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DEVELOPMENT AT A GLANCE

Toll and Toll-like receptor signalling in development

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ABSTRACT

The membrane receptor Toll and the related Toll-like receptors (TLRs) are best known for their universal function in innate immunity. However, Toll/TLRs were initially discovered in a developmental context, and recent studies have revealed that Toll/TLRs carry out previously unanticipated functions in development, regulating cell fate, cell number, neural circuit connectivity and synaptogenesis. Furthermore, knowledge of their molecular mechanisms of action is expanding and has highlighted that Toll/TLRs function beyond the canonical NF-κB pathway to regulate cell-to-cell communication and signalling at the synapse. Here, we provide an overview of Toll/TLR signalling and discuss how this signalling pathway regulates various aspects of development across species.

KEY WORDS: Toll, Tol-1, TLR, TIR-NBS-LRR, Sarm, MyD88, NF-κB, Dorsal, Wek, JNK, FoxO, Cell death, Cell survival, Cell fate, Cell proliferation, Structural plasticity, Signalling

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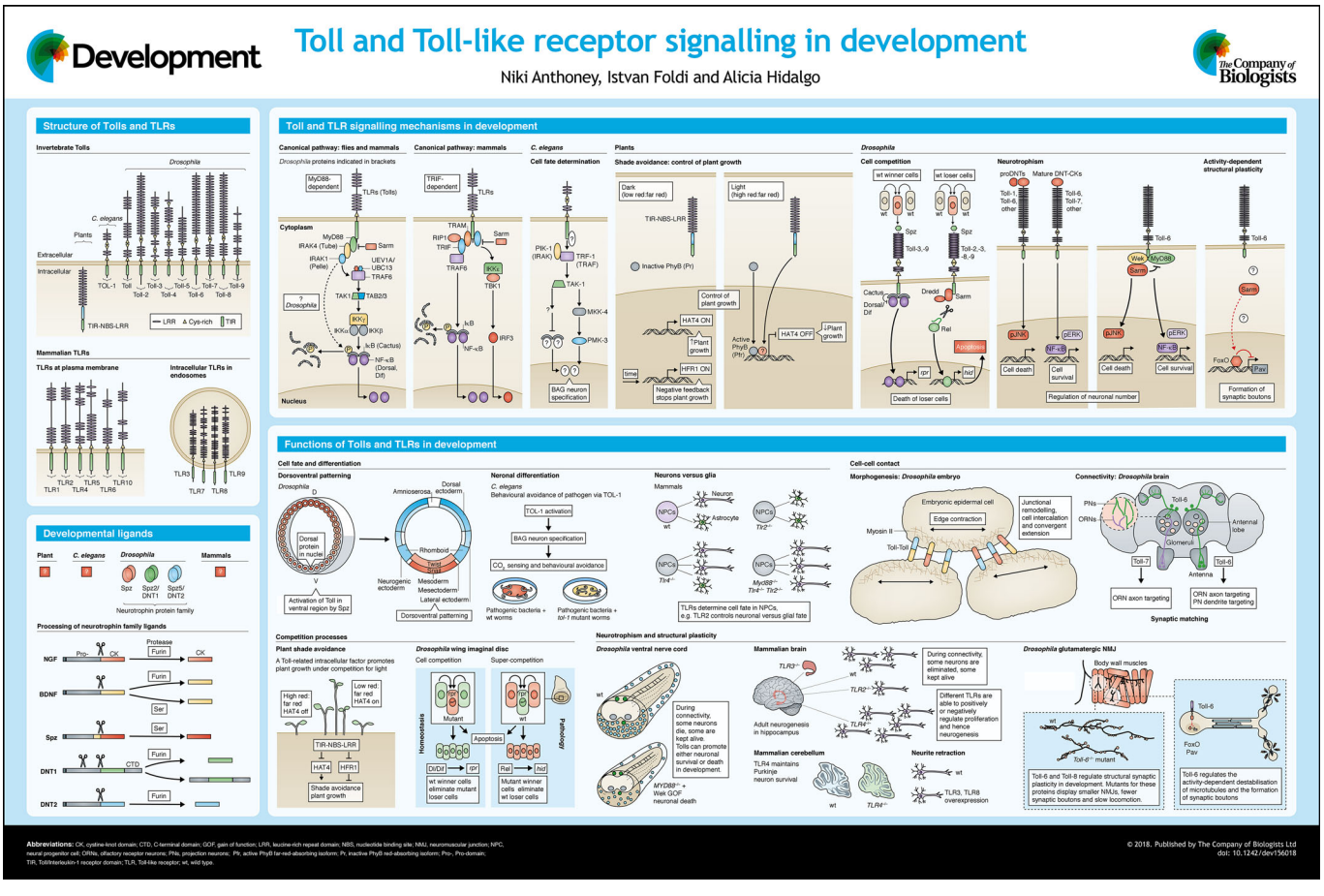
Introduction

