Developmental Biology 335 (2009) 276–287

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Tailless patterning functions are conserved in the honeybee even in the absence of Torso signaling

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ARTICLE INFO

Article history:
Received for publication 2 July 2009
Revised 21 August 2009
Accepted 1 September 2009
Available online 6 September 2009

Keywords:
Honeybee
Tailless
Terminal patterning
Evolution
Development
Torso

ABSTRACT

In *Drosophila*, the maternal Torso terminal signaling pathway activates expression of the gene *tailless* (*tll*), which is required for the patterning of anterior and posterior termini. We cloned the honeybee orthologue of *tll* (*Am-tll*) and found that embryonic expression of *Am-tll* resembles that of *Drosophila*, with expression in triangular anterior dorsal–lateral domains and a posterior cap. Functional studies revealed that *Am-tll* has an essential role in patterning the posterior terminal segments and the brain, similar to the activity of *tll* in other insects. As the honeybee genome lacks many of the components of the Torso pathway required for terminal patterning, we investigated the regulation of honeybee *tailless* (*Am-tll*). *Am-tll* is expressed maternally and, in the honeybee ovary, *Am-tll* mRNA becomes localized to the dorsal side of the oocyte, a process requiring the actin cytoskeleton. This RNA becomes redistributed in early embryos to a posterior domain. We also show that the activation of the anterior domain of *Am-tll* is dependent on honeybee *orthodenticle-1*. Together these findings indicate major differences in post-transcriptional regulation of *tailless* in the honeybee compared to other insects but that this regulation leads to a conserved expression pattern. These results provide an example of an early event in development evolving and yet still producing a conserved output for the rest of development to build upon.

Introduction

The *Drosophila tailless* gene (*tll*) encodes an orphan nuclear receptor that functions in the development of anterior and posterior structures (Pignoni et al., 1990; Strecker et al., 1988). *Tll*, and its close relative *dissatisfaction* (Finley et al., 1998), are conserved members of a group of nuclear receptors, including *nhr-67* in *C. elegans* (Schocken et al., 2008), which acts in uterus and tail development, and *Tlx* genes in vertebrates with roles in stem cell maintenance and brain development (Monaghan et al., 1995; Yu et al., 1994). *Tll*-like genes have been found in the genomes of coral (Grasso et al., 2001) and sea anemones (Putnam et al., 2007), indicating a long evolutionary history.

In *Drosophila*, *tll* is a key gene in regionalizing the early embryo, acting to repress abdominal development and to promote terminal fate (Pignoni et al., 1990; Pignoni et al., 1992; Steingrimsson et al., 1991). In later development *tll* also plays roles in patterning the brain and the hindgut (Daniel et al., 1999; Diaz et al., 1996; Kurusu et al., 2009; Younossi-Hartenstein et al., 1997). The role of *tll* in the early embryo is as one of the key effectors of the terminal patterning pathway. Terminal patterning is controlled by the Torso receptor tyrosine kinase (Klingler et al., 1988), which is activated by a C-terminal fragment of a protein encoded by the *trunk* gene (Casali and Casanova, 2001). Localized digestion of trunk into the active form at the termini of the oocyte appears to be the key triggering event of the terminal pathway, and while the mechanism of this activation is unclear, a perforin-like protein, Torso-like, is thought to play a significant role. Stimulation of the Torso receptor leads to activation of the MAPK signaling cascade and phosphorylation of target proteins leading to the derepression of *tll* and *huckebein* (another effector of terminal patterning) at the poles of the embryo (Chen et al., 2009).

Repression of *tll* expression occurs through an 11-bp element, the torso response element (tor-RE), that is normally bound by a GAGA factor encoded by the *trithorax* gene, a zinc finger protein named Tramtrack69 (Ttk69) and heatshock factor (hsp), leading to repression of *tll* expression (Chen et al., 2009; Chen et al., 2002). *Tll* expression also appears to be regulated by capicua, a repressive transcription factor phosphorylated and inactivated by MAPK (Cinnamon et al., 2004; Jimenez et al., 2000; Paroush et al., 1997) Recent evidence implies that hsp and Ttk69 are also phosphorylated by MAPK lifting repression of *tll* expression (Chen et al., 2009). The *Drosophila* terminal patterning system thus leads to restricted expression of *tll* in caps at both poles of the embryo. At the posterior this expression domain is also under the influence of posterior genes such as *nanos* and *oskar* (Cinnamon et al., 2004).

Tll appears to function as a constitutive repressor of target genes involved in patterning including *knirps* and *krüppel*, thus acting to define the developing acron and telson (Moran and Jimenez, 2006).

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doi:10.1016/j.ydbio.2009.09.002

Tll interacts with brakeless, a co-repressor protein, which is required for this repressive activity (Haecker et al., 2007). After its activation, anterior tll expression becomes restricted to two dorsal–lateral domains and expression is maintained throughout embryonic brain development where it helps shape the forming brain by modifying the proliferation of neuroblasts (Diaz et al., 1996; Kurusu et al., 2009). Posterior expression occurs in a cap that includes the regions where the A8 segment, telson and posterior gut arise (Pignoni et al., 1990).

Tll is thus a key regulator defining both anterior and posterior in *Drosophila* development. The terminal system that is required to activate *tll* expression in *Drosophila* is, however, not conserved in some insects (Dearden et al., 2006), raising the question of whether this gene still has a terminal patterning role in those insects, and if so, how it is activated.

Embryonic expression patterns for *tll* orthologues have been investigated in the Dipterans, *Anopheles* and *Musca*; a long germ Hymenopteran, *Nasonia*; and the short germ band insect, *Tribolium*. In all cases, *tll* is expressed early in a posterior cap domain and later in embryogenesis in an anterior dorsal–lateral expression domain, correlating with the position of the developing insect brain (Goltsev et al., 2004; Lynch et al., 2006b; Schroder et al., 2000). Only in Diptera (*Anopheles, Musca* and *Drosophila*) is *tll* expressed at the anterior terminal pole in early embryogenesis.

Loss of function studies for *tll* have only been carried out in two long germ band insects, *Drosophila* and *Nasonia*. *Drosophila tll* mutants lack posterior structures, including A8, telson and posterior gut and display abnormal development of anterior features, namely labrum, optic and procephalic lobes (Strecker et al., 1986). In the parasitic wasp, *Nasonia*, RNAi knockdown results in the loss of up to five posterior segments but anterior structures appear normal (Lynch et al., 2006b). In the short germ band insect *Tribolium castaneum*, a *Tc-tll* RNAi phenotype has not been reported but posterior *Tc-tll* expression is lost in *Tc-Torso* RNAi embryos (Schoppmeier and Schroder, 2005) and the posterior growth zone, which develops from the posterior terminal of the blastoderm embryo, fails to develop. This indicates that *Tc-tll* may play a role in axis elongation from the posterior growth zone. These functional studies in insects implies that *tll* has a broadly conserved role in posterior patterning in insects, a role that is not conserved in vertebrates.

RNAi against *Tribolium Torso* also reveals that *Tc-tll* posterior expression is, like *Drosophila*, regulated by a Torso-signaling cascade in this short germ band insect (Schoppmeier and Schroder, 2005). Additionally, an upstream region of the *Tc-tll* promoter can drive reporter expression at the terminal poles of the embryo in *Drosophila*, indicating conservation of the regulatory elements in the terminal pathway between *Tribolium* and *Drosophila* (Schroder et al., 2000). In *Anopheles*, activated MAPK protein can be detected at the terminal poles of *Anopheles* embryos implying that Torso signaling is also active in this Dipteran and may regulate posterior terminal expression of *Anopheles tailless* (Goltsev et al., 2004). In *Musca*, expression of *tll* is similar to *Drosophila* (Sommer and Tautz, 1991), and the regulatory sequences of the gene, despite being diverged in sequence, are regulated appropriately when placed into *Drosophila*, indicating that *Musca tll* is also under the control of Torso signaling (Wratten et al., 2006).

