Trends in Genetics

Review

The Toll gene in Drosophila pattern formation

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Toll-like receptors (TLRs) play a crucial role in innate immunity in animals. Their discovery was rewarded a Nobel Prize to Jules Hoffmann and Bruce Beutler in 2011. The name Toll stems from a Drosophila mutant that was isolated in 1980 by Eric Wieschaus and myself as a byproduct of our screen for segmentation genes in Drosophila for which we received the Nobel Prize in 1995. It was named Toll due to its amazing dominant phenotype displayed in embryos from Toll/+ females. The analysis of Toll by Kathryn Anderson in my laboratory in Tübingen and subsequently in her own laboratory in Berkeley singled out Toll as a central component of the complex pathway regulating dorsoventral polarity and pattern of the Drosophila embryo. The Drosophila Toll story provides a striking example for the value of curiosity-driven research in providing fundamental insights that later gain strong impact on applied medical research.

The discovery of dorsal

The Drosophila egg is a huge cell that is built during oogenesis in the female organism by two cell types, the somatic follicle cells surrounding the germ line-derived nurse cell–oocyte complex. Upon fertilization, it develops rapidly to form a larva by 24 h. In the first 3 h, the cleavage nuclei are not separated by cell membranes until there are about 6000 cleavage nuclei, which get included into cells forming the cellular blastoderm (Figure 1). The cuticle pattern of the Drosophila larva is richly endowed with markers for position and polarity (Figure 2A–C), and a blastoderm fate map of the larval hypoderm has been constructed [1] (Figure 2D). This pattern develops under the control of maternal genes that have been active during oogenesis and placed morphogenetic cues into the egg and the zygotic genes of the embryo itself. The long history of Drosophila genetics allowed systematic mutational approaches for the identification of maternal as well as zygotic genes affecting patterning of the larva by means of a visible phenotype displayed in the larval cuticle.

In the early 1970s, the first maternal mutants in Drosophila had been isolated, and transplantation experiments had supported the existence of ‘cytoplasmic determinants’ [2–4], which reinforced the idea that a genetic approach to an understanding of development is feasible. Although experimental embryology, mostly done in ascidian, nematode, sea urchin, amphibian, and insect embryos, was a field with long tradition [5], no molecules with morphogenetic roles had yet been identified in any system, and the postulated morphogen elements, which would determine position in the developing embryo depending on their concentration, remained elusive [6]. I joined the laboratory of Walter Gehring at the Biozentrum in Basel in 1975 with the long-term goal to discover morphogens in Drosophila. My aim was to screen for maternal mutants with defects in larval patterning and use transplantations of wild-type cytoplasm into mutant embryos as an assay for the isolation of the gene products. In a small-scale mutagenesis experiment, I fortuitously isolated the mutant dorsal with a very exciting dose-dependent phenotype: embryos from dorsal/+–mutant females lacked ventral pattern elements, the anlage of the mesoderm, and parts of the ventral denticle belts (Figure 3C), whereas the dorsal-recessive phenotype is a fully dorsalised embryo displaying dorsal tissue all around the embryo (Figure 3B).

Highlights

Toll is an exclamation of amazement that roughly translates to ‘Wow’ in English. In the dominant Toll-phenotype, the ventral segmental bands circle all around the Drosophila larva. Toll is a member of the ‘dorsal-group’ genes, named after the previously found dorsal gene, the phenotype of which displays only dorsal pattern elements.

The 12 dorsal-group genes define a single system determining the entire dorsoventral axis of the Drosophila embryo. Most components of the Toll-Dorsal pathway and much of the logic of their interactions were discovered by the powerful combination of genetics and transplantation experiments, subsequently confirmed at the biochemical level.

The Toll-Dorsal pathway also acts in the microbial defence system of Drosophila, whereas Toll-like receptors in mammals function as pattern recognition receptors covering almost the entire spectrum of innate immunity.