The *Drosophila* gene *Toll* is possibly the only gene associated with two Nobel Prizes: it was discovered as one of the key genes determining body plan, and was later rediscovered for its role in underlying innate immunity (leading to the 1995 and 2011 Nobel Prizes in Physiology or Medicine, respectively). Searches for *Toll* homologues led to the identification of Toll-like receptor (TLR) genes in complex organisms, ranging from plants to humans. Attention then focused on pathogen defence and, indeed, Toll/TLRs are now best known for their universal, evolutionarily conserved function in innate immunity. However, they are also expressed in many non-immune tissues, both throughout development and in the adult. Questions as to why Toll/TLRs are expressed in these cells and what they are doing there are thus being asked again.



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Here, we provide an overview of Toll/TLR signalling and discuss the developmental, non-immune functions of the Toll/TLR protein family.

Toll/TLRs and their ligands

Toll/TLRs are type I integral membrane receptors that dimerise to activate downstream signalling events (Gay and Gangloff, 2007). The number of Toll/TLR genes found in different species varies: *C. elegans* has one *tol-1* gene, *Drosophila* has nine *Toll* paralogues, humans have 11 TLR genes, and other species can have many more. Of the nine *Drosophila* Tolls, only Toll itself (Toll-1) has a clear function in innate immunity (Lemaitre et al., 1996). Toll-9 was reported to trigger an immune response (Ooi et al., 2002), but *Toll-9* mutants do not display immune deficiency (Narbonne-Reveau et al., 2011). Toll-7 was also reported to recognise a virus to trigger autophagy (Nakamoto et al., 2012), but this finding was subsequently refuted (Lamiable et al., 2016). Most *Drosophila* Tolls are thus not involved in immunity (Tauszig et al., 2000), and instead their developmental functions are currently being uncovered.

Toll/TLRs harbour an N-terminal extracellular ligand-binding domain that contains a number of leucine-rich repeats (LRRs) and cysteine-rich domains (Gay and Gangloff, 2007; Leulier and Lemaitre, 2008). Intracellularly, Toll/TLRs share an evolutionarily conserved C-terminal intracellular Toll/Interleukin-1 receptor (TIR) domain (Gay and Gangloff, 2007; Leulier and Lemaitre, 2008), which induces signalling by recruiting adaptor complexes via interactions between the TIR domain and Death domains (DDs) in adaptor proteins.

