PERSPECTIVES

LANDMARK

The road to Toll

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A few years ago, it would have been difficult to argue that elucidating the mechanisms of disease resistance in the fruit fly, *Drosophila melanogaster*, would provide new insights into mammalian immunity. Yet the finding that the *Drosophila* protein Toll mediates immune responses to fungal infection had a pioneering role in the identification of Toll-like receptors as essential regulators of mammalian host defence, and it fundamentally altered our understanding of innate immunity. In this Landmark article, I describe the thought processes and the experimental steps that defined Toll as a key regulator of *Drosophila* immune responses.

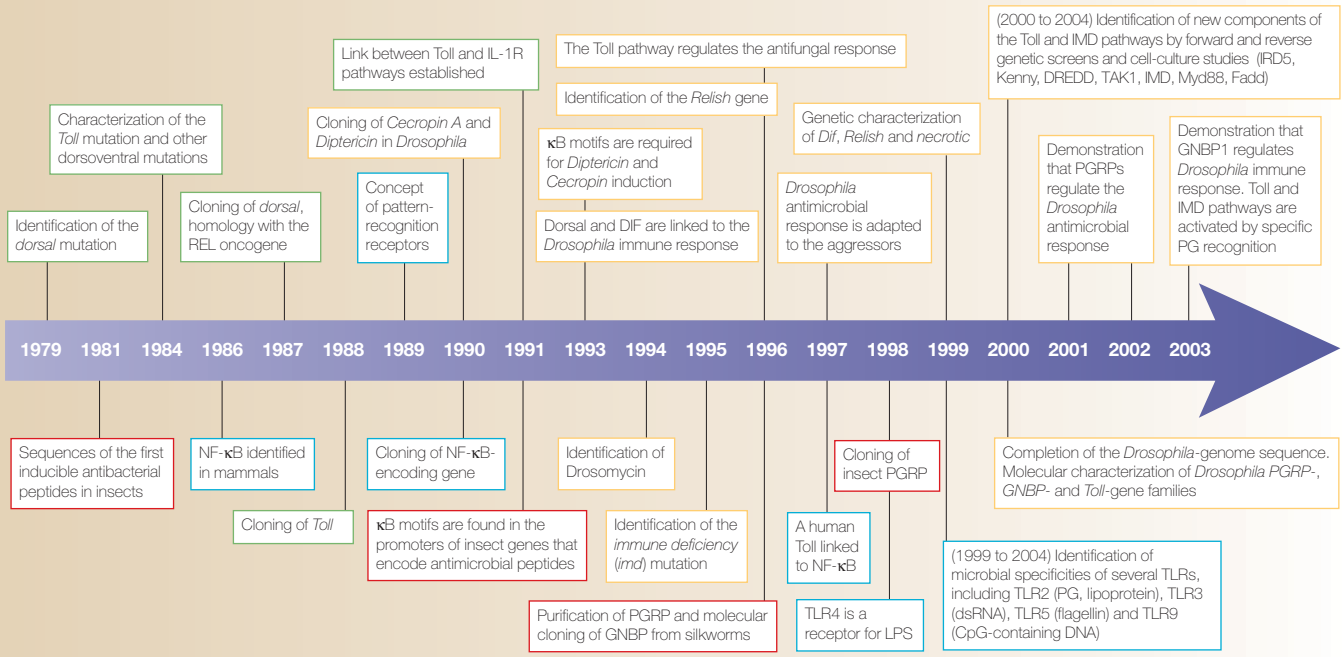
Given their relatively short lifespans, it is not obvious that insects have, or even require, a powerful immune system for fighting microbial pathogens. Nevertheless, insects are highly resistant to microbial infection. Until recently, however, the mechanisms behind this resistance were poorly understood, because insects do not have an equivalent of the vertebrate adaptive immune system. An important discovery regarding insect immunity was made in 1981, when Hans G. Boman and associates[1,2] (in Stockholm, Sweden) characterized the inducible antibacterial peptides Cecropin and Attacin from the moth *Hyalophora cecropia*. Following a septic injury, these small peptides are produced rapidly in large amounts by the insect fat body (an analogue of the mammalian liver) and then secreted into the haemolymph (insect blood) where they kill invading bacteria. By the early 1990s, several genes encoding antibacterial peptides (such as *Diptericin* and the *Cecropins*) were also identified in *Drosophila*[3–5] after they were found to be strongly induced at the transcriptional level following the injection of bacteria into the body cavity of the fly. The next challenge in the field was then to determine the molecular mechanisms that regulate these genes in response to microbial infection. Because nothing was known of the steps that lead from the recognition of microorganisms to the expression of genes that encode antibacterial peptides, my colleagues and I (in Jules Hoffmann's laboratory in Strasbourg, France) called this process 'the black box'. In this article, I describe the experiments that initiated the elucidation of the signalling pathways that control the expression of genes encoding antimicrobial peptides in *Drosophila* (TIMELINE).

Strategies for opening the black box

The first clue to what was inside the black box of antimicrobial-peptide expression was provided by the sequences of genes encoding several antibacterial peptides. Their upstream regulatory regions contain sequence motifs that are similar to the binding sites recognized by the mammalian nuclear factor-κB (NF-κB)/REL family of transcription factors[6]. Subsequently, in 1993, the use of fly lines carrying a reporter gene under the control of wild-type or mutated κB-binding motifs demonstrated that these binding sites confer the immune inducibility of the *Diptericin* and *Cecropin A1* genes in *Drosophila*[7,8], indicating that *Diptericin* and *Cecropin A1* are regulated by an NF-κB-like transcription factor. On the basis of these observations, two distinct strategies were adopted to identify the NF-κB-like transcription factor that regulates the genes encoding antibacterial peptides. The first strategy was to use biochemical techniques to purify the κB-motif binding factor(s) from extracts of *Drosophila* cell lines. For this approach, the objective was to identify the binding factor and then to work backwards, step by step, to identify the upstream elements of the signalling cascade that activate the factor. This tactic was motivated by the successful characterization of mammalian NF-κB-signalling pathways that use similar strategies[9]. Considerable efforts were made to use biochemical techniques to isolate an NF-κB-like molecule that functions in the *Drosophila* immune response; however, this approach was ultimately unsuccessful.