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2Dedicated to the memory of Kathryn Anderson (1952–2020)

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Figure 1. Oogenesis and early embryogenesis of *Drosophila melanogaster*. During oogenesis the oocyte-nurse cell complex, which is germ line derived, is surrounded by somatic follicle cells (light grey). The somatic cells produce the extraembryonic vitelline membrane and chorion, which cover the egg cell. After fertilisation, the cleavage nuclei migrate to the periphery and divide synchronously without forming cell membranes between them. In a clear cytoplasmic region at the posterior pole, the pole cells, the future germ line, emerge. At 3 h of development, the first cellular state is reached, consisting of a uniform layer of 6000 blastoderm cells.
These dramatic phenotypes, which result from a complete change of the larval fate map as ventral egg regions develop structures normally derived from more lateral or even dorsal regions, specified dorsal as the first good maternal patterning mutant. The phenotypic series suggested a morphogen gradient with a maximum at the ventral side of the egg determining the dorsoventral axis (Figure 3D) [7–9]. Pedro Santamaria later demonstrated that transplantation of wild-type cytoplasm could indeed partially rescue the mutant phenotype [10]. By that time, however, the advent of gene technology and germ line transformation in Drosophila meant that the road to the gene product would be via cloning the gene and not via protein purification.
Systematic screens for developmental mutants in *Drosophila*

My first independent position was at the European Molecular Biology Laboratory in Heidelberg, where I shared a small laboratory for 3 years with Eric Wieschaus, whom I had met in Basel. While developing screening protocols for maternal mutants, Eric Wieschaus and I also started collecting zygotic mutants affecting segmentation, which we obtained from various sources. The observed phenotypes were spectacular, and we decided first to do large-scale screens for more zygotic mutants, because they were easier than maternal screens and extremely rewarding. Indeed, these projects, in which we were later joined by an excellent geneticist, Gerd Jürgens, as a postdoc, resulted in a large and very exciting collection of patterning mutants [11,12], which provided much of the material basis for a new field of developmental genetics. We did not lose sight of the maternal mutants, however, and by chance we picked up three dominant *Toll* alleles.
by their singular ventralised phenotype (Figure 4), as well as two dominant cactus alleles and one easter allele, which shared its phenotype with dorsal. This was very encouraging, but the phenotypes were puzzling and at the time quite difficult to interpret.

In September 1980, Kathryn Anderson applied as a postdoc in my laboratory. She was a student in the laboratory of Judith Lengyel at the University of California, Los Angeles, and graduated with a thesis on RNA synthesis during Drosophila embryogenesis [13]. She must have been extremely courageous to apply to work with me, coming to Germany without knowing the language, leaving family and friends behind! I, who had worked on bacterial transcription for a thesis, was not yet well established in the field and had only published four papers on Drosophila (the screen with Eric and the rescue experiments with Pedro had not yet been published). Kathryn was fascinated by the dorsal mutation and the possibility to expand on the transplantation experiments. Although trained as a biochemist, she was keen on following a genetic approach to analyse development. She started in 1981 in my new laboratory at the Friedrich Miescher Laboratory (FML) of the Max Planck Society in Tübingen, with Gerd Jürgens as a research associate soon followed by Ruth Lehmann and Hans Georg Frohnhöfer as graduate students.

At FML, we set out to do a large-scale screen for maternal mutants on the third chromosome of Drosophila in the hope to find more genes with properties similar to dorsal. Trudi Schüpbach and Eric Wieschaus, now in Princeton, screened the second chromosome, and screens on the first chromosome had been done by Madeleine Gans and coworkers in Gif-sur-Yvette. Our screen provided us with an overwhelmingly rich yield of exciting mutants: we isolated alleles of eight genes that shared the dorsalis ed phenotype, together with torso-like, oskar, pumilio, and bicoid. One striking result that became obvious when we exchanged information with the Princeton screen group was that there was a much smaller set of observed phenotypes than identified genes, and several of these shared a common or similar phenotype. Kathryn chose the ‘dorsal-group’ of mutants for further analysis, while Ruth focused on the ‘posterior-group’ of genes, such as oskar and pumilio, and Hans Georg worked on the mutants with head defects, bicoid and torso-like.