Annotation of the honeybee genome, however, revealed that honeybee orthologues for *Torso* and *trunk* are missing, indicating that the termini are patterned through a different mechanism in the honeybee (Dearden et al., 2006; Honeybee Genome Sequencing Consortium, 2006). The honeybee *Apis mellifera* is a long germ band insect whose ancestors separated from the lineage leading to *Drosophila* about 300 million years ago (Honeybee Genome Sequencing Consortium, 2006). Recent phylogenetic and phylogenomic evidence suggests that the hymenoptera, including the honeybee, are the most basal branch of the holometabolous insects (Krauss et al., 2008; Savard et al., 2006; Zdobnov and Bork, 2007), though some controversy still exists (reviewed in Beutel and Pohl, (2006)). Here we examine the expression and function of the honeybee orthologue of *Drosophila tailless*. We find that the embryonic expression pattern of honeybee tailless *(Am-tll)* is similar to that of other insects and RNAi knockdown studies support a conserved role in anterior and posterior patterning for *tailless*. In honeybees *Am-tll* is expressed maternally, unlike other insects, and analysis of the *Am-tll* transcript suggests that post-transcriptional regulation may play an important role in *Am-tll* regulation.

Materials and methods

Cloning of A. mellifera tll and otd-1

Total RNA was extracted from honeybee embryos using the RNeasy Mini Kit (QIAGEN) and used as a template for cDNA synthesis by reverse transcription. An *Am-tll* fragment was amplified using the following oligonucleotides: 5′GGCAGCTGCGTGGTGGACAAGACG and 3′CGTCTTGTCCACCACGCAGCTGCC and cloned into the pGEM-T Easy vector (Promega) for generation of RNA *in situ* hybridization probes. *Am-otd1* was amplified from cDNA using the oligonucleotides 5′ GACCATCGGATATCCATTGTGT and 3′AGCAGGGCGAACTACTAACTC and cloned into pGEM-T Easy vector for the production of DIG-labeled RNA probes for *in situ* hybridization and pLitmus38i for the production of double stranded RNA.

Rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA amplification kit (BD Biosciences) to identify the 5′ and 3′ ends of the *Am-tll* transcript. The sequence of the oligonucleotide primer for 5′ RACE was 3′GCACGCCCTACACTGGT-TACGATGA 5′ and for 3′RACE, 5′CAGCAGGAGCAGCAACGGATCTACG 3′. RACE amplification products were cloned into pGEM-T Easy for sequencing. To clone the full-length transcript of *Am-tll*, the following oligonucleotides were used: 5′TCCACAGATGGTCAACGA3′ and 3′ GTTTTCATGTTCTTTAATTTTGTCCTA5′.

Phylogenetics

Multiple sequence alignment of proteins homologous to tll was carried out in ClustalX (Supplementary data) and analyzed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under the WAG model (Whelan and Goldman, 2001) with default settings. The WAG model was used as it was found to be most appropriate after initial experiments using mixed models. The Monte Carlo Markov Chain search was performed over 1000000 generations with trees sampled every 1000 generations, with the initial 25% of the trees discarded as "burnin." Resulting phylogenies were displayed using Dendroscope (Huson et al., 2007).

In situ hybridization and immunohistochemistry on honeybee embryos

RNA probe synthesis, *in situ* hybridization and immunohistochemistry on honeybee ovaries and embryos were carried as described previously (Dearden et al., 2009; Osborne and Dearden, 2005). Activated ERK/MAPK was detected using a monoclonal antibody raised against the activated di-phosphorylated form of ERK (Sigma M8159) at a dilution of 1 in 100.

RNAi-mediated gene knockdown in honeybee embryos

Honeybee queens were provided with Eziqueen frames (Eziqueen Systems LTD, New Zealand, http://www.eziqueen.co.nz/) in which to lay eggs. These enable removal of the eggs from the comb on plastic strips without disturbance or damage to the eggs. Plastic strips were secured to a Petri dish using Blu-Tac (Bostik) and individual eggs injected in air with double stranded RNA (dsRNA). To generate dsRNA for honeybee *tll, otd*-1 and *EGFP* (enhanced green fluorescent protein;

control), cDNA for each were subcloned into the pLitmus38i (NEB) vector, which has two T7 promoters flanking the inserted sequence. dsRNA was synthesized by *in vitro* transcription using the MEGAscript RNAi kit (Ambion) using T7 RNA polymerase, and resuspended in H_2O at 2 μg/μl. Between 200 and 400 embryos were injected with dsRNA for each RNAi experiment and then incubated at 35 °C at 80% humidity for 70 h (until hatching) or photographed at 65 h (stage 9 of development) under paraffin oil. Over half of the injected embryos die before hatching from dehydration or infection (Dearden et al., 2009).

Transgenic Drosophila generation and embryo analysis

A DNA fragment containing the 5′ UTR along with the ORF of *Am-tll* was amplified and cloned into the pUASt vector (Brand and Perrimon, 1993). A full-length clone for *Drosophila tll* was amplified using the following oligonucleotide primers: 5′GCGGAATTCCTCAGC-GAGTCCACATCG3′ and 5′GCGCTCGAGGGCTCGACTCCTGGATATGA3′. *Dm-tll* cDNA was subcloned into pUASt. P-element-mediated germ line transformation was performed as described per Rubin and Spradling (Rubin and Spradling, 1982)(1982), by injecting pUASt-*Am-tll* into *w118* embryos and selecting for red-eyed flies in the F1 generation. pUASt-*Dm-tll Drosophila* lines were generated by GenetiVision (http://www.genetivision.com/). UAS-*Am-tll* or UAS-*Dm-tll* fly lines were then crossed to *w*[*]; *P*{*w*[+mC]=GAL4-Hsp70.PB}2 line (Bloomington Drosophila Stock Center) to generate lines for heat-shock (hs) analysis. Embryos from the resulting hs-lines were collected on apple juice plates at 25 °C after 1 or 3 h and then heat-shocked by immersion in a 37 °C water bath. Embryos were either allowed to develop for a further hour before collection for antibody staining, or left for 24 h to collect for cuticle preparation. Cuticle preparations were prepared by mounting dechorionated embryos in Hoyer's medium using established methods (Stern and Sucena, 2000) and photographed under darkfield or brightfield microscopy. Immunohistochemistry was performed using the following antibodies: anti-Knirps (used at a dilution of 1 in 200; (Kosman et al., 1998)), anti-Krüppel (1 in 200; (Kosman et al., 1998)) or anti-Even-skipped (1 in 10 (Patel et al., 1994)). Secondary antibodies were diluted 1 in 300 and detected by 3,3-diaminobenzidine tetrahydrochloride (DAB) staining (Patel, 1994).

Ovary culture and treatment

Queen honeybee ovaries were dissected into culture media (Schneider's Insect media with 20% fetal bovine serum, penicillin (60 units/ml) and streptomycin (60 μg/ml)). Ovaries were cultured in 500 μl of media supplemented with either 1 μg/ml of cytochalasin D (Sigma), 100 μg/ml colchicine or equivalent volume of ethanol (control ovaries). After 2 h of culture at 35 °C, ovaries were fixed and prepared for *in situ* hybridization as described previously (Dearden et al., 2009; Osborne and Dearden, 2005).

Results

Isolation of A. mellifera tailless

Honeybee *tll* was identified by a tBlastN (Altschul and Lipman, 1990) search of the honeybee genome using the tll protein sequence from *Drosophila*. Bayesian phylogenetic analysis (Ronquist and Huelsenbeck, 2003) indicates that Am-tll is closely related to other insect tailless proteins and clusters with them against both vertebrate tll proteins and insect dissatisfaction-type proteins (Fig. 1A). Tll proteins contain a zinc finger DNA-binding domain (located at the N-terminus) and a ligand-binding domain at the C-terminus. The *Am-tll* coding sequence encodes a protein with a partial zinc-finger DNA-binding domain and a ligand-binding domain, whose sequence is similar to other tailless orthologues (Supplementary Figure 1). Both *Nasonia* and Honeybee Tll protein sequences are unusually divergent to other insect tll proteins, with long branch lengths separating them and other tll proteins from holo- and hemi-metabolous insects (Fig. 1A). Much of this divergence is in the C-terminus of the protein (Supplementary Figure 1).