A characteristic feature of Toll/TLRs is that they are promiscuous and can bind multiple ligand types (Gay and Gangloff, 2007). Mammalian TLRs, for example, are pattern recognition receptors that can bind proteins, lipopolysaccharides and nucleic acids from pathogens, or proteins released upon injury (Gay and Gangloff, 2007). Accordingly, mammalian TLRs are located at the plasma membrane or in endosomes (Gay and Gangloff, 2007). By contrast, *Drosophila* Tolls are not pattern recognition receptors (although see Nakamoto et al., 2012), and instead bind promiscuously to the protein ligands Spz, DNT1 (Spz2) and DNT2 (Spz5), which are evolutionarily conserved members of the neurotrophin (NT) protein family (Bergner et al., 1996; Foldi et al., 2017; Weber et al., 2003; Zhu et al., 2008). Like mammalian NTs (e.g. BDNF and NGF), *Drosophila* Spz, DNT1 and DNT2 are produced as pro-proteins and are subsequently cleaved to release a mature cystine-knot, which dimerises prior to binding the receptors (Arnot et al., 2010; Foldi et al., 2017; Lu et al., 2005). Cleavage of Spz involves a serine (Ser) protease cascade that terminates with the proteases Easter, which cleaves Spz in development, and Spz processing enzyme (SPE), which cleaves Spz in the context of immunity (Hong and Hashimoto, 1995; Weber et al., 2003). By contrast, DNT1 and DNT2 are cleaved by Furin proteases (Foldi et al., 2017). Mammalian NGF and BDNF are also cleaved by Furins for constitutive secretion, and BDNF is also cleaved by a Ser protease cascade for neuronal activity-regulated secretion (Lu et al., 2005). Spz induces signalling only in its cleaved form (Gay and Gangloff, 2007). By contrast, both full-length and cleaved DNT1, DNT2 and mammalian NTs can induce signalling to elicit distinct functions, such as cell death versus cell survival, respectively (Foldi et al., 2017; Lu et al., 2005).

The endogenous Toll/TLR ligands that function in development (i.e. without damage, infection or pathology), in organisms other than *Drosophila*, are currently not known. Extracellular matrix and cell adhesion proteins are endogenous ligands that can activate TLRs under pathological conditions, such as injury or cancer (Yu et al., 2010), but whether they do so during normal development is unknown. Human NTs can activate and modulate TLR signalling in cell culture (Foldi et al., 2017), but whether they do so during mammalian nervous system development is also unknown.

Tolls can also bind to one another's extracellular domains to signal across cells (Paré et al., 2014; Ward et al., 2015). They can also form heterodimers and, given that each Toll/TLR paralogue can potentially have distinct functions (Foldi et al., 2017; Meyer et al., 2014; Okun et al., 2009, 2011; Paré et al., 2014; Ward et al., 2015), it is possible that the various heterodimers perform distinct roles.

The Toll/TLR signalling pathway

The canonical signalling pathway that lies downstream of Toll/TLRs is evolutionarily conserved. The adaptor proteins that elicit Toll/TLR signalling include the TIR-bearing Myeloid differentiation primary response protein 88 (MyD88) and Sterile alpha and armadillo motif (Sarm; also known as Ect4 in *Drosophila*), an inhibitor of TLR signalling (Belinda et al., 2008; Foldi et al., 2017; Horng and Medzhitov, 2001; Imler and Hoffmann, 2001; Medzhitov et al., 1998; Peng et al., 2010; Tauszig-Delamasure et al., 2002). MyD88 recruits Tube and Pelle in *Drosophila*, and their orthologues IRAK4 and IRAK1 in mammals. Signalling results in nuclear translocation of the transcription factor NF-κB, which regulates the expression of distinct genes in immunity or development (Leulier and Lemaitre, 2008). However, several variations on this theme exist. For instance, mammalian TLRs can function via MyD88-dependent or -independent pathways, involving downstream factors that are distinct from those found in *Drosophila* (Akira and Takeda, 2004; Valanne et al., 2011). Indeed, the TIR adaptors TRIF (TICAM1), TIRAP and TRAM (TICAM2) are only found in vertebrates, whereas Weckle (Wek), a zing-finger protein, has so far only been identified in *Drosophila* (Chen et al., 2006).

Toll/TLRS also activate the MAPK pathways upstream of ERK and JNK signalling, and the latter can be activated by Sarm (Chuang and Bargmann, 2005; Foldi et al., 2017; Gay and Gangloff, 2007; Wu et al., 2015). In addition, alternative signalling pathways are being discovered, for instance those involving FoxO or cell-to-cell communication (Chuang and Bargmann, 2005; Foldi et al., 2017; McLaughlin et al., 2016; Paré et al., 2014; Ulian-Benitez et al., 2017; Ward et al., 2015), expanding the potential mechanisms of Toll/TLR function.