The dorsal-group genes: a single system determining the entire dorsoventral axis
The dorsal-group was the largest of the phenotype classes we identified. We found a recessive dorsalis ed phenotype in alleles of nudel, pipe, snake, easter, spätzle, pelle, tube, and also Toll [14]. In the Princeton screen, one more dorsal-group gene, windbeutel, as well as cactus, which as a recessive shared with Toll the ventralised phenotype, was discovered [15]. The mutant gastrulation defective (gd), which had been isolated already in the first chromosome screen [16], also turned out to have a dorsalis ed phenotype. These common phenotypes suggested that each gene product is an essential component of a single system of positional information

Figure 4. Dominant phenotype of Toll-1, which gave the gene its name. Ventral denticle belts are circling around the larva produced by a Toll-1+/ female.
determining the entire dorsoventral axis. To understand how polarity and pattern in this axis is established, it was necessary to define the function of individual components of the system, to attribute them with “personalities”, as Kathryn put it. Kathryn’s transplantation experiments revealed that partial or complete normality of the pattern could be restored upon transplantation of wild-type cytoplasm or from embryos mutant for any of the other dorsal-group genes in mutant embryos for snake, easter, Toll, spätzle, pelle, and tube but not for gd and pipe (nudel could not be tested, as the eggs frequently were collapsed). In all cases, with the exception of dorsal, the rescuing principle included maternal mRNA, which presumably was translated upon injection to produce the protein products that would find their way in the process. The response to injection, however, varied considerably among the different genes; in the case of snake, Toll, easter, and tube, a strong rescue could be obtained by injecting only about 1% of the egg volume, whereas in spätzle and dorsal, only few dorsolateral structures could be induced [14]. The transplantation assay provides a means of analysing the spatial distribution of, and requirement for, the products of the different dorsal-group genes. In contrast to the experiments on mutants affecting the anterior–posterior axis studied by Ruth and Hans Georg, who identified localised activities at the anterior and posterior pole of the egg [17], no prelocalisation of the rescuing activity could be detected in the early embryo for any of the dorsal-group genes.

The results of the transplantation experiment singled out two genes, dorsal and Toll. In the case of dorsal, cytoplasm from the ventral side of older preblastoderm embryos is more active than that from the dorsal side, and the cytoplasm has to be delivered at the ventral side to be active [10]. Supported by the strong dosage dependence, dorsal appeared the best candidate for encoding the morphogen determining the dorsoventral axis. At this time, dorsal was already being investigated by Ruth Steward, who I had met in Basel as a graduate student of Walter Gehring; she spent some time in the laboratory in Tübingen to isolate X-ray-induced dorsal alleles, which she used to isolate the dorsal gene by chromosomal walking in Princeton in Paul Schedl’s laboratory [18,19].

