Toll signaling: the enigma variations
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Experiments reported in the past year have revealed considerable diversity in Toll-mediated pathways for signal transduction in development and innate immunity. Rather than function as a well conserved signaling cassette, Toll receptors and associated factors have apparently evolved as a diverse set of configurations to defend against microbial infection in species ranging from plants to humans.

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Abbreviations
gd gastrulation defective
IKK κB kinase
IL-1 interleukin-1
IMD immune deficiency
IRAK IL-1 receptor associated kinase
lrd immune response deficient
LPS lipopolysaccharide
LRR leucine-rich repeat
NLS nuclear localization signal
Tlr Toll-like receptor

Prelude
The Toll signaling pathway was discovered independently in the biochemical investigation of cytokine responses in cultured mammalian cells and in the molecular genetic dissection of embryonic patterning in Drosophila [1–3]. In both systems, transmembrane receptors of the Toll family signal through adaptor molecules and protein kinases to effect nuclear localization of a transcription factor (Figure 1). Evolutionary conservation of the systems first became apparent with the discovery that the mammalian transcription factor NF-κB and the Drosophila morphogen Dorsal, the targets of these pathways, were both homologous to the products of the vertebrate proto-oncogene c-rel.

With the characterization of additional components and the elaboration of more mechanistic details, it appeared that the Toll-family receptors and associated factors constituted a conserved signaling cassette employed for distinct purposes at different times and in different tissues. The Toll pathway would thus take its place alongside other conserved signaling systems, such as those described for Wnt and Ras [4,5]. Recent evidence, however, indicates that there are in fact several different Toll pathways. These pathways carry out signal transduction by distinct routes, yet are largely restricted to a single function, that of defense against infection.

One Toll pathway involves direct activation of the receptor by a pathogen-encoded macromolecule. A second functions as part of a signal relay system, mediating intracellular signaling in response to activation of a host-encoded ligand. Others involve a limited subset of pathway components. Together, these divergent forms of the Toll-signaling cassette constitute an intriguing series of variations based on a common molecular and functional theme. Here I review these variations, bringing into a common context recent results from biochemical experiments, genetic studies, and genome analyses.

Statement of the theme: Toll-like receptors in mammalian innate immunity
Upon binding with bacterial lipopolysaccharide (LPS) or interleukin-1 (IL-1), mammalian cell-surface receptors initiate a signal-transduction pathway that directs release of NF-κB (a p50/p65 heterodimer) from its inhibitor IκB (Figure 1b) [6]. Receptor activation drives formation of a complex that includes the IL-1 receptor associated kinase (IRAK) and two adaptor proteins, TRAF6 and MyD88. This multiprotein assembly mediates activation of the IκB kinase (IKK) complex, which targets IκB for degradation via phosphorylation of specific serine residues [7]. The subsequent ubiquitination and proteolysis of IκB exposes the nuclear localization signal (NLS) on NF-κB, allowing nuclear import and activation of gene expression.

In the innate immune response to infection, the critical receptors for NF-κB regulation are homologues of the Drosophila Toll protein. Toll and Toll-like receptors (Tlrs) from flies and mammals contain extracellular binding surfaces comprising leucine-rich repeats (LRRs), a single membrane-spanning domain, and an intracellular domain that mediates signal transduction but lacks apparent catalytic activity [8,9]. Conservation between the Toll cytoplasmic domain and that of both the IL-1 receptor and some plant disease resistance) proteins led to the designation of this region as the TIR domain.

The demonstration that a transfected Tlr gene could activate NF-κB and the identification of Tlr4 mutations in LPS-resistant mice established Tlr4 as a critical mediator of LPS signaling [10,11]. Moreover, the Tlr4 gene from a given mammal (human, mouse, or hamster) confers on transfected cells the ability to respond to the specific LPS derivative to which that species is sensitive [12*,13**]. Such results suggest strongly that LPS interacts directly with Tlr4 to activate signal transduction.

Given the existence of more than a half dozen mammalian Tlr genes, there is the potential for different family members to be specific for different sets of microbial pathogens [14*]. Indeed, several studies reveal that Tlr2 and Tlr4 recognize distinct microbial products: Tlr2 is specific for Gram-positive bacteria, whereas Tlr4 recognizes Gram-negative species.
The Toll pathway in defense and pattern formation

Variation 1: Toll signaling in antifungal defense and pattern formation

The Toll pathway in *Drosophila* was first identified on the basis of its role in establishing embryonic dorsoventral polarity (Figure 1a) [2]. Homodimers of the Dorsal protein are initially present throughout the embryonic cytoplasm, where they are retained by an inhibitor, Cactus. Following fertilization, a localized source of the ligand Spätzle is required to induce the Dorsal/Dif pathway, which then relays information to the Dorsal/Cactus complex via an adaptor, Tube, and a protein kinase, Pelle. The result is Cactus degradation and formation of a nuclear concentration gradient of Dorsal that defines dorsoventral polarity.