Overall, the outcome of Toll/TLR signalling appears to be cell type-, context- and time-specific, depending, for instance, on the adaptors available to a cell at a given time. However, it is possible that discrepancies between different data sets might have arisen due to as yet undiscovered links. Below we examine current evidence of how the diversification of Toll/TLR signalling may explain their diverse functions in development.

Toll/TLR signalling in cell fate determination and differentiation

Toll was discovered for its role in determining the dorsoventral axis of the *Drosophila* embryo (Anderson et al., 1985a,b). All ectodermal cells in the early *Drosophila* embryo express Toll at the membrane and an inactive form of the transcription factor Dorsal (NF-κB) in the cytoplasm. However, they receive a localised source of Spz in only the most ventral half of the embryo: Spz produced by the mother is secreted as a pro-protein into the perivitelline space, and a Ser protease cascade culminates in the cleavage of Spz and hence the activation of Toll only ventrally (Stathopoulos and

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Levine, 2002). Toll recruits an adaptor protein complex that includes MyD88, Tube and Pelle, and the autophosphorylation of Pelle results in its dissociation from the complex and the degradation of Cactus (Iκ-B), which is bound in a complex with Dorsal (Sun et al., 2002). This provokes the nuclear translocation of Dorsal, forming a gradient of nuclear Dorsal along the ventral half of the embryo (Stathopoulos and Levine, 2002). The Dorsal gradient drives the ventralmost expression of *snail*, a broader expression pattern of *twist*, and, more laterally, *rhomboid* expression (Stathopoulos and Levine, 2002). Snail and Twist specify mesoderm, forming the muscle; Twist alone specifies mesectoderm, forming the ventral midline; and Rhomboid determines the neuroectoderm (Stathopoulos and Levine, 2002). A complementary gradient represses ventral genes and enables dorsal patterning. Through these pathways, the embryo forms a central nervous system (CNS) nerve cord ventrally and a heart dorsally.

Neuronal cell fate determination and differentiation in *C. elegans* also require Toll/TLR signalling. *tol-1* is expressed in BAG neurons – a population of sensory neurons that sense CO_2 released by pathogenic bacteria. In these cells, TOL-1 determines cell fate via TRAF, IRAK and Iκ-B, and, accordingly, BAG neurons in *tol-1* mutant worms do not differentiate appropriately (Brandt and Ringstad, 2015). *C. elegans* lacks an *NF-κB* homologue, so TOL-1 signalling presumably activates an alternative transcription factor to determine cell fate (Irazoqui et al., 2010). By regulating BAG neuronal fate, TOL-1 enables worms to smell pathogenic bacteria and trigger a behavioural escape response – an intriguing pathogen avoidance function carried out via the nervous system.

TLRs also regulate neuronal proliferation and differentiation in mammals (Okun et al., 2011). In mice, TLRs function in neural progenitor cells (NPCs) to regulate cell proliferation and neuronal versus glial (astrocyte) cell fate determination (Lathia et al., 2008; Okun et al., 2010b; Rolls et al., 2007; Shechter et al., 2008; Sloane et al., 2010). In the absence of TLR2, for example, NPCs can produce only astrocytes, whereas the overexpression of *Tlr2* results in only neurons being produced (Rolls et al., 2007). By contrast, $Tlr4^{-/-}$ mutant NPCs produce more progeny cells but most of them are neurons (Rolls et al., 2007). In addition, $Myd88^{-/-}$ mutant or $Tlr4^{-/-};Tlr2^{-/-}$ double-mutant NPCs only produce neurons at the expense of astrocytes. Thus, MyD88 and TLR4 are required for astrocyte fate specification, and TLR2 for neuronal specification. Altogether, the influence of TLRs on cell proliferation and cell fate determination can vary between distinct TLRs and progenitor cell types.