**Toll is key gene for the establishment of polarity and pattern**

Kathryn found that for tube, snake, easter, spätzle, and pelle, the normalised patterns upon injection of wild-type cytoplasm always form in the normal orientation as given by the curvature of the egg, no matter where the cytoplasm is removed from the wild-type donors and no matter where it is injected. Toll was an exception; in the dorsalsed embryos lacking the Toll product, a complete dorsoventral pattern can be locally induced by the injection of wild-type cytoplasm, and, in this case, the site of injection determines the ventral side of the embryo. This very clear response indicated that Toll embryos lack any residual dorsoventral polarity [20]. Small amounts of cytoplasm were sufficient to produce a complete dorsoventral pattern, implying a vast excess of the Toll product in wild-type embryos. The rescue was spatially restricted to the site of injection, indicating that the Toll product had a limited ability to diffuse. Cytoplasm from both the dorsal and ventral sides of wild-type embryos was equally able to induce ventral structures when transplanted into Toll embryos. Thus, in wild-type embryos, Toll at the dorsal side was inhibited, and this inhibition depended on Toll being active at the ventral side. Kathryn, in discussions with our next-door neighbour, the renowned theoretical biologist Hans Meinhardt, speculated that Toll was a component of a patterning system explaining the formation of morphogen gradients from weakly polarized starting conditions. This was consistent with the Gierer-Meinhardt model, which is based on local activation by autocatalysis coupled to long-range inhibition [21], and predicted that a second activated site could not be induced in the same embryo. Siegfried Roth, a graduate student in my laboratory, later tested this by performing injections of wild-type cytoplasm into two sites of Toll embryos and found, to his and Meinhardt’s dismay, that a second Toll activation peak could be easily induced at any distance to the first peak [22], ruling out this elegant interpretation.
In a ‘tour de force’, Kathryn Anderson and Gerd Jürgens genetically analysed the properties of Toll [23] and provided remarkably accurate insights into how Toll functions in the complete absence of molecular information. Toll alleles showed various phenotypes, a ventralised dominant phenotype but lateralisated or dorsalised patterns in recessive alleles. It turned out that the first Toll alleles had already been isolated in 1973 in an unpublished screen by Tom Rice in the laboratory of Alan Garen at Yale (T. Rice, PhD thesis, Yale University, 1973), without recognition of these phenotypes. Thankfully, the mutants were still available, and Kathryn identified two recessive Toll alleles with lateralisated phenotypes among them together with alleles of easter, tube, and pelle. The dominant Toll phenotype is characterised by an expansion of ventral pattern elements at the expense of dorsal pattern elements (Figure 4). This pattern aberration is caused by a gain of function of the gene, as revertants of the dominant sterility, which are caused by a second mutation inactivating the gene, display the dorsalised recessive phenotype. Surprisingly, some dominant alleles in trans with a null allele displayed a dorsalised null phenotype, which means that the dominant phenotype required the presence of a wild-type gene product. Kathryn and Gerd predicted that the Toll protein should dimerise, a prediction that was later confirmed at the biochemical level. The dominance is not due to overproduction, as duplications carrying an extra copy of the Toll gene partially suppressed the phenotype. Kathryn and Gerd proposed that Toll exists in an active and an inactive state, with the dominant alleles producing constitutively activated product that does not require activation. It was likely that some of the dorsal-group genes were involved in activating Toll, whereas others acted downstream and were induced by activated Toll to produce the ventral and lateral pattern elements. To determine which of the dorsal-group genes was required for Toll activation, double mutants of a dominant Toll with loss-of-function alleles of the dorsal-group genes were constructed. Creating double mutants with a dominant female sterile is a challenge, and as many of the dorsal-group genes including Toll are located on the third chromosome, recombination had to be induced by X-rays in males (in Drosophila there is no recombination during male meiosis). It turned out that combinations of dominant Toll with loss-of-function nudel, pipe, gd, snake, and easter alleles produced some lateral pattern elements, whereas Tl;dorsal double mutants were completely dorsalised. This was interpreted in a genetic cascade model with Toll in the centre, requiring activation via the upstream genes nudel, pipe, gd, snake, and easter, whereas dorsal was placed downstream, absolutely required to produce ventral and lateral pattern elements. The genes spätzle, pelle, and tube could not be tested as no recombinants could be obtained because they are very closely located to Toll; in later experiments in the Anderson laboratory, based on transplanting transcripts of dominant alleles, spätzle was placed upstream and pelle and tube downstream of Toll between Toll and dorsal. In retrospect, all these genetic parameters pointed to Toll as a membrane-bound receptor, but, at the time, there was still no paradigm for signalling cascades, and the roles of the other dorsal-group genes could not yet be distinguished. Kathryn presented this work at a Gordon Conference in 1985. Subsequently, she accepted an offer for an assistant professorship at Berkeley to clone Toll and the other dorsal-group genes that could be rescued in her own laboratory. In my laboratory in Tübingen, we focused on the ventralised mutant cactus, which we generously obtained from the Princeton group [15], as well as nudel, pipe, and gd that could not be rescued.

The molecular analysis: Toll is a membrane-bound receptor
At this time the first Drosophila patterning genes had been cloned, among them the bithorax complex and the first segmentation genes, which, excitingly, turned out to encode transcription factors. The first dorsal-group gene cloned was snake [24]. During their project to clone the bithorax complex of homeotic genes, Pierre Spierer in Dave Hogness’s laboratory had created a genomic walk across the rosy-Ace-region of the third chromosome, where snake happened to map. snake homologs in vertebrates suggested that it was a member of a protease cascade
similar to blood clotting enzymes. A protease cascade might be involved in activating Toll by proteolytic cleavage. Excitingly, in 1987, *dorsal* was shown to encode a protein with homology to the avian oncogene v-rel [25], which later turned out to be a transcription factor with homology to NF-κB. Toll was cloned by the Anderson laboratory in 1988 [26].