To determine the 5′ and 3′ end sequences of the *Am-tll* transcript and map the transcriptional start site, RACE (rapid amplification of cDNA ends, (Wang and Young, 2003)) was carried out. A full-length *Am-tll* mRNA clone was isolated by RT-PCR and the predicted gene structure (depicted in Fig. 1B) was confirmed by sequencing. 5′ RACE revealed that the *Am-tll* transcript has a long (1091 bp) 5′ untranslated region (UTR) with thirteen potential initiating AUG codons before the first in-frame AUG codon. In comparison the *Drosophila melanogaster* is 232 bp long. UTR length has not been examined in other species. Analysis using UTRscan (http://www.ba.itb.cnr.it/UTR/) (Pesole and Liuni, 1999) indicated the presence of a putative internal ribosome entry site (IRES) at the 3′ end of the 5′ UTR, immediately before the in-frame ATG (Fig. 1B). IRESs allow internal initiation of translation despite the presence of upstream secondary structure and multiple AUG codons, such as in the case here. They have been identified in some viral and eukaryotic mRNAs (Komar and Hatzoglou, 2005) and may play a role in developmentally regulating translation (Ye et al., 1997). Additionally, *Am-tll* has a larger 3′ UTR (688 bp) than that in *D. melanogaster* (347 bp), which may be important for mRNA localization or regulation by microRNAs. A number of mRNAs contain mRNA localization signals in their 3′UTR (Brunel and Ehresmann, 2004; Irion and St Johnston, 2007; Russo et al., 2006). No sequences similar to the tor-REs (Liaw et al., 1995) were found immediately upstream of the *Am-tll* transcriptional start site, suggesting that *Am-tll* expression may not be regulated by the torso signaling mechanism found in *Drosophila, Musca* and *Anopheles*.

Expression of Am-tll in honeybee ovaries and embryos

To determine if *Am-tll* acts to pattern terminal regions of the honeybee embryo, we examined its expression pattern in honeybee queen ovaries and during embryogenesis. In queen ovaries, *Am-tll* mRNA was detected in the posterior nurse cells (those that lie adjacent to the anterior of the oocyte) and in the developing oocyte. Initially *Am-tll* RNA is distributed throughout the cytoplasm of immature oocytes in the vitellarium (Fig. 2A) but quickly becomes localized to the dorsal side of the oocyte and is associated with the oocyte nucleus in more mature oocytes (Figs. 2A and B). Mid-oogenesis, *Am-tll* RNA remains localized to the dorsal side of the oocyte, across the entire length of the developing egg (Fig. 2C). Just prior to egg laying, dorsally localized *Am-tll* RNA becomes enriched towards the posterior end of the oocyte and appears to be lost from the anterior end (Fig. 2D). This pattern of maternal localization of *Am-tll* RNA is unexpected as *tll* expression in the ovary has not been detected in other insects (Lynch et al., 2006b; Pignoni et al., 1990). The unusual RNA localization pattern implies that the RNA is transported and tethered in the oocyte.

In newly laid eggs (pre-blastoderm, stage 1, Staging from (DuPraw, 1967)), maternal *Am-tll* mRNA can still be detected along the dorsal side of the embryo, enriched towards the posterior end of the embryo (Fig. 2E). As energids divide, rise through the yolk and populate the egg in anterior to posterior sequence, maternal *Am-tll* RNA is transported towards the posterior pole in association with nuclei that migrate posteriorly after arriving at the egg surface (Fig. 2F). *Am-tll* RNA is then located at the posterior terminus of the embryo (Fig. 2G) either through RNA transport, or de-novo synthesis. Following cellularization, *Am-tll* RNA expression is present in an anterior–dorsal stripe of cells and in cells around the posterior cap (Fig. 2H). At gastrulation (stage 6, 32–36 h after laying) the anterior domain is split into two stripes of cells along the dorsal midline (Figs. 2I to K) and *Am-tll* expression at the posterior pole ceases. Anterior expression is eventually limited to sets of anterior cells associated

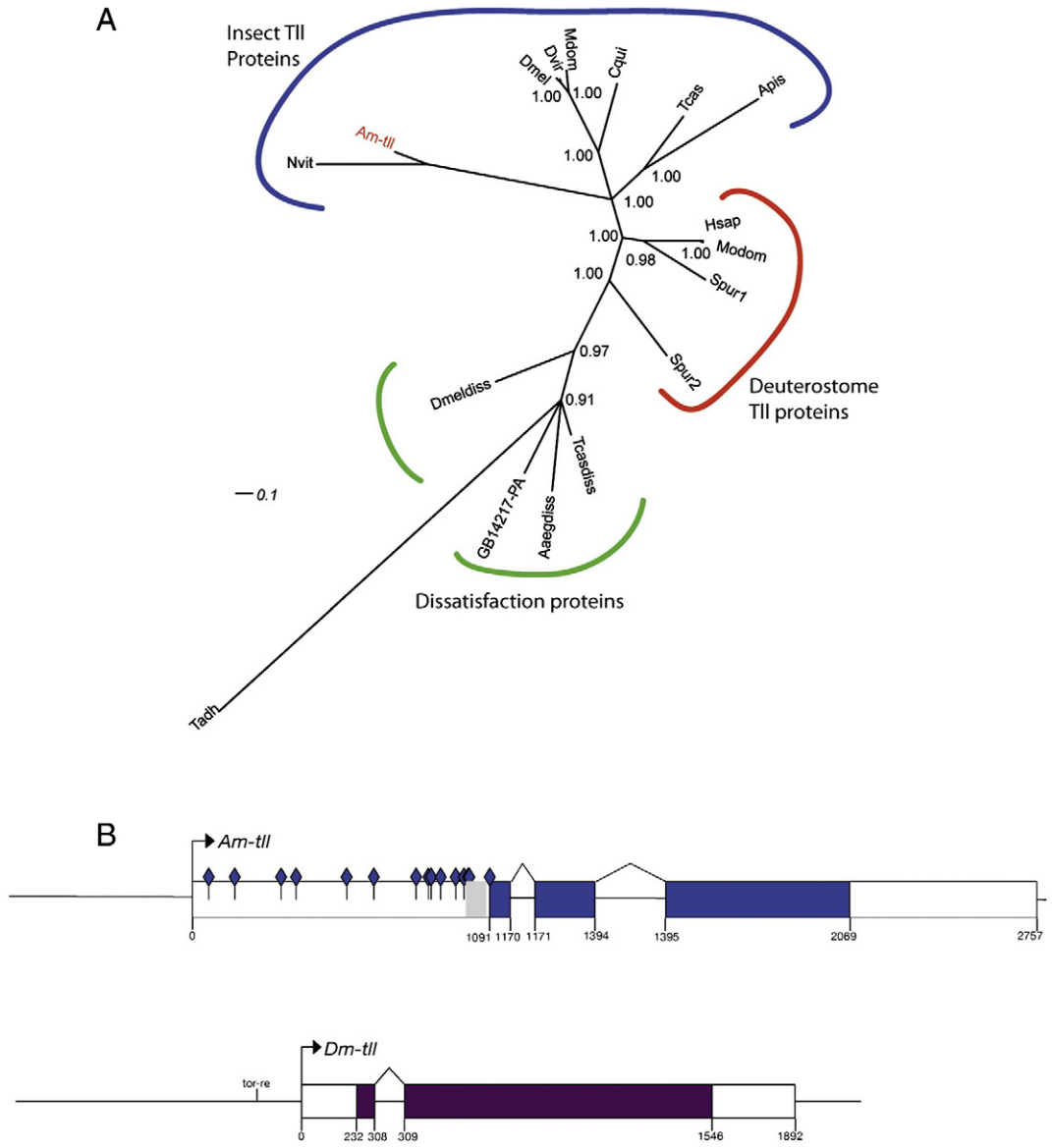

Fig. 1. (A) Unrooted phylogram drawn from Bayesian phylogenetic analysis of tll protein sequences. Numbers at tree nodes are posterior probabilities. Am-tll clusters with other insect tll proteins to the exclusion of deuterostome tll and dissatisfaction proteins. Abbreviations are Nvit (*Nasonia vitripennis*), Am-tll (Honeybee tailless), Dmel (*Drosophila melanogaster*), Dvir (*Drosophila virilis*), Mdom (*Musca domestica*), Cqui (*Culex quinquefasciatus*), Tcas (*Tribolium castaneum*), Apis (*Acyrthosiphon pisum*), Hsap (*Homo sapiens*), Modom (*Monodelphis domestica*), Spur1 and Spur2 (*Strongylocentrotus purpuratus* tll proteins 1 and 2), Tcasdiss (*Tribolium castaneum* dissatisfaction), Aaegdiss (*Aedes egyptii* dissatisfaction), GB14217-PA (Honeybee gene identifier for dissatisfaction), Tadh (*Tricoplax adherans* tll-like protein), Dmeldiss (*Drosophila melanogaster* dissatisfaction). (B) Structure of the *Am-tll* and *Dm-tll* genomic regions. Blue boxes represent coding sequence, clear boxes UTRs. AUG codons are labelled, with the initiator codon in red. Shaded box is location of predicted internal ribosome entry site (IRES).

with patterning of the brain primordium. Late in embryogenesis (stage 9), *Am-tll* RNA expression is maintained in the neuroblasts of the procephalic lobes (Figs. 2L and M).