Cell-cell signalling via Toll/TLRs

Tolls can also use their extracellular domains to mediate Toll-to-Toll contact across cells. In cell culture and *in vivo*, Toll-2 and Toll-8 can function as heterophilic cell adhesion molecules and facilitate cell migration (Keith and Gay, 1990; Kleve et al., 2006). Toll-1 was also proposed to function as a cell adhesion molecule in the interaction between muscle and motoneurons, and in heart development (Halfon et al., 1995; Rose et al., 1997; Wang et al., 2005). However, whether these are direct functions, and the mechanisms underlying them, remain to be determined.

In the *Drosophila* embryonic epidermis, the pair-rule transcription factors Eve and Runt regulate the expression of *Toll-2*, *Toll-6* and *Toll-8*, which direct cell-cell interactions and cell behaviour during convergent extension (Paré et al., 2014). Disruption of these Tolls results in defective planar polarity, cell intercalation and convergent extension (Paré et al., 2014). In neural circuit formation in the pupal fly brain, Toll-6 and Toll-7 are involved in the connectivity between olfactory receptor neuron axons and projection neuron dendrites at antennal lobe glomeruli (Ward et al., 2015). Specifically, Toll-7 is primarily involved in olfactory receptor neuron axon targeting and Toll-6 in projection neuron dendrite targeting, and both are involved in synaptic matching (Ward et al., 2015). These events do not appear to depend on ligand binding, the Toll-6 and Toll-7 cytoplasmic domains or downstream nuclear signalling, meaning that they must depend on cell-to-cell contact (Ward et al., 2015). However, connectivity does not depend on direct contact between Toll-7 and Toll-6 either, so how it comes about is unclear. Conceivably, the cell-to-cell functions of Tolls could involve both homophylic and heterophylic contacts, but whether other factors are involved is unknown.

Toll/TLR function in competition processes

Tolls and Toll-related factors are involved in different types of competition processes in both plants and animals. *Arabidopsis* has multiple non-canonical cytosolic Tolls, bearing LRR and TIR domains that flank a nucleotide binding site (NBS) (Faigon-Soverna et al., 2006). These proteins, like other Toll/TLRs, underlie immunity against pathogens. However, during development, TIR-NBS-LRR factors regulate plant competition for light by responding to the ratio of red to far-red light (Faigon-Soverna et al., 2006). If a plant grows with no other plants around, it senses a high ratio, causing Phytochrome B (PhyB) and an unknown factor downstream of TIR-NBS-LRR to translocate to the nucleus to repress the expression of genes such as *HAT4*, thereby slowing plant growth. If a plant grows in a densely populated area, it senses a lower ratio, as the neighbouring plants create shade. In darkness, PhyB and TIR-NBS-LRR signalling remain in the cytoplasm, and *HAT4* expression is derepressed, thus promoting plant growth. Plants continue to grow higher to compete for light until feedback takes effect, as genes such as *HFR1* that repress growth are also switched on. It is known that TIR-NBS-LRR functions independently of PhyB, but whereas PhyB is known to be activated by light, the factor that activates TIR-NBS-LRR, and the identity of the nuclear factor(s) that is activated downstream, are unknown (Faigon-Soverna et al., 2006). Thus, plant Tolls regulate growth in response to the environment.

In *Drosophila*, Tolls are involved in epithelial cell competition. During development, cells compete for proliferation and space, and this can be put to the test with mutations that result in 'winner' wild-type cells eliminating 'loser' mutant cells by apoptosis, or 'super-competitor' genotypes that cause the elimination of neighbouring loser wild-type cells (Meyer et al., 2014). In the *Drosophila* wing disc epithelium, Tolls are involved in these processes. Genetic inference indicates that Spz may function via Toll-3 and Toll-9 to trigger, via Dorsal and Dif, the Reaper-induced apoptosis of loser mutant cells, or via Toll-2, -4, -8 and -9 to induce the apoptosis of loser wild-type cells via Sarm, Rel and Hid (Meyer et al., 2014). However, how these distinct mechanisms are engaged in each case is unclear. The sources of Spz and its protease, and whether Spz can bind other receptors than Toll-1, still need to be established. These findings also raise the question of what enables signalling via Tolls to activate Rel – a typical Imd target (Ganesan et al., 2011) – downstream of Sarm, an inhibitor of MyD88–NF-κB signalling in other contexts (Foldi et al., 2017; Peng et al., 2010).