The Anderson laboratory used a standard procedure for positional cloning of *Drosophila* genes by producing a *Toll* allele due to the insertion of a transposable P-element; in the case of *Toll* it was straightforward to screen for revertants of the dominant female sterility. The P-insertion site and adjacent sequences were cloned and used to screen for mRNAs. A long transcript of 5.3 kb was found to rescue the dorsalised phenotype of *Toll* alleles in the same polarity-inducing manner as cytoplasm from wild-type donor embryos. The amino acid sequence revealed a signal peptide as well as a membrane-spanning domain separating the transcript into an 803-amino acid extracellular domain and a 269-amino acid cytoplasmic domain. This suggested that Toll might be a receptor protein that was activated by a molecule present in the extracellular compartment surrounding the egg cell, the perivitelline space. At that time, only a limited repertoire of cloned genes, mostly from mammalian cell culture systems, was known. The leucine-rich sequences in the extracellular domain were similar to those in some membrane proteins, without much explanatory value. However, after this publication, a strong homology of the cytoplasmic domain of Toll with that of the interleukin receptor (IL-1R), later called TIR domain, which has an important role in immunity in mammals, was found [27]. In tissue culture cells, IL-1R activates nuclear uptake of the transcription factor NF-κB after its release from the inhibitor IκB [28], thus suggesting an interesting homology of the Toll-Dorsal and the IL-1R-NF-κB pathway.

**A gradient of Dorsal nuclear localisation determines the dorsoventral pattern**

In my laboratory, Siegfried Roth and Robert Geisler investigated the recessive ventralising *cactus* mutants, and *cactus* turned out, as expected, to encode the *Drosophila* homolog of IκB [29,30]. Double mutants of *cactus* with the dorsal-group genes indicated that none of them, with the exception of *dorsal* itself, abolishes the faculty in *cactus* mutants to produce lateral and ventral pattern elements, supporting the notion of *cactus* acting immediately upstream of *dorsal*, the latter encoding the morphogen. In the laboratory, Wolfgang Driever had just discovered the morphogen gradient of the transcription factor Bicoid, produced by the anteriorly localised mRNA that spreads posteriorly and determines the pattern in a concentration-dependent manner [31,32]. Although *dorsal* RNA is uniformly distributed in the egg, the first experiments using antibodies to detect the protein described a graded distribution of the Dorsal protein and hypothesized it to be due to translational control or localized protein degradation [33]. Subsequent, more refined experiments by Siegfried Roth and by Christine Rushlow in Michael Levine’s laboratory indicated instead that the Dorsal gradient arose through regulation of nuclear import [34–36]. Crucial for this conclusion was the observation that protein levels were identical on the dorsal and ventral sides of the embryo during mitosis, the gradient only becoming apparent as Dorsal entered ventral nuclei after each mitosis (Figure 5A). In mutants of any of the dorsal-group genes, Dorsal protein remains in the cytoplasm at all positions along the dorsoventral axis, whereas it is located in the nuclei in dominant *Toll* and *cactus* mutant embryos (Figure 5B,C). The central role for nuclear localization suggested an interesting parallel with the action of NF-κB/IκB. Siegfried Roth also demonstrated a strong correlation of the nuclear localisation of Dorsal and the expression of zygotic target genes *twist*, expressed at high levels of nuclear concentration, and *zen*, which seems to be repressed by Dorsal and is transcribed only at the low levels found on the dorsal side [34] (Figure 6B,C). These important findings nevertheless left a number of key questions unanswered. How to distinguish between the seven genes *nudel*, *pipe*, *gd*, windbeutel, *snake*, easter, and *spätzle* that act upstream of *Toll*? Which gene encodes the ligand? What is the role
of the downstream genes tube and pelle in transmitting the activated Toll to Cactus and the Dor-sal nuclear uptake gradient?

Spätzle, processed by Snake and Easter in the perivitelline fluid, is the Toll ligand
As the experiments of Roth had ruled out the formation of a Toll gradient by autocatalysis and lat-eral inhibition, the big question arose: where does the polarity-inducing signal come from in an egg cell surrounded by the dead vitelline membrane composed of extracellular matrix? An impor-tant cue came from the studies of another system involved in patterning the anterior–posterior ends of the Drosophila embryo. In my laboratory, Frank Sprenger and Leslie Stevens had just cloned the gene torso, the key gene of the system determining the terminal ends of the embryo. torso, for which, as for Toll, dominant gain-of-function alleles had been identified, turned out to encode a receptor tyrosine kinase (RTK) [37]. A gene with the same phenotype, torso-like, was

Figure 5. Dorsal antibody staining in cross sections of blastoderm-stage embryos, dorsal side up. (A) Wild-type. The Dorsal protein is distributed in a nuclear concentration gradient with a maximum at the ventral side. (B) pelle embryo. Dorsal is excluded from the nuclei all around the blastoderm. (C) Dominant Toll embryo. The protein is localized in the nuclei all around the blastoderm. Reproduced, with permission, from [34].