The second strategy for identifying a *Drosophila* NF-κB-like transcription factor that regulates immune responses was a genetic approach, which I undertook with colleagues in the Hoffmann laboratory. The power of using genetic techniques to dissect complex biological processes had previously been illustrated by the mutant screens that Eric Wieschaus and Christiane Nusslein-Volhard carried out (in Heidelberg, Germany) to identify *Drosophila* genes that regulate early embryogenesis. In the early 1990s, several research groups identified parallels between the establishment of the dorsoventral axis by the Toll pathway in *Drosophila* embryos (BOX 1) and the cytokine-induced expression of several immune genes by the interleukin-1 receptor (IL-1R)–NF-κB-signalling cascade in mammals[10–12]. These groups noted that in both pathways, a Toll/IL-1R (TIR)-domain-containing transmembrane receptor — *Drosophila* Toll or mammalian IL-1R (FIG. 1) — activates intracellular signalling, which culminates in the nuclear translocation of an NF-κB/NF-κB-like transcription factor. In *Drosophila*, the NF-κB-like factor regulated by the Toll pathway during embryonic patterning is known as Dorsal[13,14], and Dorsal regulates target genes through κB-binding motifs[15–17].

Timeline | **Discoveries relevant to the function of the Toll and Imd pathways in *Drosophila* immunity**



The discovery of the role of Toll in the *Drosophila* immune response (yellow) was influenced mainly by research in three fields: insect immunity (red), vertebrate immunology and signalling (blue) and developmental genetics (green). DIF, Dorsal-related immunity factor; DREDD, Death-related ced-3/Nedd2-like protein; ds, double-stranded; GNBP, Gram-negative-bacteria-binding protein; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1R, interleukin-1 receptor; IMD, Immune deficiency; IRD5, Immune-response deficient 5; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PG, peptidoglycan; PGRP, PG-recognition protein; serpin, serine-protease inhibitor; TAK1, Transforming growth factor-β-activated kinase 1; TLR, Toll-like receptor.

The parallels between the Toll pathway and the IL-1R pathway raised the obvious question of whether the Toll pathway, in addition to its role in dorsoventral polarity, controls the expression of antibacterial peptides in differentiated tissues. Furthermore, although the genes encoding components of the Toll pathway were initially described as maternal-effect genes (which regulate early embryogenesis), it was soon apparent that these genes are also expressed in larvae and adults[18–20]. With Jean Marc Reichhart and other colleagues[21] in the Hoffmann laboratory, we determined that the expression of the *dorsal* gene is upregulated and that in fat-body cells, the *Dorsal* protein translocates rapidly to the nucleus in response to bacterial infection. These results suggested that Dorsal was the NF-κB-like factor that mediated *Drosophila* immune responses, and they stimulated a wave of enthusiasm for further studies of Dorsal. The next goal was to determine whether Dorsal regulated the expression of the genes encoding antimicrobial peptides and whether this Dorsal activity was linked to the Toll pathway. This project was facilitated by the numerous *dorsal* and *Toll* mutants from the Wiechaus–Volhard screens, which were available at the Tübingen stock centre (Germany). The results that I obtained, however, were frustrating: although, in fat-body cells, Dorsal was activated by the Toll pathway in response to bacterial infection, none of the mutations affecting either Dorsal or the Toll pathway significantly altered the induction of *Diptericin* expression after infection[22]. During this period, Tony Ip and colleagues (in Michael Levine's laboratory in San Diego, United States) identified a second *Drosophila* NF-κB-like gene, which they called *Dorsal-related immunity factor* (*Dif*). However, they stopped studying *Dif* when they realized that it was not involved in dorsoventral patterning of the embryo. Further studies of *Dif* started when Ylva Engström (in Stockholm) pointed out a potential link between DIF and the expression of the genes encoding antimicrobial peptides. They showed that DIF is expressed by the fat body, that it can bind to the κB-like sequence motifs in the *Cecropin A* promoter and that its translocation into the nucleus is regulated by Toll[23]. However, because there were no fly lines carrying a mutation in the *Dif* gene, they were unable to test for DIF function in immune responses. A clue to how antimicrobial-peptide expression is regulated was provided eventually by Dan Hultmark's group (in Stockholm), when they observed that overexpression of an active form of Toll increased the expression of a *Cecropin A* transgene in a cell-culture assay[24]. However, although our studies of Dorsal, the identification of DIF, and the Toll overexpression studies all indicated a link between the Toll pathway and the expression of antimicrobial peptides, they still did not identify the exact function of Toll in the immune response.

There are two pathways

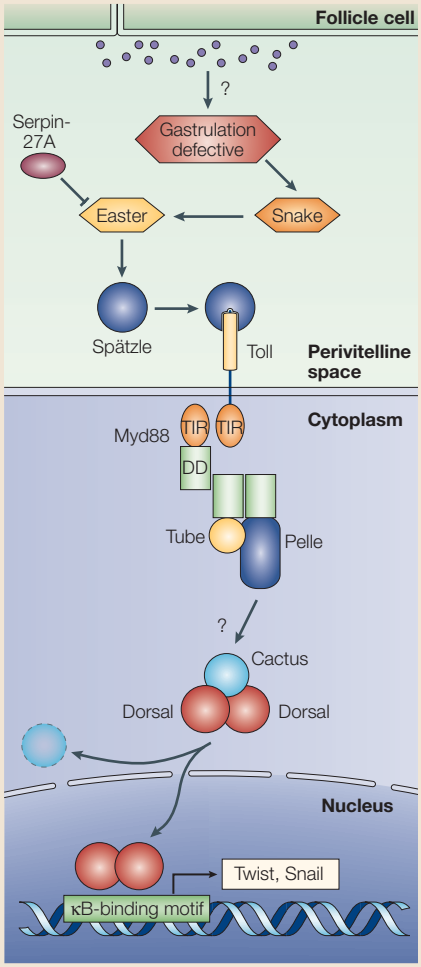
The initial lack of success using genetic strategies refocused attention on the biochemical approach, and it left me at an impasse in my attempts to use genetics to decipher the signalling pathway that regulates the expression of antimicrobial-peptide genes. The way out of this quandary was revealed by two unexpected discoveries made in the Hoffmann laboratory. The first breakthrough happened in 1994, when Philippe Bulet (a talented biochemist) and his colleagues carried out a differential screen to identify *Drosophila* peptides that are induced specifically by bacterial infections. In their screen, one of the proteins that was highly induced by bacterial infection was a peptide, which they called DIF-30. DIF-30 did not, however, have any antibacterial activity, and interest in DIF-30 declined until Bulet and colleagues (with the help of the plant pathologist Willem F. Broekaert, from Leuven, Belgium) showed that DIF-30 has strong inhibitory effects against various filamentous fungi. DIF-30 was renamed Drosomycin, and

publication of these studies provided the first description of an inducible antifungal peptide identified in insects[25]. Drosomycin then became a key molecule in my attempts to identify immune-signalling pathways in fruit flies (discussed later).