Competition also plays a role during the development of the CNS, as many neurons and glia normally die during circuit formation, and cells compete for ligands that maintain their survival. In mammals, both neuronal elimination and protection are regulated by NTs,

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which function as pro-NTs to promote neuronal death and as cleaved NTs to promote neuronal survival by engaging distinct receptor types (Lu et al., 2005). Similarly, in *Drosophila*, DNT1 and DNT2 can also function either as pro-DNTs or as cleaved forms to regulate neuronal death or survival (Foldi et al., 2017; McIlroy et al., 2013; Zhu et al., 2008). Distinct Toll receptors may have a preferential pro-apoptotic or pro-survival function, and the same Toll receptor can induce either neuronal survival or death, depending on the adaptors available to that particular cell type at a given developmental time (Foldi et al., 2017); for example, Toll-6 can promote neuronal survival via MyD88 in the embryonic CNS, but if Wek and Sarm are also present, as in the pupal CNS, then Toll-6 can also promote neuronal death. How pro-DNT1 may induce apoptosis, whether this depends on particular Tolls, or whether other co-receptors are also involved, are not known. Nonetheless, cell number control in the developing *Drosophila* CNS depends on the balance of antagonistic pro-apoptotic and pro-survival functions involving Tolls.

Whether NTs function in concert with TLRs to regulate neuronal number in mammals is also unknown. Human BDNF and NGF can induce and modify TLR signalling in cell culture (Foldi et al., 2017), but whether they do so in the CNS *in vivo* has not been explored. Importantly, TLRs in mammals can regulate the number and survival of both NPCs and neurons during embryonic development (Okun et al., 2011; Zhu et al., 2016). MyD88, TLR2, 3 and 4, for example, can repress NPC proliferation, while TLR4 is required to maintain Purkinje neuron survival and TLR8 can promote neuronal death (Ma et al., 2007; Okun et al., 2011; Zhu et al., 2016). The expression of both TLR3 and TLR8 in the nervous system is dynamic during development, so both might function in nervous system development (Okun et al., 2011). Potential functions of Tolls in cell proliferation have not been explored in *Drosophila* and, conversely, the ligands and mechanisms mediating TLR function in neuronal number control in mammals are unknown.

Structural nervous system plasticity mediated by Toll/TLRs

Changes in neuronal and glial number, neurite growth and retraction, and synapse formation and elimination continue in the adult brain and can be linked to changes in neuronal activity. Activity-dependent structural plasticity enables the brain to change in response to experience, and in the hippocampus it is thought to enable learning and the encoding of memory. A number of studies indicate that Toll/TLRs could play a role in structural brain plasticity. For example, TLRs can regulate adult hippocampal neurogenesis (Okun et al., 2011). Indeed, *Tlr2*$^{-/-}$ mutant mice exhibit reduced neurogenesis, whereas *Tlr4*$^{-/-}$ mutants show increased neurogenesis, independently of neuronal survival. On the other hand, the overexpression of TLR8 promotes neuronal death, and both TLR3 and TLR8 can also induce neurite retraction (Ma et al., 2007; Okun et al., 2011). These alterations in structural plasticity also correlate with cognitive deficits, as highlighted by the finding that *Tlr3*$^{-/-}$ mutant mice have impaired spatial recognition and contextual memory (Okun et al., 2010a).