Figure 6. Antibody staining in cross sections of blastoderm-stage embryos, dorsal side up. (A) Cactus antibody in wild-type embryos. The Cactus protein is distributed in a concentration gradient with a maximum at the dorsal side, it is excluded from the nuclei. Reproduced, with permission, from [52]. (B) Dorsal antibody in wild-type embryos. The distribution of the Dorsal protein in the cytoplasm reflects that of the cactus protein, which binds the cytoplasmic Dorsal protein but is degraded ventrally. (C) Twist and Zen antibody. The transcription factor Twist is expressed at the ventral side at high nuclear Dorsal concentrations, and Zen is expressed at the dorsal side at low nuclear Dorsal concentrations. Reproduced, with permission, from [34].
shown to be required in the follicle cells, apparently involved in providing a ligand for the Torso RTK at the anterior and posterior egg pole [38]. David Stein and Siegfried Roth asked whether a signal, in analogy with the signal for Torso activation, could come from the follicle cells and become deposited into the vitelline membrane and the extracellular perivitelline fluid surrounding the egg cell. By creating mosaics in which the germ line is mutant and the somatic follicle cells are wild type (Figure 1) and vice versa, they discovered that nudel, pipe, and windbeutel are required in the soma in contrast to gd, easter, snake, and spätzle that must be expressed in the germ line. This suggests that the cue determining the dorsoventral asymmetry is produced in the follicle cells. To detect an activity (presumably the Toll ligand) that induced polarity in these mutants, they recovered perivitelline fluid from Toll−/− mutant embryos and injected this into the perivitelline fluid of pipe−/− mutant embryos (Figure 7A,B). They could locally restore a complete dorsoventral pattern, whereby the site of injection determined ventral (Figure 7D,E). The activity could only be detected in the perivitelline fluid of eggs that lack the Toll product. This was expected if the Toll ligand is produced in very limited quantities and normally sequestered by the extracellular domain of the Toll receptor protein but accumulates in the absence of Toll protein [39]. Perivitelline fluid taken from embryos that expressed Toll could rescue easter, snake, or spätzle embryos but not embryos lacking the pelle, tube, or dorsal products. In these cases, the orientation of the normalised pattern corresponded to the normal orientation as given by the curvature of the egg. Taken together, in addition to the presumed active Toll ligand, three separable activities present in the perivitelline fluid required for the production of the Toll ligand were identified, presumably the protein products

Figure 7. Transplantation of perivitelline fluid. (A) Recovery of a drop of perivitelline fluid from Toll−/− embryos at gastrulation by pricking the vitelline membrane. (B) Transplanting of fluorescent bovine serum albumin (BSA) into the perivitelline space at the dorsal side (arrow) 2 and 10 min after injection illustrates the rapid diffusion of proteins in the perivitelline space. (C) Wild-type larva, dorsal side up, anterior left. (D) Uninjected pipe embryo (E) injected with perivitelline fluid from Toll−/− embryos at the dorsal side develops a larva with reversed polarity, with the denticle belts at the dorsal side. Reproduced, with permission, from [39].
of easter, snake, and spätzle [40]. easter had been cloned and shown to encode a secreted serine protease [41], as does snake and gastrulation defective [24,42], suggesting that these were involved in a serine protease cascade processing the Toll ligand. In the Anderson laboratory, the spätzle gene was isolated from a P-element-induced allele and shown to encode a novel protein that appears to occur in several precursor forms, the active form being a 23-kD peptide with no apparent homologies to any known ligands [43]. The active Spätzle peptide and its precursors could indeed be purified from the perivitelline fluid of Toll− embryos, demonstrating its role as a Toll ligand [44]. All three proteins Snake, Easter, and Spätzle, apparently are produced in the early embryo from maternal mRNA and secreted in the perivitelline fluid, where they can freely diffuse. Processing of the Spätzle precursor, which is present at much lower levels than the Snake and Easter serine proteases, occurs at the ventral side of the egg by the Easter protein [41]. This site is determined by the restricted expression of pipe, a sulfotransferase that transfers sulfate to several vitelline membrane proteins in the ventral follicle cells [45,46]. The ventral expression of pipe is established by another signalling cascade acting during oogenesis via the epidermal growth factor receptor [47]. Activation of Toll is passed on via a complex of Tube and Pelle, with Tube acting upstream of Pelle [48]. Pelle, a serine-threonine kinase [49], induces the degradation of Cactus and the release of Dorsal, now free to enter the nucleus [48,50,51]. Cactus protein is distributed in a gradient with a maximum at the dorsal side, inverse to that of nuclear Dorsal [52] (Figure 6A). The nuclear concentration gradient of the transcription factor Dorsal with a maximum at the ventral side determines the dorsoventral pattern, consistent with its proposed role as a morphogen (Table 1).