The second breakthrough was my serendipitous discovery of the *Drosophila immune deficiency* (*imd*) mutation. After failing to link *Diptericin* expression with mutations in the Toll pathway, I decided to stop focusing solely on Toll and broaden my search. Consequently, I began to measure the level of *Diptericin* expression after infection of any fly line that carried a mutation in a gene that could be linked to immune responses. To my delight, I found that *Diptericin* expression was significantly reduced in stock 1046 from the Bloomington Stock Center (United States). This fly line originates from an EMS (ethyl methane sulphonate) mutagenesis carried out by Ellsworth Grell in 1969 and has a mutation, *Black cells* (*Bc*), that affects *Drosophila* blood cells known as a crystal cells[26]. Crystal cells are implicated in the prophenoloxidase cascade, an enzymatic reaction that leads to the deposition of melanin around invading pathogens and has an important role in arthropod immune defence[27]. Our first analysis indicated that the *Bc* mutation affected *Diptericin* expression because a chromosomal deficiency that spanned *Bc* also reduced *Diptericin* expression. A link between a melanization cascade and the expression of antibacterial peptides was an attractive concept, and we were preparing a publication on the role of *Bc* in *Diptericin* regulation when we realized that we were on the wrong track: first, I noticed that other fly lines carrying mutations that reduced the melanization reaction did not affect *Diptericin* expression; and second, Michael Levine informed Jules Hoffmann that in their studies, the *Bc* mutation did not affect *Diptericin* expression. So, faced with the absence of detectable defects in the anti microbial-peptide expression of Toll-deficient mutants, and after hearing about my results during a visit from Jules Hoffmann, Joe Corbo and Michael Levine had also started to study stock 1046 (the *Bc* mutant). Using a different set of deficiencies than I had used, they identified a deficiency spanning the mutation that reduced *Diptericin* expression but not the *Bc* mutation, demonstrating that these two mutations were at different loci. Using this new information, we then determined that the mutation that blocked *Diptericin* expression actually mapped to a locus 3.5 centimorgans from *Bc*, which we called *imd*, and we used genetic recombination to generate an *imd*-mutant line that lacked the *Bc* mutation[28].

When published in 1995 (REF. 28), *imd* was the first reported mutation to affect the expression of genes encoding antibacterial peptides, and its identification demonstrated the potential of genetic approaches for analysing immune-signalling pathways. Interestingly, although *imd*-mutant flies are perfectly viable, they are highly susceptible to Gram-negative bacterial infection, and this phenotype provided the first functional evidence that antimicrobial peptides are important for fighting infections *in vivo*. I also made the crucial observation that the level of *Drosomycin* expression is nearly normal in *imd* mutants, which indicated that more than one signalling pathway regulates the expression of antimicrobial peptides and that the expression of the genes encoding the antibacterial peptide Diptericin and the antifungal peptide Drosomycin are regulated by separate pathways. Corbo and Levine[29] published their own work on the *imd* mutation one year later, but they did not analyse *Drosomycin* expression in the *imd* mutant. The ultimate identification of *imd* as a second mutation in stock 1046 was, for me, a good lesson in genetics, and it also reminded me that science often progresses when unexpected help and stimulation is provided by potential competitors.

Box 1 | Toll and establishment of polarity in the *Drosophila* embryo



Semisaturating screens carried out by Christiane Nüsslein-Volhard's group (in Tübingen, Germany), as well as by Trudy Schüpbach and Eric Wieschaus (in Princeton, United States), identified numerous maternal-effect mutations that disrupt embryonic polarity. Some of these mutations affect 12 genes that are involved in establishing dorsoventral polarity[67,68]. Among these, numerous mutations in the *Toll* (which means 'cool' in German) locus were analysed in further detail by Kathryn V. Anderson in Nüsslein-Volhard's group[69]. In 1988 , the *Toll* gene was cloned in the Anderson laboratory (in Berkeley, United States) and shown to encode a transmembrane receptor[70]. The molecular characterization of the other dorsoventral patterning genes, carried out by several research groups, has defined the components of a signalling pathway. Recently, two additional genes, *Myd88* and *Serpin-27A*, which were identified initially for their immune phenotype, were shown to function in dorsoventral patterning[71–74]. The Toll pathway is also used at later developmental stages, including morphogenetic movement, muscle attachment[75] and haemocyte (blood cell) proliferation[76].

During oogenesis, a molecular cue localized on the ventral side of follicle cells initiates a proteolytic cascade in the perivitelline space outside the fertilized embryo, which is mediated by the proteases Gastrulation defective, Snake and Easter. The activity of Easter is inhibited by the serine-protease inhibitor, Serpin-27A. This cascade results in the ventral processing of Spätzle in a graded manner. The cleaved form of Spätzle then functions as a ligand for Toll. Localized activation of Toll leads to the activation of an intracellular pathway that involves the adaptors Tube and Myd88 and the kinase Pelle. The result of this activation is the phosphorylation and degradation of the IκB orthologue Cactus. Cactus interacts with and inhibits the transcription factor Dorsal. Degradation of Cactus allows Dorsal to enter the nucleus, where it regulates the genes that organize dorsoventral patterning, such as *twist* and *snail*.

DD, death domain; Serpin, serine-protease inhibitor; TIR domain, Toll/interleukin-1 receptor domain.

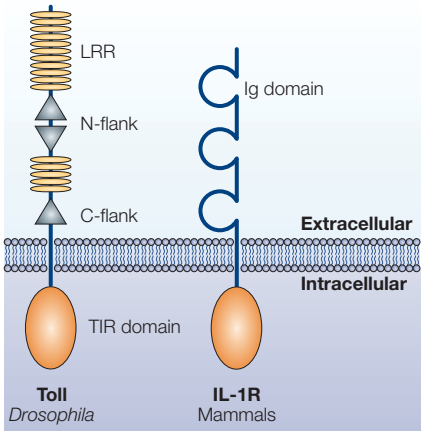


Figure 1 | **Structure of the Toll and IL-1 receptors.** The ectodomain of Toll comprises leucine-rich repeats (LRRs) that are flanked by cysteine-rich motifs (known as the N- and C-flanks). The ectodomain of the interleukin-1 receptor (IL-1R) comprises three immunoglobulin (Ig) domains. The intracellular Toll/IL-1R (TIR) domain of both Toll and the IL-1R interacts with TIR-domain-containing adaptor proteins (for example, *Drosophila* Myd88 or the mammalian MyD88) and signals through NF-κB or NF-κB-like molecules (FIG. 3).