Absence of Torso signaling in honeybee terminal patterning

Sequence analysis of the honeybee genome found that the honeybee lacks orthologues of the *Drosophila* proteins Trunk and Torso (Dearden et al., 2006), implying that *tll* expression and terminal patterning of the honeybee embryo is not controlled through the Torso signaling pathway as described from *Drosophila*. To further investigate this, we used an antibody raised specifically against the activated form of ERK (dpERK), the MAP kinase activated in response to Torso signaling, to detect activation of this pathway in honeybee embryos. No dpERK activation was detected in early stage honeybee embryos (Fig. 3A) but activated ERK could be detected at later stages during gastrulation along the ventral midline (Fig. 3B). In *Drosophila*, dpERK can be detected at the terminal ends of the early embryo (Fig. 3C) where Torso signaling is active. This confirms that while MAPK signaling is active in the honeybee, it is not associated with terminal pattering, further demonstrating the absence of the *Drosophila* type terminal signaling pathway in honeybee.

Maternal Am-tll mRNA localization requires the actin cytoskeleton

Localization and mobilization of RNA transcripts in insect oocytes often occurs by means of anchoring and movement along cytoskeletal networks (Steinhauer and Kalderon, 2006). Maternal RNAs in *Drosophila* are localized via polarized microtubule networks and anchored by actin filaments (Kloc and Etkin, 2005). In *Nasonia*, both mechanisms have been shown to be involved in maternal RNA localization; microtubules control anterior localization of at least two mRNAs,

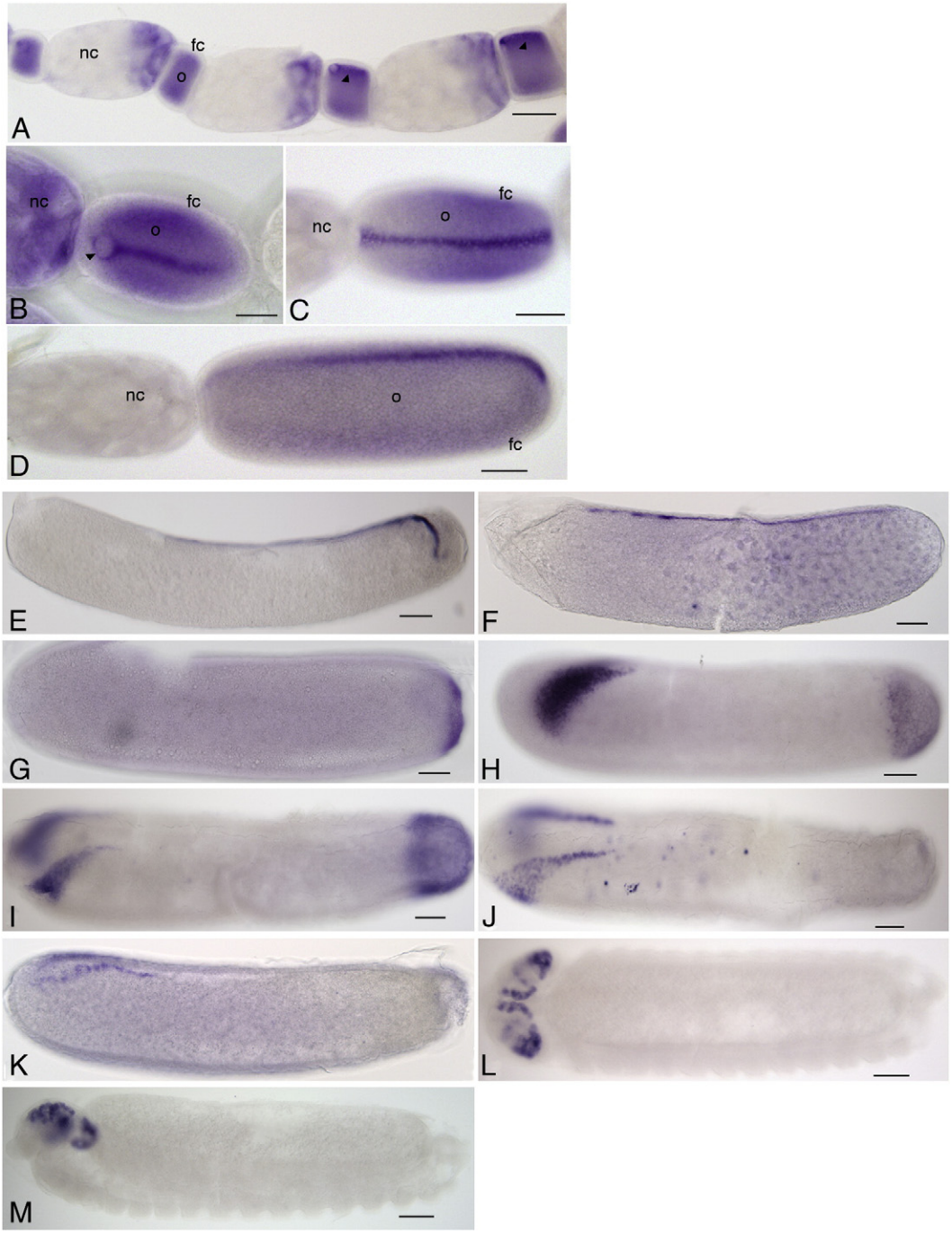

Fig. 2. A developmental expression series of *Am-tll* RNA expression through honeybee oogenesis and embryogenesis as detected by *in situ* hybridisation. *Am-tll* RNA is stained in blue. Scale bars represent 100 μm. All ovarioles and embryos are displayed with anterior to the left. (A) The early vitellarium of a single honeybee ovariole stained for *Am-tll* expression. Early in oogenesis, *Am-tll* mRNA is expressed by the posterior nurse cells and transported into the neighboring oocyte. No staining is detected in the follicle cells that surround the oocyte, or in anterior nurse cells. Expression in the oocyte is initially broadly distributed but as ooctyes become older (to the right in the image), *Am-tll* RNA becomes localized (arrowheads) to the dorsal surface of the oocyte (oriented towards the top of this image) associated with the oocyte nucleus. (B) Dorsal view of an early stage oocyte; localized *Am-tll* RNA surrounds the oocyte nucleus (arrowhead) and spreads along the dorsal surface towards the posterior of the oocyte. (C) Dorsal view of a mid-stage oocyte *Am-tll* RNA is localized in a stripe along the dorsal surface. As the oocyte matures expression in the posterior nurse cells ceases. (D) Lateral view of an oocyte and nurse cells in late oogenesis. At this time dorsally localized *Am-tll* RNA has been lost at the anterior dorsal end of the oocyte and becomes enriched towards the dorsal–posterior end. No expression of *Am-tll* RNA can be seen in the nurse cells. (E) Lateral view of a newly-laid embryo. Maternal *Am-tll* RNA can be seen in a dorsal domain, enriched at the posterior with no RNA visible in anterior regions. (F) Lateral view of a stage 2 embryo. At this stage *Am-tll* RNA moves to the posterior of the syncytial embryo in association with migrating nuclei and becomes located at the posterior terminal by stage 3 (G, dorsal view), when the embryo cellularizes. (H, I and J) Lateral views of embryos at stages 5 to 7 stained for *Am-tll*. During these stage *Am-tll* RNA expression becomes strongly upregulated in two triangular dorsal–lateral domains at the anterior of the embryo and at the posterior pole, where expression has now expanded to a terminal cap (H). This expression is maintained through stage 6 (I lateral view) to stage 7 (J lateral view), where expression is lost in the posterior domain. (K) Lateral view of a stage 8 embryo, at this stage *Am-tll* RNA expression has been lost from the posterior domain and the anterior expression domain has shrunk to a small number of cells at the dorsal anterior on the edges of the gastrulation furrow. *Am-tll* continues to be expressed in the developing brain at late stages. (L) ventral and (M) lateral view of stage 9 embryo. Abbreviations: nurse cells (NC), oocyte (o) and follicle cells (fc).