Dual function of Toll in dorsoventral patterning and innate immunity

Indications that Toll is a gene with additional functions in the larva were reported by the Anderson laboratory already in 1988. Strong loss-of-function alleles are zygotic semilethal without significant morphological defects. But the Toll transcript is detected in various regions of the embryo. These late transcripts were shown to be of zygotic rather than maternal origin, but when isolated as polyA+ RNA and injected into mutant embryos, they rescued the maternal phenotype [53]. This means that the same Toll product used maternally to establish the dorsoventral axis also has functions later in the embryo itself. A first hint that the homology of the Toll-Dorsal and the IL-1R-NF-κB pathway was based on an involvement of the Toll-Dorsal pathway in innate immunity in Drosophila was the detection of the presence of κB-type binding sites within the transcriptional regulatory sequences of genes encoding antimicrobial peptides in Drosophila [54,55]. In the laboratory of Michael Levine, a Dorsal homolog, Dorsal-related immunity factor (Dif), was identified. Dif is predominantly expressed in the fat body, the functional equivalent of the mammalian liver [56]. Upon microbial infection, the Dif protein is translocated into the nuclei, in analogy to NF-κB. Significantly, Dif is located in the nuclei in uninfected dominant Toll-mutant larvae, indicating that its nuclear location is mediated by the Toll pathway [56]. Bruno Lemaître in the laboratory of Jules Hofmann studied the involvement of the Toll-Dorsal pathway genes in the response of adult flies upon microbial infection. They monitored the expression of the antifungal peptide drosomycin and discovered that the signalling chain, including spätzle, Toll, tube, pelle, and cactus, is active in innate immunity in Drosophila [57,58]. These genes all are semilethal, indicating that they are required in the zygote as well as maternally. dorsal, which is fully viable and required mainly maternally, is largely replaced by Dif in the immune response. The homology of Toll, the interleukin receptor, and NF-κB/κB with Dorsal/Dif and Cactus suggested that the ancient function of the pathway might have been in innate immunity, conserved between insects and vertebrates.

In 1997, the first mammalian Toll-like receptor (TLR) was cloned and found to be involved in signalling in the adaptive immune system [59]. By human expressed sequence tag (EST) library screening, more TLRs were found, including a total of 10 in humans and 12 in mice. The
recognition of the key role of TLRs in innate immunity in mammals was founded on a mouse mutant strain deficient in innate immunity. This strain is resistant to a strong endotoxin, such as lipopolysaccharide (LPS). A single locus, lps, was positionally cloned in the Beutler laboratory and found to encode Tlr4, which was shown to physically interact with LPS [60,61]. Subsequently, knockout mutations in other TLR genes showed that each was responsible for a subset of microbial agents, thereby covering almost the entire spectrum of innate immunity. The TLRs in mammals function as pattern recognition receptors, whereas in Drosophila immunity, activation via the processing of the Spätzle ligand seems to prevail [62]. To Kathryn’s dismay, the Toll pathway in mammalian systems does not appear to have a role in embryonic development.

Nine Toll homologs have been identified in Drosophila, but most of the pathogen defence relies on the original Toll-1. In a large-scale screen in germ line clones in my laboratory, a small number of genes participating in the Toll-Dorsal pathway, which have been missed in previous screens, were identified [63]. Among them was krapfen, which turned out to be the dMyd88 gene, encoding a protein with a TIR domain and serving as an adaptor for the Toll receptor, upstream of the Tube-Pelle complex [64]. dMyd88 is the only gene in the pathway that has been discovered first in the mammalian system, but also tube and pelle homologs have been identified independently in the innate immunity pathway as IRAK4 and IRAK1, respectively [65–67].