Yet, little did I know how competitive and stimulating the genetic studies of *Drosophila* immunity would become.

Toll regulates the antifungal response

Freshly armed with the discovery that *imd* regulates *Diptericin* but not *Drosomycin* expression, I postulated that the Toll pathway could be a regulator of *Drosomycin*. So, in my previous experiments, I had selected the wrong target gene! This time, by checking the expression of a series of genes encoding antimicrobial peptides in *Toll* and Toll-pathway mutants, my colleagues and I determined that this prediction was correct: after microbial infection, *Drosomycin* expression is regulated by the Toll pathway, whereas *Diptericin* expression is regulated by *imd*[30]. It was also shown that not all of the components of the Toll pathway that regulate embryogenesis (BOX 1) have an immune function. For example, Easter, a serine protease that cleaves Spätzle and activates the Toll pathway during embryonic development[31], does not regulate Toll during immunity. Similarly, several lines of evidence indicated that the NF-κB-like factor Dorsal, which is activated by the Toll pathway during development, is not required for the expression of antimicrobial peptides. Initially, Tony Ip and colleagues[32] (in Worcester, United States) generated a fly line carrying a small deletion that spans both *Dif* and *Dorsal*. By re-introducing transgenes encoding DIF or Dorsal into this deletion line, they demonstrated that DIF, but not Dorsal, was the main regulator of the expression of genes encoding antimicrobial peptides in adult fruit flies. Subsequently, the identification of mutations that only affected the *Dif* locus confirmed this result[33].

Another important finding that we made after our return to studying Toll, was that in contrast to *imd* mutants, which die after Gram-negative bacterial infection, fruit flies carrying mutations in the Toll pathway are highly susceptible to fungal infection (FIG. 2). In addition, flies that lack both IMD and Toll fail to express any antimicrobial peptides and are susceptible to both bacterial and fungal infections. The marked and complementary phenotypes of the *Toll* and *imd* mutants indicated that Toll and IMD were components of the two main signalling pathways that regulate both the expression of antimicrobial peptides and resistance to bacterial and fungal infection. Our demonstration of Toll function in the antifungal immune response was published in 1996 (REF. 30) and provided the first evidence that Toll has an important role in animal host defence. In this paper, we suggested a basic model in which Toll and IMD control the expression of genes encoding antimicrobial peptides, and we extended the parallels between the cytokine-induced activation of NF-κB and the Toll pathway, thereby showing that the regulation of NF-κB/NF-κB-like molecules is an ancient mechanism for fighting infection.

An adapted innate immune response?

When it was first shown that *Drosomycin* and *Diptericin* are not regulated by the same signalling pathways, I wondered whether the expression of each gene is induced in response to different types of infection. To test this hypothesis, the levels of *Drosomycin* and *Diptericin* expression were compared after infecting fruit flies with different types of microorganism. The results were clear: the gene encoding the antibacterial peptide, *Diptericin*, was most highly induced by Gram-negative bacteria, whereas Gram-positive bacteria and fungi were the strongest inducers of the gene encoding the antifungal peptide, *Drosomycin*. The simplest interpretation of these results is that *Toll* and *imd* are activated differentially by different types of microorganism. I consider that the best experiment supporting this interpretation was the demonstration that flies dusted with spores of *Beauveria bassiana* (a fungus that infects insects) specifically express the antifungal peptide Drosomycin but do not express antibacterial peptides, indicating the selective activation of the Toll pathway[34]. Furthermore, Toll-deficient flies succumb rapidly to *B. bassiana* infections. This experiment demonstrates that flies mount immune responses that are adapted to the invading microorganism. It was also the first demonstration, using a natural route of infection, that showed that Toll signalling is required to combat a true insect pathogen.

These observations — that the Toll pathway is more responsive to Gram-positive bacteria and fungi, whereas IMD regulates responses to Gram-negative bacteria — challenged the prevailing dogma that innate immune mechanisms provide an entirely nonspecific response to infection. On the contrary, the separation of Toll- and IMD-mediated responses enables the fly to mount an immune response that is, to some extent, adapted to the species of aggressor. The existence of a degree of specificity in innate immune responses is not restricted to *Drosophila*, and determining how infections by distinct microorganisms shape the innate immune responses of vertebrates is currently the focus of intense study. When we published the data on selective *Toll* and *imd* activation in 1997 (REF. 34), we used the term 'adapted immune response', rather than 'specific immune response', to indicate how *Drosophila* uses several signalling pathways to discriminate between microorganisms and mount microorganism-specific immune responses.

Our experiments on selective *Toll* or *imd* activation also taught us that the types of microorganism used, as well as the infection procedure (natural versus artificial infection), influence immune responses. Therefore, some inconsistencies in the reports on *Drosophila* immunity can probably be attributed to the way immune responses are triggered — a common observation in immunology. For example, I now realize that our success in identifying the function of Toll

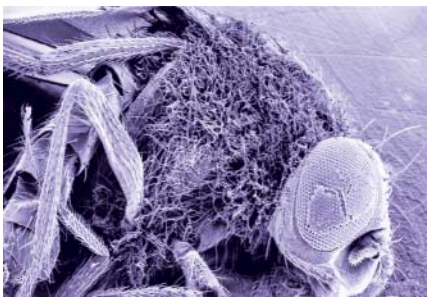


Figure 2 | ***Toll* mutants are highly susceptible to fungal infection.** Toll-deficient fruit flies (shown), but not wild-type fruit flies, succumb rapidly to infection with the fungus *Aspergillus fumigatus*. This image is reproduced with permission from REF. 30 © (1996) Cell Press.

in the *Drosophila* immune response was partly because we routinely used a mixture of Gram-negative and Gram-positive bacteria to infect flies, whereas other groups only used Gram-negative bacteria. The Gram-positive bacteria strongly activated the Toll pathway and enabled us to discern the role of Toll in inducing *Drosomycin* expression.