Fig. 3. Detection of activated ERK (black) in honeybee and *Drosophila* embryos. Scale bars are 100 μm, embryos are arranged with anterior to the left. (A) Stage 1 honeybee embryo stained for activated ERK. No activated ERK protein can be detected by immunohistochemistry using conditions in which it is detectable in older honeybee embryos (B) and in *Drosophila* embryos (C and D). (B) In later Honeybee embryos (stage 6 shown, ventral view), dpERK staining is observed in the ventral ectoderm, in a few cells that line ventral midline (VE, arrows) and in the tracheal primordia (TP, arrow) of the developing segments. (C) The activated form of ERK, phosphorylated in response to Torso-signaling (Gabay et al., 1997), is detected at the poles of a blastoderm *Drosophila* embryo (asterisks) using the same reaction conditions as for (A). (D) Stage 10 *Drosophila* embryo (ventral view), dpERK is detected in the tracheal placodes (TP) and ventral ectoderm (VE).

while the actin cytoskeleton is required for anchoring of *nanos* and *otd-1* to the posterior pole of the oocyte (Olesnicky and Desplan, 2007). To determine what cytoskeletal structures are important for anchoring of *Am-tll* maternal mRNA to the dorsal side of the oocyte, dissected honeybee Queen ovaries were cultured in the presence of chemical inhibitors (Fig. 4). Treatment with cytochalasin D, which causes breakdown of the actin cytoskeleton, resulted in loss of *Am-tll* mRNA localization (Figs. 4A and B) , whereas culturing ovaries with colchicine, which depolymerizes microtubule networks, had no affect on *Am-tll* localization (Figs. 4C and D). These results indicate that the actin cytoskeleton is required to anchor *Am-tll* RNA to the dorsal side of the honeybee oocyte.

Am-otd1 is required for anterior but not posterior Am-tll expression in honeybee embryos

As in honeybee, there is no evidence for the involvement of a Torso signaling pathway in terminal development in *Nasonia vitripennis,* another hymenopteran. In this insect, anterior and posterior *tll* expression, which is first detected at the late blastoderm stage, requires *Nv-orthodenticle-1 (Nv-otd1)* (Lynch et al., 2006b). *Nv-otd1* is expressed maternally, localizing to the posterior and anterior poles of the oocyte. Both anterior and posterior expression domains of *Nv-tll* expression were found to be dependent upon *Nv-otd1* (Lynch et al., 2006b). We investigated whether the honeybee orthologue of *Nv-otd1, Am-otd1*, could regulate *Am-tll* expression in place of the Torso signaling pathway. Maternal *Am-otd* expression is detected throughout the oocyte (data not shown). In blastoderm stage honeybee embryos, *Am-otd1* mRNA is localized to the anterior half of the embryo (Fig. 5A). Later *Am-otd1* expression is upregulated in two lateral–anterior domains from which the procephalic lobes develop, similar to the anterior expression of *Am-tll* (Fig. 5B). Weak expression of *Am-otd1* is detected at the posterior pole of the honeybee embryo, unlike *Nasonia* where strong expression is associated with the posteriorly located oosome (Lynch et al., 2006a). This posterior domain is activated AFTER the expression of *Am-tll* in the posterior of the embryo. To test the potential role for *Am-otd1* in regulating *Am-tll* we used RNAi to knockdown expression of the *Am-otd1* transcript and assayed the effect of that knockdown on *Am-tll* expression using *in situ* hybridization. In all surviving *Am-otd1* RNAi embryos examined (number surviving = 96, number examined = 48), *Am-tll* expression is normal at the posterior pole but absent in the anterior, implying that while *Am-otd1* expression is required for activation of the anterior domain of *Am-tll*, it is not required for expression in the

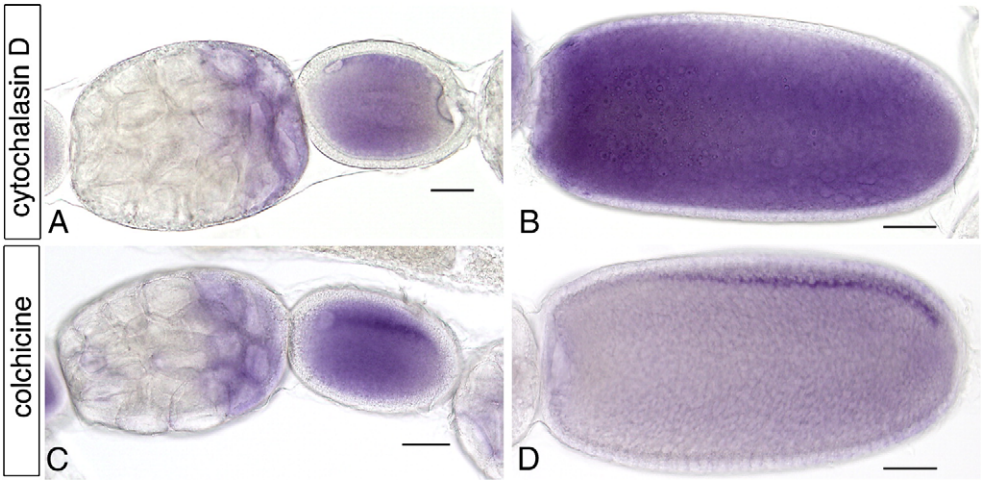

Fig. 4. Maternal anchoring of *Am-tll* RNA requires the actin cytoskeleton. Dissected Queen ovaries were cultured in the presence of cytochalasin D, colchicine or vehicle alone. Dissected ovarioles are shown with anterior of the oocytes to the left, scale bars represent 100 μm. (A and B) Ovaries cultured in the presence of cytochalasin D showed loss of *Am-tll* RNA anchoring to the dorsal side of the oocyte. (A) shows an early oocyte, (B) a later one. In both cases *Am-tll* RNA is not localized within the oocyte but uniformly distributed. (C and D) Ovaries cultured with colchicine retain *Am-tll* localization. (C) Early and (D) late oocytes stained for *Am-tll* RNA show normal localization of that RNA in the presence of colchicine.

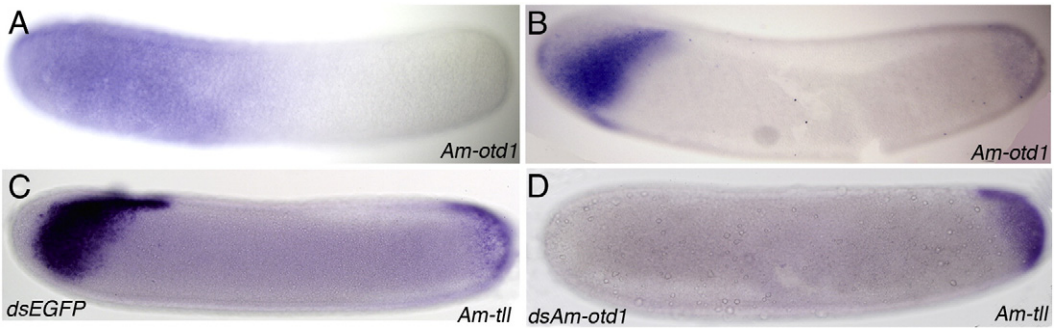

Fig. 5. *Am-otd1* is required for anterior *Am-tll* expression. Scale bars represent 100 μm, embryos are oriented anterior to the left, dorsal up. (A and B) Expression of *Am-otd1* as detected by *in situ* hybridisation (blue). (A) Maternal *Am-otd1* RNA is detected throughout the anterior half of the syncytial blastoderm embryo (stage 1). (B) *Am-otd1* RNA expression is upregulated in two anterior-lateral patches similar to those seen for *Am-tll* RNA (Fig. 2H). (C) Detection of *Am-tll* RNA in an embryo injected with double-stranded RNA made from the eGFP gene. *Am-tll* RNA expression is unaffected by this treatment (D) Detection of *Am-tll* RNA in an embryo injected with double-stranded RNA made from the *Am-otd1* gene. RNAi knockdown of *Am-otd* results in loss of *Am-tll* RNA expression from the anterior of the embryo but not from the posterior terminus.

posterior domain (Figs. 5C and D). Additionally, resulting embryos and larvae for *Am-otd1* or *Am-orthodenticle-2* (*Am-otd2*) RNAi show no posterior segmentation defects (unpublished data) supporting the hypothesis that honeybee *otd* genes, unlike those of *Nasonia*, regulate only anterior development. These results indicate that posterior *Am-tll* expression is achieved by a mechanism different to that of *Nasonia*. The expression pattern of *Am-tll* RNA implies that this mechanism is likely to involve maternal expression and transport of honeybee *tailless* mRNA to the posterior of the embryo via an RNA localization mechanism.