Table 1. The Toll-Dorsal pathway

| Location of protein | Protein | Protein type |
|--------------------|---------|--------------|
| Follicle cells     | WINDBEUTEL [15] | Chaperone for pipe [76] |
| Ventral follicle cells, localised | PIPE [14] | Sulfotransferase [46] |
| Follicle cells     | NUDEL [14] | Secreted serine protease, extracellular matrix protein [77] |
| Ventral perivitelline space, localised | GASTRULATION-DEFECTIVE [16] | Secreted serine protease [42] |
| Perivitelline space | SNAKE [14] | Secreted serine protease [24] |
| Perivitelline space, ventral→dorsal gradient | EASTER [11,14] | Secreted serine protease [41] |
| Perivitelline space, ventral→dorsal gradient | SPÄTZLE [14] | Secreted growth factor [43] |
| Egg cell membrane, ventral→dorsal gradient of activated Toll | TOLL [11,14] | Transmembrane receptor [26] |
| Egg cell cytoplasm | WECKLE [83] | Adapter [78] |
| Egg cell cytoplasm | MYD88/KRAPFEN [63] | Adapter [64] |
| Egg cell cytoplasm | TUBE [14] | Adapter [79] |
| Egg cell cytoplasm | PELLE [14] | Serine/threonine kinase [49] |
| Egg cell cytoplasm, dorsal→ventral gradient | CACTUS [11,15] | Inhibitor, binds Dorsal, homolog to IκB [29] |
| Blastoderm nuclei, ventral→dorsal gradient | DORSAL [8] | Transcription factor, homolog to NF-κB [25] |

*Left column: distribution of active components in the compartments of the follicle and egg. Middle column: dorsal-group genes starting the signalling cascade with the localized expression of the Pipe protein in the ventral follicle cells to the Dorsal nuclear concentration gradient determining the dorsoventral pattern at the blastula stage. The references refer to the discovery of the gene. The genes discovered in the Nüsslein-Volhard laboratory are named after long or hollow objects with the exception of easter (discovered on Easter). Spätzle is a pasta specialty in southern Germany, Windbeutel, Weckle, and Krapfen are variations of a bun, and Pelle is a peel. The original dorsal was unfortunately named after its dorsalised phenotype, which often gives rise to confusion. Right column: biochemistry of the proteins encoded by the dorsal-group genes. The references refer to the isolation and sequencing of the gene.
In recent years, the *Drosophila* Toll genes were increasingly found to play multiple roles in other developmental processes [68]. Strikingly, in several of those, the Toll proteins function as cell adhesion molecules and provide heterophilic cell contacts. An interesting example is their surprising involvement in germ band elongation by convergent extension in *Drosophila*, which is conserved in diverse arthropods [69,70]. In this system, three different Toll proteins interact directly via their extracellular leucine-rich domains to induce the intercalation between cells by changing the actin–myosin skeleton. They are expressed in segmental or pair-rule stripes under the control of the segmentation genes *even-skipped* and *runt*, genes that were discovered in the same screen that led to the identification of Toll; in this manner, the connection to these genes brings the story of Toll back to its origin, the screen for segmentation genes [11].

Kathryn Anderson had already argued in 1996 that the involvement of Toll-like genes in insect and vertebrate innate immunity as well as in plant disease resistance made it likely that Toll genes arose before the divergence of plants and animals, serving as an ancient defence system against pathogens in multicellular organisms [71]. How the pathway has been adopted for dorsoventral patterning in *Drosophila* is still a mystery. This process is not highly conserved even among arthropods [72]. However, the antimicrobial defence mechanism, including the Toll pathway, is particularly active in the serosa, the extraembryonic membrane protecting the growing embryo in most insects [73,74]. From there, it might have found its way into dorsoventral egg patterning (S. Roth, personal communication).

For a comprehensive review on the *Drosophila* Toll-Dorsal pathway see Stein and Stevens, 2014 [75].

**Concluding remarks**

The Toll story, starting with the inquisitiveness on how the fly embryo develops, helped tremendously to unravel the pathways by which humans defend themselves against pathogens. It is a striking example of the rule that curiosity-driven research into fundamental biological phenomena does lead to novel insights that not only increase our understanding about life but also will often be of value for human health.

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**Declaration of interests**

No interests are declared.

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