The Toll and IMD paradigm

All of these findings established a model of two potentially independent pathways that regulate the expression of the *Drosophila* genes encoding antimicrobial peptides. This model was tested rapidly when several groups began to use the power of *Drosophila* genetics to identify new factors that regulate *Drosophila* immune responses. After *imd*, the next gene identified to control antibacterial responses was characterized in Dan Hultmark's laboratory (in Umea, Sweden) in 1999 (REFS 35,36). His group demonstrated that a deletion of the *Relish* gene — which encodes a third *Drosophila* NF-κB-like protein — produces phenotypes that are similar to those of fruit flies carrying the *imd* mutation[35,36]. Subsequently, several successful forward genetic screens identified other mutations that, similar to the *imd* and *Relish* mutations, render flies highly susceptible to Gram-negative bacterial infections[37–43]. Surprisingly, none of these mutations affect any detectable functions of the Toll pathway. Genetic epistasis studies and molecular analysis of gene function show that *imd*, *Relish* and these other genes encode components of a signalling pathway, which is completely distinct from the Toll pathway and is essential for combating Gram-negative bacterial infection[38,39,42,44–48] (FIG. 3).

Today, the Toll and IMD pathways have emerged as a simple paradigm of innate immune-response regulation in animals, showing how two distinct signalling cascades can modulate the expression of a complex transcriptional programme in response to different pathogens (FIG. 3). This model disputes prevailing views of innate immunity by indicating the existence of specificity and the absence of redundancy in *Drosophila* innate immune responses. There was initially some resistance to a simple genetic model of two separate NF-κB-like signalling pathways in fruit flies, perhaps because studies of mammalian NF-κB regulation (in cultured cells) indicate intricate and convergent networks of signalling cascades. Although it is possible that genetic analysis has simplified our vision of the *Drosophila* immunoresponsive-signalling pathways, I suspect that complexity exists in the capacity of these two pathways to integrate

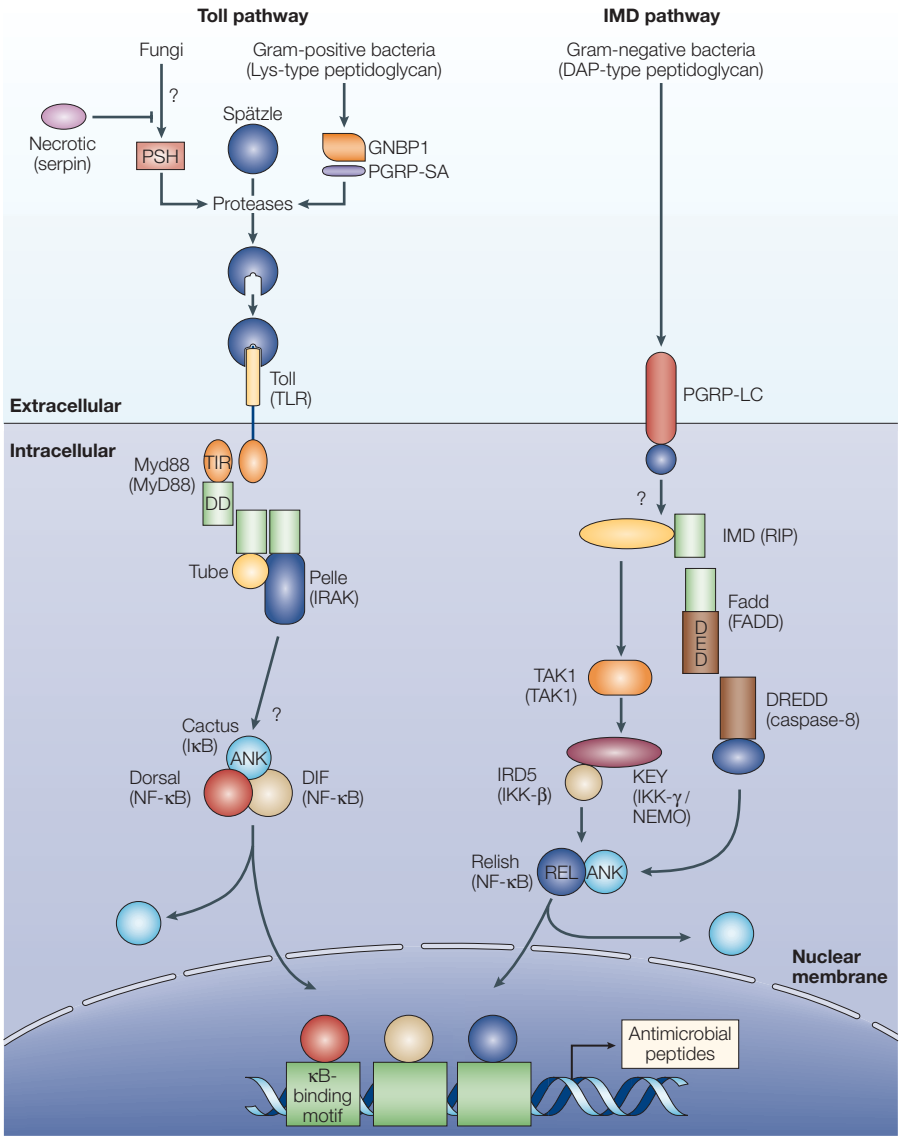


Figure 3 | **The Toll and Imd pathways.** The genes that encode antimicrobial peptides are regulated by a balance between two signalling pathways: the Toll pathway, which is activated mainly by fungi and Gram-positive bacteria, and the Immune deficiency (IMD) pathway, which is activated mainly by Gram-negative bacteria. Depending on the variant of the κB-binding motif present in the promoter, the genes encoding antimicrobial peptides are more sensitive to the Toll–DIF cascade (for example, *Drosomycin*), the IMD–Relish cascade (for example, *Diptericin*) or are co-regulated. Toll is activated by binding to a cleaved form of Spätzle, which is processed by proteolytic cascades that are activated by secreted recognition molecules (such as the peptidoglycan-recognition protein PGRP-SA and Gram-negative-bacteria-binding protein 1, GNBP1). PGRP-SA might bind to a lysine-type peptidoglycan found in Gram-positive bacteria. Intracellular signal transduction (BOX 1) regulates the nuclear translocation of the nuclear factor-κB (NF-κB)-like proteins DIF and Dorsal. The IMD pathway is probably triggered by an interaction between the transmembrane receptor PGRP-LC and peptidoglycan from Gram-negative bacteria (diaminopimelate (DAP)-type peptidoglycan). Following PGRP-LC activation, the death-domain (DD) adaptor protein, IMD, is recruited and binds to Fadd, which interacts with the caspase DREDD (Death-related ced-3/Nedd2-like protein). DREDD has been shown to associate with Relish (which it might cleave directly) after Relish has been phosphorylated by the *Drosophila* IKK (inhibitor of NF-κB (IκB)-kinase) complex, which comprises Immune response deficient 5 (IRD5) and Kenny (KEY). The IKK complex is itself activated by TAK1 (Transforming-growth-factor-β-activated kinase 1) (a mitogen-activated protein kinase kinase kinase) in an IMD-dependent manner. After cleavage, the REL domain of Relish moves to the nucleus, where it regulates the transcription of genes with immune function[77,78]. Vertebrate homologues are indicated in parentheses. ANK, ankyrin-repeat domain; DED; death-effector domain; DIF, Dorsal-related immunity factor; FADD, FAS-associated death domain; IRAK, interleukin-1-receptor-associated kinase; MyD88, myeloid differentiation primary-response protein 88; NEMO, NF-κB essential modulator; PSH, Persephone; RIP, receptor-interacting protein; serpin, serine-protease inhibitor; TIR domain, Toll/interleukin-1-receptor domain; TLR, Toll-like receptor.