Tolls also regulate structural synaptic plasticity, both under normal conditions and in response to increased neuronal activity, at the *Drosophila* glutamatergic neuromuscular junction (NMJ). *Toll-6* and *Toll-8* mutant larvae have smaller NMJs, with fewer synapses and, consequently, larvae exhibit a slow crawling behaviour (Ballard et al., 2014; McIlroy et al., 2013; Ulian-Benitez et al., 2017). Genetic evidence indicates that Toll-8 promotes NMJ growth independently of NF-κB signalling and via JNK instead (Ballard et al., 2014). Toll-6 can also promote activity-dependent NMJ growth via JNK and FoxO, and downstream of Sarm, repressing the expression of the kinesin *pavarotti* and causing destabilisation of microtubules, to enable new synaptic bouton formation (McLaughlin et al., 2016). This is consistent with other reports that JNK stimulates NMJ growth downstream of Wallenda (Collins et al., 2006). However, how Tolls activate JNK at the NMJ has not been determined. In CNS neurons, Toll-6 does not directly interact with Sarm and instead Toll-6 signalling via Sarm and JNK depends on Wek (Foldi et al., 2017), but *wek* mutants do not seem to affect NMJ growth (McLaughlin et al., 2016), which would imply that Wek is not involved. An attractive possibility is that Sarm could function at the synapse like in *C. elegans*, sensing postsynaptic CaMKII activation to activate JNK signalling (Chuang and Bargmann, 2005).

The *C. elegans* Sarm adaptor protein TIR-1 determines neuronal fate through a mechanism that links synapse formation with right-left asymmetry. *C. elegans* initially has two identical sensory neurons known as AWC neurons at the left and right side of the embryo (Chuang and Bargmann, 2005). During synapse formation, Ca^{2+} signalling causes activation and binding of CaMKII to Sarm/TIR-1 postsynaptically, resulting in MAKKK activation, which in turn represses *str-2* expression (giving rise to an AWC OFF neuron). Through a stochastic interaction between the AWC neurons, CaMKII is inhibited in one neuron and *str-2* is derepressed (giving rise to an AWC ON neuron). As a consequence, olfactory receptor genes are expressed differentially in each neuron, enabling AWC OFF neurons to detect pentanedione and AWC ON cells to detect butanone (Chuang and Bargmann, 2005). However, there is no evidence that this mechanism involves TOL-1.

In *Drosophila*, NMJ growth also depends on the retrograde factor DNT2 produced from the muscle, which binds a receptor complex formed of Toll-6 and Kek6, whereby Kek6 causes the activation of CaMKII (Ulian-Benitez et al., 2017). However, the mechanism might differ from that of *C. elegans*, as Toll-6 and Kek6 function presynaptically. Toll-6 and Kek6 positively cooperate as co-receptors, but whether they regulate Sarm at the NMJ has not been explored. Solving this might not be simple, as Sarm can also have opposite functions, driving axonal degeneration via JNK as well (Gerdts et al., 2016). In fact, Sarm seems to have multiple functions, some of which are independent of Toll/TLRs.

Perspectives

As we have highlighted, a number of recent studies indicate that Toll/TLRs have widespread functions in regulating cell fate, cell number and cell shape. General properties of Toll/TLRs are that they are promiscuous receptors, and that their function depends on context, i.e. on the ligands, proteases, other Toll/TLRs, co-receptors and downstream factors available to distinct cells at particular times. Given the prevalent evolutionary conservation yet diversity of biological form, both shared and distinct mechanisms may exist, and it is equally important to identify both. However, much remains unknown. For instance, little is known about the ligands that bind and activate Toll/TLRs in a developmental context. In addition, while numerous downstream signalling pathways have been identified in different contexts, this has led to many contradictions: it is unclear whether such discrepancies are due to context-dependent mechanisms, experimental deficits, or missing links yet to be discovered. Further work is clearly needed to better understand the signalling mechanisms that lie downstream of Toll/TLRs and how they might be utilised in different scenarios. Nonetheless, the complexity of the Toll/TLR signalling system that

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functions in a developmental context is awe inspiring. Through answering these open questions, the prophetic Toll – which means ‘amazing’ in German – will continue to reveal its greatness.

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Competing interests

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A high-resolution version of the poster is available for downloading in the online version of this article at http://dev.biologists.org/content/145/9/dev156018/F1.poster.jpg

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