Spätzle, Toll, Tube, Pelle, and Cactus function in concert again in both larvae and adults as part of the invertebrate innate immune response [18]. Upon fungal challenge, wild-type *Drosophila* express Drosomycin, a potent antifungal peptide. This immune response is protective, because flies mutant for *spätzle*, *toll*, *tube*, *pelle*, or *cactus* fail to induce the *drosomycin* gene and succumb to fungal infection much more readily than the wild type. The direct inducer of *drosomycin* in adults is not Dorsal, but rather Dif, the *Drosophila* immunity factor [19**].

Despite the fact that Toll, Pelle, Cactus, Dorsal and Dif all have structural and functional counterparts in vertebrates, the Dorsal/Dif pathway differs from the NF-κB pathway in several significant respects. First, Spätzle, the Toll ligand, is not a fungal product but is instead a host-encoded protein that is activated by proteolysis [20,21**]. In dorsoventral patterning, Spätzle is activated by a proteolytic cascade involving the products of the *gastrulation defective* (*gd*), *snake*, and *easter* genes [22]. A different proteolytic pathway is required in the antifungal defense because mutations in a serine protease inhibitor (serpin) gene result in constitutive Drosomycin expression even in the absence of *gd* or *snake* gene [21**]. Thus it appears that fungal infection triggers a proteolytic pathway leading to activation of Toll, which then relays information to the nucleus via Dif.

A second way in which the *Drosophila* Toll pathway differs from the mammalian Tlr4 pathway is in the proteins required to induce Cactus degradation (Table 1). Cactus proteolysis is regulated, at least in part, by motifs similar to those targeted by vertebrate IKK proteins and by a conserved pathway for signal-induced ubiquitination [23,24,25*]. Nevertheless, the *Drosophila* homologues of the kinase IKKβ and the regulatory subunit IKKγ are dispensable for dorsoventral patterning and the anti-fungal response (J Hoffmann, personal communication; K Anderson, person-
al communication). A remaining candidate for the Cactus kinase is DmIKKε, for which mutations have not been described. DmIKKε is a counterpart to IKKε and related vertebrate kinases, which transduce signals to NF-κB in T-cell activation and perhaps other processes [26*–29*].

The Dorsal/Dif pathway also differs from the NF-κB pathway in the adaptor proteins that act downstream of the receptor. MyD88 contains a TIR domain that interacts with the TIR domain of Toll family receptors, whereas Tube protein has a novel repeat that mediates binding to Dorsal [30–32]. Furthermore, although MyD88 and Tube interact with IRAK and Pelle, respectively, via interaction motifs termed death domains [33,34,35**], the binding of Tube to Pelle involves sequences outside the death domain that are not conserved in either MyD88 or IRAK [35**].

A remaining difference observed in the Drosophila system is an apparent bifurcation in the dorsoventral pathway, such that there is signaling from Toll, Tube, and Pelle not only to Cactus but also to Dorsal. Specifically, a nuclear concentration gradient of Dorsal persists, albeit in an attenuated form, in the absence of either Cactus or a Dorsal–Cactus interaction [24,36*]. This residual gradient might reflect regulation of Dorsal by Relish, the only Drosophila protein other than Cactus with an IκB-like domain (see below). It seems more likely, however, that the observed signal-dependent phosphorylation of Dorsal and the binding of Pelle to Dorsal in embryos reflect a pathway for Cactus-independent signal transduction to Dorsal [31,37,38].

**Variation 2: Drosophila anti-bacterial responses**

*Drosophila* mount an immune response not only against fungi but also against bacteria, inducing expression of anti-bacterial peptides such as Diptericin, Attacin, and Cecropin [39*]. The pathway leading to Diptericin induction is distinct from the anti-fungal pathway because it is unaffected by mutations in *dorsal, Dif*, or the genes that regulate these factors [18,40*]. Furthermore, a number of genes specific for this pathway have been identified, including the *imd* (immune deficiency) locus and the *ird* (immune response deficient) genes [39*,41,42] (Table 1).