many external factors (nature of infectious agents, mode of infection) and internal factors (tissue identity, physiological state) and to transduce these variables into a complex output (the sequential expression of many genes with immune function) that is far from understood.

Distinct functions for Toll and TLRs

The studies of Toll stimulated research on Toll-like receptors (TLRs) in mammals. In 1997, the identification of a human Toll homologue in expressed sequence tag (EST) databases and the analysis of its function indicated that, similar to Toll, TLRs were linked to NF-κB signalling and were probably important regulators of immunity[49]. Subsequently, a series of remarkable studies with mutant mice clearly demonstrated that TLRs function as recognition receptors for many microbial and viral ligands and control numerous aspects of both the innate and adaptive immune responses[50,51]. Interestingly, although TLRs function as direct recognition receptors for microbial components, the recognition of lipopolysaccharide by TLR4 is mediated by a complex that includes CD14 and MD2, in addition to TLR4. The identification of TLRs as receptors for microbial and viral ligands was not predicted by studies carried out in flies because Toll clearly does not interact directly with microbial products but is activated by an endogenous ligand, Spätzle, during the immune response[30,52]. Although the *Drosophila* genome encodes eight additional Toll molecules, surprisingly, none of these has yet been shown to participate directly in microbial sensing, and only one (Toll-9) has been implicated in the regulation of immune responses (using a cell-culture assay)[53–55].

In contrast to the rapid identification of TLRs and their ligands, the *Drosophila* molecules involved in microbial recognition remained unknown until recently. At present, we know that some aspects of microbial recognition in flies are mediated by peptidoglycan-recognition proteins (PGRPs) and Gram-negative-bacteria-binding proteins (GNBPs)[43,56–60] — two protein families identified, initially in other insects, by their capacity to bind microbial components[61–64] (FIG. 3). Furthermore, unlike mammals, the *Drosophila* immune system recognizes Gram-negative bacteria by detecting a specific form of peptidoglycan and not by sensing lipopolysaccharide[65]. Therefore, it seems that insects and mammals might use different strategies to detect microorganisms and that functional differences exist between the *Drosophila* Toll molecules and the human TLRs, although they both have important roles in innate immune responses that are mediated by NF-κB/NF-κB-like molecules.

Continued value of *Drosophila* models

The conservation of some immune responses in insects and mammals has produced an exchange of results and ideas that have invigorated the field of innate immunity. The discovery of TLRs was a turning point in the study of the mammalian immune system and opened numerous avenues of research. This discovery also validated the fruit fly as a model for analysing immune-response pathways. One advantage of the *Drosophila* model is that it offers a different perspective on the battle between pathogens and their hosts, and it allows fundamental questions in immunity to be addressed, without the added complexity of an adaptive immune system. A second advantage of the *Drosophila* model is illustrated by the studies of *Toll* and *imd* that have been described here, during which about 100,000 flies were infected. This seems reasonable when one understands that 300–400 flies can be infected in one hour. Clearly, such exhaustive studies are not possible in vertebrate immunology. Furthermore, comparing the strategies that different species have developed to fight microbial infection is essential if we want to fully understand the immune system and not become confused by its intrinsic complexity. These comparisons, however, also need to consider the varied methods that are used to study immune responses in different species, because different approaches can influence the models that we build. As discussed in this Landmark, the immune-signalling pathways in *Drosophila* were elucidated as an extension of the discovery of antimicrobial peptides. However, it is not difficult to imagine that a simple screen for mutations that caused susceptibility to infection by pathogens would also have identified the *Drosophila* NF-κB-like pathways. Such a project was technically possible several decades ago, but at that time, fly geneticists had mostly deserted the field of physiology and were focusing their attention on *Drosophila* development. Finally, although the black box of antimicrobial-peptide gene expression has lost some of its mystery, antimicrobial peptides are only part of the large arsenal of insect immune responses to pathogens. As a result, the future holds the promise of many exciting discoveries that will probably further impact on mammalian studies.

