hosts and their microbiota is challenging but essential; advances in this area might help us to understand complex diseases such as inflammatory bowel disease, and to develop treatments for some intestinal infectious and autoinflammatory diseases. Indeed, flies harbouring symbiotic intracellular bacteria of the *Wolbachia* genus have been found to be less susceptible to viral infections[172,173,179]. This discovery has led to field trials involving the release of *Wolbachia*-colonized mosquitoes to break the transmission cycle of dengue virus in Australia[180]. Recent studies have also highlighted that successful immunity to infection requires two facets: first, targeting the microbe for elimination, termed resistance; and second, inducing tissue-protective responses, termed disease tolerance[174–176]. Mechanistically, they are defined by their effects on both organism survival and pathogen load. Resistance mechanisms promote survival by decreasing bacterial numbers, whereas tolerance mechanisms improve survival without necessarily affecting bacterial load. Recent studies have provided evidence that tolerance is a feature that is conserved across species from insects to humans[177], and work in *D. melanogaster* has found that active repair mechanisms and metabolic adaptations are

key to surviving infection[62,136]. Undoubtedly, the power of *D. melanogaster* genetics will further our understanding of the genetic basis for tolerance mechanisms and the means by which a host adapts to infection-associated damage. How the *D. melanogaster* holobiont interacts with different environmental insults — such as altered diets, toxins and more virulent pathogens — will be the focus of future work. Such studies will provide new insights into the physiological consequences of microbial colonization and how immunity interacts with behaviour, metabolism, physiology and hormonal regulation. Further study of infections, immune defences and their systemic regulators will continue to open up new routes for translational research and new treatment strategies, and result in a more complete understanding of health and disease.

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Competing interests statement
The authors declare no competing interests.