The target of the *imd/ird* pathway is Relish, a p105 homologue active in humoral immunity [43**]. Like the mammalian p105 protein, Relish contains both a Rel homology domain and a set of IκB-like ankyrin repeats. A null mutation in relish renders flies highly susceptible to bacterial infection and eliminates induction of *diptericin* in response to infection. Surprisingly, processing of Relish is quite different from that of p105. Whereas signaling in mammalian cells leads to proteasome-mediated cleavage of p105 and degradation of the IκB-like domain [44], signal-dependent cleavage of Relish is proteasome-independent and results in stable Rel and IκB-like fragments (D Hultmark, personal communication).

### Table 1

**Components of Toll signaling pathways in Drosophila.**

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Antifungal (Dorsoverential)</th>
<th>Antibacterial (Diptericin)</th>
<th>Accession nos.†/other names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toll</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>18-wheeler</td>
<td>–</td>
<td>+</td>
<td>MetProx</td>
</tr>
<tr>
<td>Toll-3</td>
<td>–</td>
<td>+</td>
<td>CG18241</td>
</tr>
<tr>
<td>Toll-4</td>
<td>+</td>
<td>–</td>
<td>CG7250</td>
</tr>
<tr>
<td>Toll-5</td>
<td>–</td>
<td>+</td>
<td>CG8595</td>
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<tr>
<td>Toll-6</td>
<td>–</td>
<td>+</td>
<td>Tollo</td>
</tr>
<tr>
<td>Toll-7</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Toll-8</td>
<td>–</td>
<td>+</td>
<td></td>
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<tr>
<td>Adaptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube</td>
<td>+</td>
<td>–</td>
<td>CG2078</td>
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<tr>
<td>DmMyd88</td>
<td></td>
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<tr>
<td>dTraf1</td>
<td>–</td>
<td>+</td>
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<tr>
<td>dTraf2</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Kinasas</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pelle</td>
<td>+</td>
<td>–</td>
<td>Ird-5, dLak, Ik</td>
</tr>
<tr>
<td>DmIKKβ</td>
<td>–</td>
<td>+</td>
<td>Kenny</td>
</tr>
<tr>
<td>DmIKKγ</td>
<td>+</td>
<td>–</td>
<td>Ik2</td>
</tr>
<tr>
<td>DmIKKε</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Effectors/inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cactus</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>+*</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Dif</td>
<td>+*</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Relish</td>
<td>–</td>
<td>+</td>
<td></td>
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</tbody>
</table>

For each gene for which mutations have been characterized, symbols indicate whether the gene is required (+) or not (–) for the pathways listed. *Dif* is required for the antifungal pathway in adults; Dorsal is required for the dorsoventral pathway in embryos. These two loci are redundant for the antifungal response in larvae [40*]. The accession numbers and alternate names are archived in Flybase (http://flybase.bio.indiana.edu/).

DmIKKβ and DmIKKγ, dispensable in fighting fungal infection, are both active in the anti-bacterial immune response. Mutations in either locus block Diptericin induction but have no effect on Drosomycin expression (J Hoffmann, personal communication; K Anderson, personal communication). Furthermore, an activated complex of DmIKKβ and DmIKKγ phosphorylates Relish *in vitro* (N Silverman, D Hultmark, T Maniatis, personal communication). Given the involvement of an IKK complex, it seems likely that the Diptericin pathway will also involve the *Drosophila* homologues of TRAF6 [45,46] and MyD88 (S Wasserman, unpublished data).

At least one Tlr protein, 18-Wheeler, is expressed in the larval fat body, the focus of the humoral immune response, and appears to be active in the *Drosophila* antibacterial response [47]; however, because *diptericin* expression is relatively unaffected in an 18-wheeler mutant, it is likely that further genetic analyses will place additional Tlr proteins in the *imd/ird* pathway. Given that *Drosophila* encodes a repertoire of eight Toll family receptors (see Table 1), there is the potential, as in mammals, for substantial specificity in pathway activation and function.
Although the imd\textsubscript{iird} pathway of \textit{Drosophila} bears substantial similarity to that of mammals, the mechanism of signal transduction is most likely different. In the NF-xB pathway, the IRAK kinases are necessary for signal transduction [56•]; a readily recognizable homolog exists in worms, but not flies. In contrast, a mammalian TRAF6 interactor termed ECSI (evolutionarily conserved signaling intermediate in Toll pathways) has a \textit{Drosophila} homologue which binds to dTRAF1, the fly TRAF6 counterpart [57•]. Furthermore, dTRAF1 also interacts with Pelle, which binds not only to Tube and Dorsal, but also to Toll and Pellino, a novel fly protein [46,58,59]. There are, thus, clearly additional networks of interactions that need to be sorted out.