Am-tll is required for brain and posterior terminal patterning of honeybee embryos

RNAi-mediated knockdown of *Am-tll* was performed to determine its role in honeybee embryogenesis. Newly-laid embryos were microinjected with dsRNA against *Am-tll* and allowed to proceed through to larval stages. Surviving *Am-tll* RNAi larvae ($n=66$) displayed defects in both anterior and posterior terminal regions (Figs. 6A and B). All survivors lacked terminal posterior segments (A9-A10), indicating that *Am-tll* is required for differentiation of terminal structures. The majority (72.7%, $n=48$) of *Am-tll* RNAi larvae also had defects in the head region (Fig. 6). Examination of earlier stage embryos (stage 9) was carried out to determine how the head region was affected by loss of *Am-tll*. *Am-tll* knockdown resulted in complete loss of the brain primordium, though it did not affect formation of mandibles and maxillae head structures (Figs. 6C, D). The labrum is still present but has shifted dorsally from its normal location, probably due to the loss of the procephalic lobes (Fig. 6D). This correlates well with sites of *Am-tll* anterior expression, which is expressed in procephalic neurectoderm but not along the anterior-ventral surface of the embryo from which the mouth structures arise (Fig. 2M).

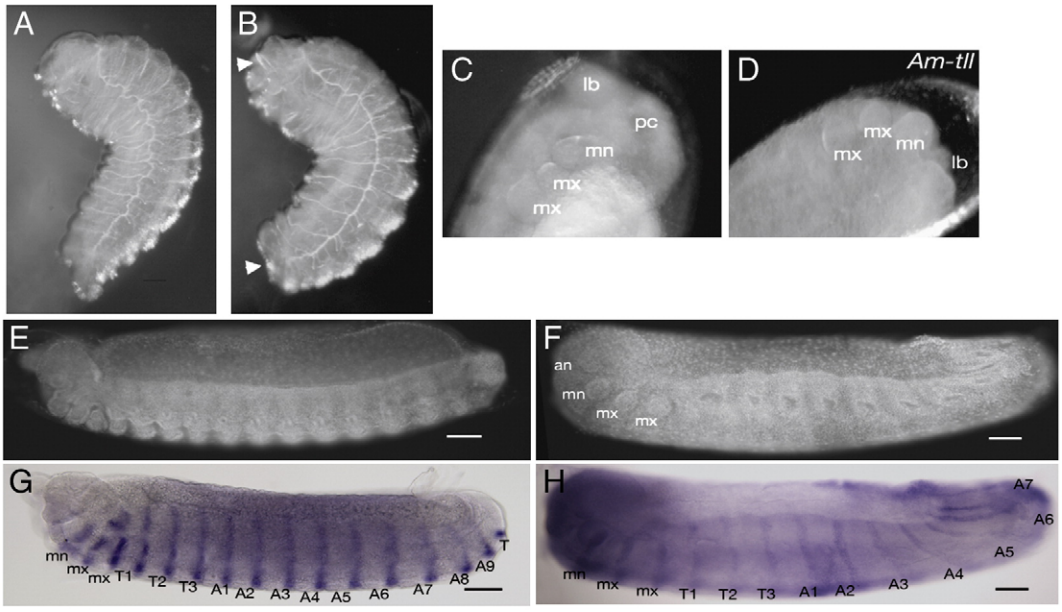

Fig. 6. The embryonic and larval phenotype of *Am-tll* RNAi treated embryos. Larvae and embryos are displayed anterior up, dorsal to the right. Scale bars represent 100 μm. (A) Larva of an embryo injected with dsRNA against eGFP. eGFP dsRNA injected larvae are identical to wild-type larvae, showing that treatment with dsRNA against non-targeted sequences does not affect embryo development. (B) Microinjection of dsRNA for *Am-tll* results in deformation of the head region (arrowhead) and loss of terminal segments, A9 and the telson (arrowhead). (C) Wild-type stage 9 embryo showing normal head development. (D) *Am-tll* RNAi knock-down results in complete loss of the procephalic neurectoderm. *Am-tll* is required for posterior terminal segments and development of the procephalic lobes. (E) DAPI stained stage 9 embryo injected with eGFP control double stranded RNA showing normal embryo morphology. (F) DAPI stained embryo of the same age as (E) injected with *Am-tll* double stranded RNA showing defects in embryo morphology at both anterior and posterior ends. The posterior segments of the embryo have extended into dorsal regions. (G) eGFP double stranded RNA injected embryo from (E) stained for *e30* (Osborne and Dearden, 2005; Walldorf et al., 1989), the honeybee orthologue of *engrailed,* RNA (Blue). Segments are labelled. (H) *Am-tll* double stranded RNAi injected embryo stained for *e30* RNA to indicate missing segments. Expression of e30 in the head is unaffected. A single stripe of cells stripes expressing e30 RNA can be seen posterior of A7 indicating the loss of A9 and A10. Abbreviations: labrum (lb), mandibles (mn), maxillae (mx), thoracic segments (T1–3), abdominal segments (A1–9), 10th abdominal segment (T), procephalic lobes, (pc).

To confirm the identity of the missing segments in *Am-tll* RNAi embryos we stained stage 9 control and *Am-tll* knockdown embryos for *e30* (Walldorf et al., 1989), the honeybee orthologue of *engrailed* (Figs. 6E–H). Expression patterns for this gene have previously been published and are similar to the *engrailed* gene of *Drosophila* (Osborne and Dearden, 2005). In *Am-tll* knockdown embryos, stripes of cells expressing *e30* are disrupted in posterior regions. A single stripe posterior to the stripe in the A7 segment is present indicating the loss of A9 and A10 consistent with the larval phenotype. The spacing of stripes of cells expressing e30 in the posterior is also slightly disrupted, and the posterior of the embryo extends dorsally, a phenotype not seen in embryos injected with double stranded RNA from eGFP. Anterior expression of *e30* is not affected.

Overexpression of Am-tll in Drosophila

The protein sequences encoded by both *Apis* and *Nasonia tll* genes are more divergent from those of other insects. These sequence changes may indicate that the function of this protein differs in these species. To test if *Am-tll* can regulate patterning pathways in a similar way to *Drosophila tll*, despite the differences in protein sequence between them, we used the UAS-GAL4 ectopic expression system (Brand and Perrimon, 1993) to overexpress *Am-tll* in *Drosophila* embryos. Ubiquitous blastoderm expression of *Dm-tll* by heat-shock has been shown to lead to deletions in thoracic and abdominal regions and loss of *kni* and *Kr* central expression domains in *Drosophila* (Moran and Jimenez, 2006; Steingrimsson et al., 1991) (Fig. 7). Ectopic expression of *Am-tll* in *Drosophila* represses abdominal expression of the gap genes, *kr* and *Kni* (Figs. 7D, E), leading loss of the pair-rule gene *even-skipped* stripe 5 and fusion of *eve* stripes 2 to 3 (Fig. 7F). Cuticle preparations revealed hs-*Am-tll Drosophila* embryos lacked trunk and abdominal segments compared to heat-shocked controls (Figs. 7J, K and L). The formation of ectopic Filzkörper structures was also observed in hs-*Am-tll* embryos (Figs. 7M and N). Filzkörper structures are terminal structures normally derived from the A8 segment and are occasionally observed ectopically in embryos over-expressing *Drosophila tailless* (Moran and Jimenez, 2006; Steingrimsson et al., 1991). Over-expression of *Am-tll* in *Drosophila* produces very similar phenotypes to over-expression of *Dm-tll*, both in the expression of downstream factors regulated by *tll* (Figs. 7G–I)