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1. Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. & Boman, H. G. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**, 246–248 (1981).
2. Hultmark, D. *et al*. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. *EMBO J.* **2**, 571–576 (1983).
3. Samakovlis, C., Kimbrell, D., Kylsten, P., Engstrom, A. & Hultmark, D. The immune response in *Drosophila*: pattern of *Cecropin* expression and biological activity. *EMBO J.* **9**, 2969–2976 (1990).
4. Kylsten, P., Samakovlis, C. & Hultmark, D. The *cecropin* locus in *Drosophila*; a compact gene cluster involved in the response to infection. *EMBO J.* **9**, 217–224 (1990).
5. Wicker, C. *et al*. Insect immunity. Characterization of a *Drosophila* cDNA encoding a novel member of the Diptericin family of immune peptides. *J. Biol. Chem.* **265**, 22493–22498 (1990).
6. Sun, S. C., Lindstrom, I., Lee, J. Y. & Faye, I. Structure and expression of the *attacin* genes in *Hyalophora cecropia*. *Eur. J. Biochem.* **196**, 247–254 (1991).
7. Kappler, C. *et al*. Insect immunity. Two 17bp repeats nesting a κB-related sequence confer inducibility to the *Diptericin* gene and bind a polypeptide in bacteria-challenged *Drosophila*. *EMBO J.* **12**, 1561–1568 (1993).
8. Engstrom, Y. *et al*. κB-like motifs regulate the induction of immune genes in *Drosophila*. *J. Mol. Biol.* **232**, 327–333 (1993).
9. Li, Q. & Verma, I. M. NF-κB regulation in the immune system. *Nature Rev. Immunol.* **2**, 725–734 (2002).
10. Gay, N. & Keith, F. *Drosophila* Toll and IL-1 receptor. *Nature* **351**, 355–356 (1991).
11. Schneider, D. S., Hudson, K. L., Lin, T. Y. & Anderson, K. V. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal–ventral polarity in the *Drosophila* embryo. *Genes Dev.* **5**, 797–807 (1991).
12. Heguy, A., Baldari, C. T., Macchia, G., Telford, J. L. & Melli, M. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the *Drosophila Toll* protein are essential for IL-1R signal transduction. *J. Biol. Chem.* **267**, 2605–2609 (1992).
13. Nusslein-Volhard, C. in *Determinants of Spatial Organization* (eds Subtelny, S. & Konigsberg, I. R.) 185–211 (Academic, New York, 1979).
14. Steward, R. *Dorsal*, an embryonic polarity gene in *Drosophila* is homologous to the vertebrate proto-oncogene *c-rel*. *Science* **238**, 692–694 (1987).
15. Ip, Y. T., Kraut, R., Levine, M. & Rushlow, C. A. The Dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* **64**, 439–446 (1991).
16. Thisse, C., Perrin-Schmitt, F., Stoetzel, C. & Thisse, B. Sequence-specific transactivation of the *Drosophila twist* gene by the *dorsal* gene product. *Cell* **65**, 1191–1201 (1991).
17. Pan, D. J., Huang, J. D. & Courey, A. J. Functional analysis of the *Drosophila twist* promoter reveals a dorsal-binding ventral activator region. *Genes Dev.* **5**, 1892–1901 (1991).
18. Gerttula, S., Jin, Y. S. & Anderson, K. V. Zygotic expression and activity of the *Drosophila Toll* gene, a gene required maternally for embryonic dorsal–ventral pattern formation. *Genetics* **119**, 123–133 (1988).
19. Letsou, A., Alexander, S., Orth, K. & Wasserman, S. A. Genetic and molecular characterization of *tube*, a *Drosophila* gene maternally required for embryonic dorsoventral polarity. *Proc. Natl Acad. Sci. USA* **88**, 810–814 (1991).
20. Hecht, P. M. & Anderson, K. V. Genetic characterization of *tube* and *pelle*, genes required for signaling between Toll and Dorsal in the specification of the dorsal–ventral pattern of the *Drosophila* embryo. *Genetics* **135**, 405–417 (1993).
21. Reichhart, J. M. *et al*. Expression and nuclear translocation of the rel/NF-κB-related morphogen Dorsal during the immune response of *Drosophila*. *C. R. Acad. Sci. III, Sci. Vie* **316**, 1218–1224 (1993).
22. Lemaitre, B. *et al*. Functional analysis and regulation of nuclear import of Dorsal during the immune response in *Drosophila*. *EMBO J.* **14**, 536–545 (1995).
23. Ip, Y. *et al*. *Dif*, a *dorsal*-related gene that mediates an immune response in *Drosophila*. *Cell* **75**, 753–763 (1993).
24. Rosetto, M., Engstrom, Y., Baldari, C. T., Telford, J. L. & Hultmark, D. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a *Drosophila* hemocyte cell line. *Biochem. Biophys. Res. Commun.* **209**, 111–116 (1995).