**Coda: perspectives**

In recent years, we have grown used to conservation in mechanistic detail in concert with functional diversity. The Wingless and Ras signaling cassettes are highly conserved at the biochemical level but function in a diverse set of developmental processes [4,5]. In the case of the Tlr pathways, the opposite is true: function is largely constant but the pathways have diverged. The contrast is apparent even at the level of individual components — sequence identity between \textit{Drosophila} and human components of the Toll pathway is in general much lower than that of components in the Wingless or Ras pathway.

Conserved function and diverged mechanism makes sense for a pathway that evolved to carry out immune function. Distinct pathogens present distinct problems for infected organisms, synthesizing diverse sets of macromolecules and disrupting growth and metabolism by disparate routes. In this context, variations in the Toll-signaling pathway could be means of linking specific ligands to specific responses. How such immune pathways were co-opted for development in \textit{Drosophila}, and perhaps other organisms [60], remains an open and fascinating evolutionary question.
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


8. An excellent review on the role of IKK in mammalian innate immunity.


15. A clear and broad review of the role of the Toll pathway in the mammalian response to LPS.


22. Genetic and biochemical data reveal that Spätzle is proteolytically activated in the antifungal response and confirm that the pathway for Spätzle proteolysis differs from that required for dorsal patterning. A mutation inactivating the Spr43Ac gene, which encodes a serpin (serine protease inhibitor), is shown to constitutively activate expression of the gene for the antifungal peptide Drosomycin. This constitutive activation is dependent on the Toll pathway and the Toll ligand Spätzle. Furthermore, loss of Spr43Ac function results in cleavage, and hence activation, of Spätzle in the absence of infection.


27. The authors identify Slimb/Tb1-TrCP as a specificity determinant for signal-dependent ubiquitination of both Cactus and IκB.


29. See annotation [29].

30. Pomerantz JL, Baltimore D: NF-κB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J 1999, 18:6694-6704. See annotation [29].


33. These findings, along with those reported in [15*], provide direct support for the hypothesis that different members of the mammalian Trl family recognize distinct classes of pathogens. Inactivation of Trr function by mutation [Takeuchi et al. [15*]] or by expression of dominant negative molecules [Underhill et al. [16*]] is used to demonstrate that Trl2 is required for a normal response to Gram-positive, but not Gram-negative, bacteria, whereas Trl4 exhibits the opposite specificity.
Differentiation and gene regulation


The amino-terminal domains of Pelle and Tube are shown to have the same fold as death domains but to interact in vitro in a novel manner. In addition, compensatory mutations based on the crystal structure are used to demonstrate for the first time that Tube and Pelle interact in embryos.


The authors use a form of Dorsal incapable of binding Cactus to investigate Cactus-independent signaling to Dorsal.


An up-to-date review on the antimicrobial peptides of Drosophila and the role of the Toll pathway in their regulation.


Dorsal and DIF are shown to have redundant functions in the immune response of larvae. See also [19**].


The first characterization of relish mutants. Flies deficient for relish are shown to be highly susceptible to bacterial infection but to be wild-type in hematopoietic development as well as encapsulation and phagocytic responses.


The authors of this paper report the unexpected finding that the catalytic activity of IRAK is dispensable for its role in transducing a signal from the IL-1 receptor to NF-κB. Rather than make targeted gene knockouts, the authors have used site-directed mutagenesis to disrupt IRAK and authors have used selection schemes in combination with somatic cell genetics to identify mutations altering the cellular cytokine responses. Having found an IRAK mutation by this route, the authors demonstrate that either a wild-type IRAK gene or an IRAK gene that has a site-directed mutation eliminating catalytic function can restore signal responsiveness.


54. Rathjen JP, Chang JH, Staskawicz BJ, Michelmore RW: Constitutively active Pto induces a Ptd-dependent hypersensitive response in the absence of avrPto. EMBO J 1999, 18:3322-3340. Evidence is provided that the Pto homolog Pto acts upstream of the Toll homolog Prf. In particular, the authors demonstrate that signaling by a constitutively active form of the tomato Pto kinase is independent of avrPto, the normal pathway inducer synthesized by the Pseudomonas strain that confers bacterial speck disease. They further demonstrate, however, that such signaling requires the activity of Prf, a Toll-like molecule with a leucine zipper motif at its amino terminus, suggesting an order of action distinct from that seen in animals.


Patent