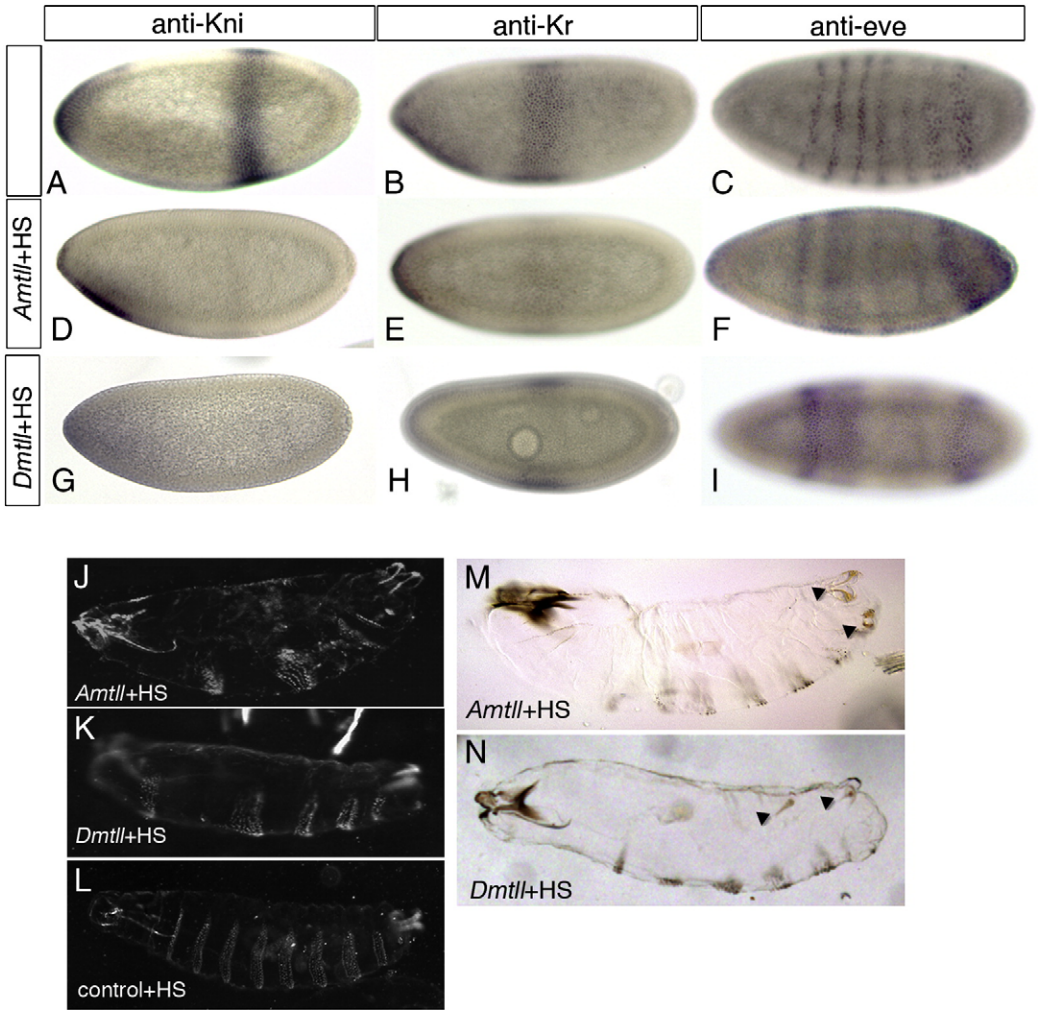

Fig. 7. Ectopic expression of *Am-tll* and *Dm-tll* in *Drosophila* embryos. Embryos are oriented anterior to the left, dorsal up. (A–C) Wild-type expression of Kni (A), Kr (B) and eve (C) imaged using immunohistochemistry in heatshocked hs-GAL4 embryos. (D–F) Expression of Am-tll protein induced by heat shock in hs-GAL4;UAS *Am-tll* embryos. Expression of kni (D) is absent in its central domain. (E) Kr expression is reduced and (F) *eve* stripe five and fusion of *eve* stripes two and three has occurred in these embryos. (H–J) Expression of Dm-tll protein induced by heat shock in hs-GAL4;UAS *Dm-tll* embryos. The expression of Kr, kni and eve are affected in the same was as for *Am-tll*. Heat shock (hs)-induced expression of *Am-tll* or *Dm-tll* in *Drosophila* blastoderm embryos results in loss of *kni* and *Kr* expression from the center of the embryo. This resulted in the loss of *eve* stripe five and fusion of *eve* stripes two and three as correct establishment of *eve* stripe expression requires both of these gap segmentation genes (Nibu et al., 1998; Struffi and Arnosti, 2005). (K–M) Cuticle preparations of larvae expressing *Am-tll* (K), *Dm-tll* (L) or controls revealed loss or fusion of most of the abdominal segments in both hs-*Am-tll* and hs-*Dm-tll* embryos. (N and O) Bright-field image of heat-shocked, hs-GAL4;UAS *Am-tll* (N) and hs-GAL4;UAS Dm-tll (O) larvae with Filzkörper structures labeled (arrowheads) in both normal and ectopic positions.

and in the observed larval morphology (Figs. 7J, K and L). These results imply that the Am-tll protein has similar biochemical function to *Dm-tll* at least when ectopically expressed in *Drosophila*, despite protein sequence differences in the DNA binding domain (Supplemental Figure 1). It seems likely that these changes have little effect on DNA binding specificity, when overexpressed in this way, suggesting, but not proving, that Am-tll may bind sequences similar to those Dm-tll binds to. The ability of *Am-tll* to repress the expression of abdominal patterning factors, such as *kni* and *Kr* is consistent with its role in promoting posterior fate in the honeybee, as observed in our RNAi experiments.

Discussion

Terminal patterning in the honeybee is not carried out by the Torso cell signaling pathway described from *Drosophila*. Components of the pathway are not present in the genome (Dearden et al., 2006), nor is there evidence for MAP kinase activation at the termini of the embryo (Fig. 3). Despite this the function of *Am-tll*, a critical effector of this pathway in *Drosophila*, seems similar to that of other insect *tll* genes. RNAi targeting *Am-tll* produces phenotypes that are similar to the RNAi of *tll* in Nasonia (Lynch et al., 2006b) and *Drosophila* mutants (Steingrimsson et al., 1991).

Am-tll encodes a derived member of the insect tll protein family and it is possible that the protein has evolved a new function that allows it to carry out a conserved function in the absence of Torso signaling. Over-expression of *Am-tll* in *Drosophila*, however, produces phenocopies of over-expression of *Drosophila tll*, including repression of kni and Kr (Fig. 7). In this system then, Am-tll protein function is very similar to that of *Drosophila* tll. It seems that *Am-tll* functions in a similar way to *Drosophila tll* both biologically and molecularly. The regulation of *Am-tll* is, however, different from other insects and allows this conserved component of terminal patterning to act in this process despite the absence of Torso signaling.

Regulation of Am-Tll expression

Tll genes in insects appear to have conserved roles in patterning the terminal ends of the embryo, with subsequent roles in brain and posterior development. We found that both the zygotic expression pattern of honeybee *tailless* and its role in honeybee development (based on RNAi data) is very similar to that of *tll* in *Drosophila* and other insects, but that its regulation is different from *tll* in any other insect from which it is described.

Maternal expression of *Am-tll* RNA results in its localization to a dorsal stripe on the oocyte, another point of difference between *Am-tll* and *tll* from other insect species. Our experiments indicate that *Am-tll* RNA is localized in this region by tethering to the actin cytoskeleton. In other systems, untranslated regions (UTRs) within the RNA transcripts are often involved in RNA localization (Brunel and Ehresmann, 2004; Irion and St Johnston, 2007; Russo et al., 2006). This may be the case for the *Am-tll* transcript, which has much longer UTRs than those seen in its *Drosophila* orthologue, which lacks the same maternal expression pattern. The regulation of *Am-tll* RNA localization is clearly complex. A system is required to capture and move *Am-tll* transcripts dorsally in the oocyte, in association with the oocyte nuclei. *Tll* RNA is then lost or moved in a regulated way from the anterior dorsal of the oocyte and from around the nucleus. Finally, dorsal anchoring to the actin cytoskeleton is lost, presumably on reorganization of the latter in early embryogenesis. It has been shown in other organisms that stable anchoring of localized RNAs requires actin microfilaments in the oocyte and, following oocyte-to-embryo transition, the anchored mRNA is released (Kloc and Etkin, 2005; Weil et al., 2008; Yisraeli et al., 1990). After dorsal anchoring, *Am-tll* RNA becomes associated with and probably travels with nuclei to the posterior of the oocyte. In the honeybee, a number of maternal transcripts are localized to dorsal or ventral regions of the oocyte, and are then redistributed in the early embryo though interactions with energids/nuclei (Wilson and Dearden, unpublished data).