25. Fehlbaum, P. *et al*. Insect immunity: septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J. Biol. Chem.* **269**, 33159–33163 (1994).
26. Rizki, T., Rizki, R. & Grell, E. A mutant affecting the crystal cells in *Drosophila melanogaster*. *Rouxs Arch. Dev. Biol.* **188**, 91–99 (1980).
27. Söderhäll, K. & Cerenius, L. Role of prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **10**, 23–28 (1998).
28. Lemaitre, B. *et al*. A recessive mutation, immune deficiency (*imd*), defines two distinct control pathways in the *Drosophila* host defense. *Proc. Natl Acad. Sci. USA* **92**, 9465–9469 (1995).
29. Corbo, J. C. & Levine, M. Characterization of an immunodeficiency mutant in *Drosophila*. *Mech. Dev.* **55**, 211–220 (1996).
30. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. & Hoffmann, J. The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983 (1996).
31. Chasan, R. & Anderson, K. V. The role of Easter, an apparent serine protease, in organizing the dorsal–ventral pattern of the *Drosophila* embryo. *Cell* **56**, 391–400 (1989).
32. Meng, X., Khanuja, B. S. & Ip, Y. T. Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-κB factor. *Genes Dev*. **13**, 792–797 (1999).
33. Rutschmann, S. *et al*. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity* **12**, 569–580 (2000).
34. Lemaitre, B., Reichhart, J. & Hoffmann, J. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl Acad. Sci. USA* **94**, 14614–14619 (1997).
35. Dushay, M., Asling, B. & Hultmark, D. Origins of immunity: *Relish*, a compound *Rel*-like gene in the antibacterial defense of *Drosophila*. *Proc. Natl Acad. Sci. USA* **93**, 10343–10347 (1996).
36. Hedengren, M. *et al*. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol. Cell* **4**, 827–837 (1999).
37. Wu, L. P. & Anderson, K. V. Regulated nuclear import of *Rel* proteins in the *Drosophila* immune response. *Nature* **392**, 93–97 (1998).
38. Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M. & Lemaitre, B. The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO Rep.* **1**, 353–358 (2000).
39. Rutschmann, S. *et al*. Role of *Drosophila* IKKγ in a Toll-independent antibacterial immune response. *Nature Immunol.* **1**, 342–347 (2000).
40. Elrod-Erickson, M., Mishra, S. & Schneider, D. Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr. Biol*. **10**, 781–784 (2000).
41. Lu, Y., Wu, L. P. & Anderson, K. V. The antibacterial arm of the *Drosophila* innate immune response requires an IκB kinase. *Genes Dev.* **15**, 104–110 (2001).
42. Vidal, S. *et al*. Mutations in the *Drosophila dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-κB-dependent innate immune responses. *Genes Dev.* **15**, 1900–1912 (2001).
43. Choe, K. M., Werner, T., Stoven, S., Hultmark, D. & Anderson, K. V. Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*. *Science* **296**, 359–362 (2002).
44. Silverman, N. *et al*. A *Drosophila* IκB kinase complex required for Relish cleavage and antibacterial immunity. *Genes Dev.* **14**, 2461–2471 (2000).
45. Stöven, S., Ando, I., Kadalayil, L., Engström, Y. & Hultmark, D. Activation of the *Drosophila* NF-κB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep.* **1**, 347–352 (2000).
46. Georgel, P. *et al. Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev. Cell* **1**, 503–514 (2001).
47. Leulier, F., Vidal, S., Saigo, K., Ueda, R. & Lemaitre, B. Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in *Drosophila* adults. *Curr. Biol.* **12**, 996–1000 (2002).
48. Naitza, S. *et al*. The *Drosophila* immune defense against Gram-negative infection requires the death protein dFADD. *Immunity* **17**, 575–581 (2002).
49. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A human homologue of the *Drosophila Toll* protein signals activation of adaptive immunity. *Nature* **388**, 394–397 (1997).
50. Akira, S. Mammalian Toll-like receptors. *Curr. Opin. Immunol.* **15**, 5–11 (2003).
51. Beutler, B., Hoebe, K., Du, X. & Ulevitch, R. J. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukoc. Biol.* **74**, 479–485 (2003).
52. Weber, A. N. *et al*. Binding of the *Drosophila* cytokine Spatzle to Toll is direct and establishes signaling. *Nature Immunol.* **4**, 794–800 (2003).
53. Tauszig, S., Jouanguy, E., Hoffmann, J. A. & Imler, J. L. Toll-related receptors and the control of antimicrobial peptide expression in *Drosophila*. *Proc. Natl Acad. Sci. USA* **97**, 10520–10525 (2000).
54. Imler, J. L. & Hoffmann, J. A. Toll receptors in *Drosophila*: a family of molecules regulating development and immunity. *Curr. Top. Microbiol. Immunol.* **270**, 63–79 (2002).
55. Ooi, J. Y., Yagi, Y., Hu, X. & Ip, Y. T. The *Drosophila* Toll-9 activates a constitutive antimicrobial defense. *EMBO Rep.* **3**, 82–87 (2002).
56. Michel, T., Reichhart, J. M., Hoffmann, J. A. & Royet, J. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756–759 (2001).
57. Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. & Ezekowitz, R. A. Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. *Nature* **416**, 644–648 (2002).
58. Gottar, M. *et al*. The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature* **416**, 640–644 (2002).
59. Gobert, V. *et al*. Dual activation of the *Drosophila* Toll pathway by two pattern recognition receptors. *Science* **302**, 2126–2130 (2003).
60. Pili-Floury, S. *et al*. *In vivo* RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in *Drosophila* adults. *J. Biol. Chem.* **279**, 12848–12853 (2004).
61. Yoshida, H., Kinoshita, K. & Ashida, M. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.* **271**, 13854–13860 (1996).
62. Lee, W., Lee, J., Kravchenko, V., Ulevitch, R. & Brey, P. Purification and molecular cloning of an inducible Gram-negative bacteria-binding protein from the silkworm, *Bombyx mori*. *Proc. Natl Acad. Sci. USA* **93**, 7888–7893 (1996).
63. Ochiai, M. & Ashida, M. A pattern recognition protein for peptidoglycan. Cloning the cDNA and the gene of the silkworm, *Bombyx mori*. *J. Biol. Chem.* **274**, 11854–11858 (1999).
64. Kang, D., Liu, G., Lundstrom, A., Gelius, E. & Steiner, H. A peptidoglycan regonition protein in innate immunity conserved from insects to humans. *Proc. Natl Acad. Sci. USA* **95**, 10078–10082 (1998).
65. Leulier, F. *et al*. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nature Immunol.* **4**, 478–484 (2003).
66. Rubin, G. M. *et al*. Comparative genomics of the eukaryotes. *Science* **287**, 2204–2215 (2000).
67. Anderson, K. V. & Nusslein-Volhard, C. in *Pattern Formation: A Primer in Developmental Biology* (eds Malacinski, G. M. & Bryant, S. V.) 269–289 (Macmillan, New York, 1984).
68. Anderson, K. V., Bokla, L. & Nusslein-Volhard, C. Establishment of dorsal–ventral polarity in the *Drosophila* embryo: the induction of polarity by the *Toll* gene product. *Cell* **42**, 791–798 (1985).
69. Anderson, K. V., Jurgens, G. & Nusslein-Volhard, C. Establishment of dorsal–ventral polarity in the *Drosophila* embryo: genetic studies on the role of the *Toll* gene product. *Cell* **42**, 779–789 (1985).
70. Hashimoto, C., Hudson, K. & Anderson, K. The *Toll* gene of *Drosophila*, required for dorsal–ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269–279 (1988).
71. Hashimoto, C., Kim, D. R., Weiss, L. A., Miller, J. W. & Morisato, D. Spatial regulation of developmental signaling by a serpin. *Dev. Cell* **5**, 945–950 (2003).
72. Ligoxygakis, P., Roth, S. & Reichhart, J. M. A serpin regulates dorsal–ventral axis formation in the *Drosophila* embryo. *Curr. Biol.* **13**, 2097–2102 (2003).
73. Kambris, Z. *et al*. *DmMyD88* controls dorsoventral patterning of the *Drosophila* embryo. *EMBO Rep.* **4**, 64–69 (2003).
74. Charatsi, I., Luschnig, S., Bartoszewski, S., Nusslein-Volhard, C. & Moussian, B. Krapfen/dMyd88 is required for the establishment of dorsoventral pattern in the *Drosophila* embryo. *Mech. Dev.* **120**, 219–226 (2003).
75. Belvin, M. P. & Anderson, K. V. A conserved signaling pathway: the *Drosophila* Toll–Dorsal pathway. *Annu. Rev. Cell Dev. Biol.* **12**, 393–416 (1996).
76. Qiu, P., Pan, P. C. & Govind, S. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development* **125**, 1909–1920 (1998).
77. Hultmark, D. *Drosophila* immunity: paths and patterns. *Curr. Opin. Immunol.* **15**, 12–19 (2003).
78. Ferrandon, D., Imler, J. L. & Hoffmann, J. A. Sensing infection in *Drosophila*: Toll and beyond. *Semin. Immunol.* **16**, 43–53 (2004).

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Competing interests statement

The author declares that he has no competing financial interests.

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