After the maternal phase of expression, zygotic *Am-tll* expression becomes similar to that of other insects. This consists initially of a cap of expression at the posterior, appearing later in development relative to *Dm-tll* where the maternal Torso signaling cascade derepresses *tll* expression at the embryonic poles at the blastoderm stage. Expression in the anterior appears later still, unlike *tll* genes in dipterans but very similar to those of *Nasonia* and *Tribolium*. Expression in this anterior domain, like that of *Nasonia,* appears to be activated by an orthodenticle protein, Am-otd1. Our RNAi experiments indicate that removal of *Am-otd1* causes complete loss of expression of this anterior domain. The timing (at blastoderm stage) and mode of activation (via *Am-otd1*) of the anterior *Am-tll* domain indicates that regulation of this expression domain uses no common components to that of the *Drosophila* terminal patterning system. The oocyte RNA localization, and timing of the appearance of the posterior cap also implies that a terminal patterning pathway like that of *Drosophila* does not regulate *Am-tll* expression. Nor is *Am-otd1* involved in initial posterior expression as its expression domain appears after the expression of *Am-tll,* and *Am-otd1* RNAi knockdowns do not show posterior defects.

A conserved role for tailless proteins in brain patterning

Am-tll is expressed at the anterior end of the embryo in two large dorso-lateral stripes, in a similar fashion to *Drosophila*, *Anopheles* and *Tribolium.* This expression corresponds to the location of the developing procephalic lobes, which subsequently develop into the cerebral lobes of the brain. *Am-tll* continues to be expressed by brain neuroblasts through to hatching, and possibly beyond. A conserved role in patterning the procephalic lobes of the developing brain is also apparent, at least for *Drosophila* and honeybee, as these structures are lost in *tll* mutant and knockdown embryos in both species (Fig. 4D; (Strecker et al., 1986; Younossi-Hartenstein et al., 1997)). *Drosophila tll* mutants also show a reduction in the size of labrum (Strecker et al., 1986). In our studies, the labrum in honeybee *Am-tll* knockdown embryos is still present, but it was impossible to accurately determine if its size was affected by loss of the entire procephalic region in these embryos altering the positioning of the labrum.

The involvement of tll proteins in brain patterning and/or neurogenesis is conserved across many phyla. In both vertebrates and insects *tll* has often been found to be co-expressed in the embryonic brain with *orthodenticle* (*otd*/*otx*) genes (Lynch et al., 2006b; Monaghan et al., 1995; Schoppmeier and Schroder, 2005). In *Drosophila*, *otd* is not required for early procephalic *Dm-tll* expression (Rudolph et al., 1997), whereas in the honeybee and *Nasonia otd* is expressed in two anterior dorsal–lateral domains preceding *tailless* expression during embryogenesis. RNAi studies presented here and in *Nasonia* (Lynch et al., 2006b) have shown that this anterior expression of *otd-1* is required for upregulation of *tll* in the anterior of the embryo in both of these insects. In honeybees it is important to point out that, unlike other studied insects, this regulatory link between *otd* and *tll* in the anterior is entirely different to the regulation of the posterior domain of *Am-tll*, We hypothesize that this anterior domain regulation may reflect an ancestral regulatory linkage in brain development between *otd*/*otx* genes and *tll* genes, and that this linkage has been inherited from the common ancestor of vertebrates and arthropods.

Strategies to achieve posterior tailless expression varies between insects

Tailless function in posterior patterning is conserved in holometabolous insects. Knockdown or mutant studies in honeybee, *Nasonia* and *Drosophila* all produce a loss of posterior segments upon loss of *tll* (Fig. 6B; (Lynch et al., 2006b; Strecker et al., 1986). However, the

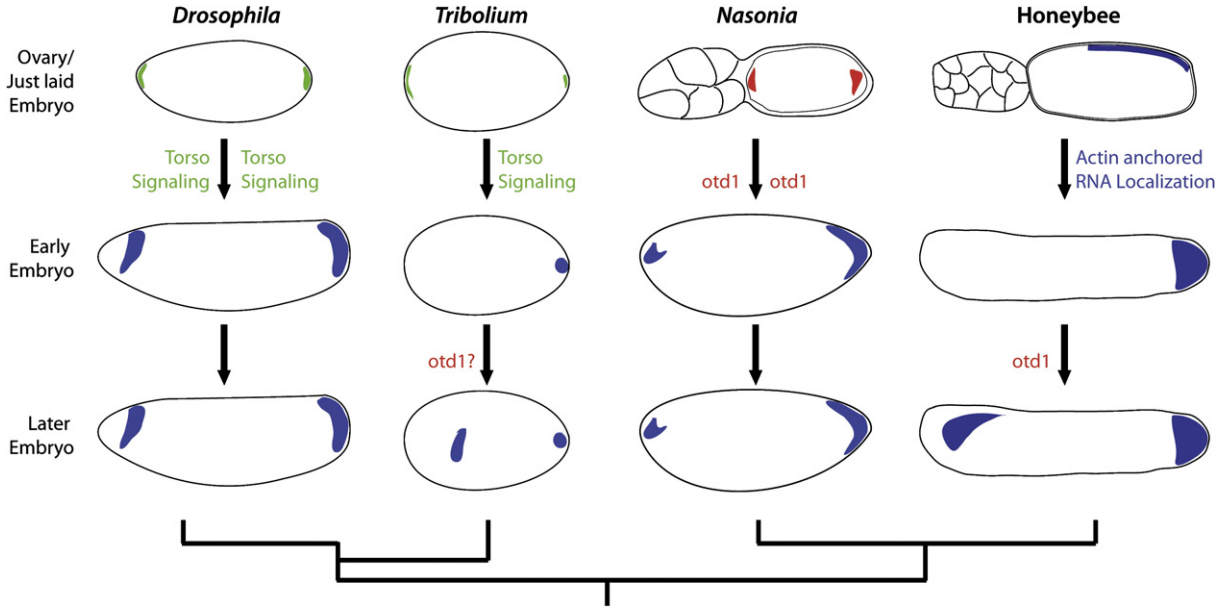

Fig. 8. Diversity of mechanisms to localize *tll* expression in holometabolous insects. Likely relationships between the species is indicated at the bottom of the figure. In *Drosophila*, *tll* expression at both anterior and posterior of the embryo is activated by terminal signaling through the Torso receptor (Pignoni et al., 1990; Pignoni et al., 1992; Steingrimsson et al., 1991). In *Tribolium* signaling through the Torso receptor is required for posterior expression of *tll* (Schoppmeier and Schroder, 2005). This expression domain appears before an anterior domain, which may be activated by *otd1* as the expression domains of these genes overlaps (Li et al., 1996; Schinko et al., 2008; Schroder, 2003). In *Nasonia*, *tll* expression is activated by *otd1*. *Otd1* RNA is localized to both the anterior and posterior poles of the oocyte (Lynch et al., 2006a; Lynch et al., 2006b). In honeybees, *tll* expression in the posterior occurs through a complex series of RNA localization events that transports maternal RNA to the posterior of the embryo. Anterior *tll* expression is activated later and depends on *otd1* expression.

upstream events leading to early embryonic posterior *tll* expression differs between these insects (Fig. 8). A MAPK cell-signaling pathway (the Torso signaling pathway) is required for derepression of posterior *tll* expression in Diptera (*Drosophila* and *Anopheles*) and in the short germ band insect, *Tribolium* (Schoppmeier and Schroder, 2005). *Nasonia* uses maternal RNA localization of an activator (*otd-1*) to the posterior of the oocyte to activate *tll* expression at the posterior of the embryo (Lynch et al., 2006a), possibly redeploying an ancestral regulatory linkage in a new part of the embryo. The honeybee also uses maternal RNA localization but in this case the honeybee *tll* transcript itself is expressed maternally and appears to localizes to the posterior pole of the early embryo after laying, bypassing the need for localizing an activator such as otd.

Recent phylogenetic studies on holometabolous insects suggest that the Hymenoptera may be the most basal group of the holometabolous insects (Krauss et al., 2008; Savard et al., 2006; Zdobnov and Bork, 2007), in contrast to previous studies suggesting the Coleoptera may be more distant (Beutel and Pohl, 2006). Both Diptera and *Tribolium* use Torso signaling to activate *tll* expression, while in the hymenopterans *Nasonia* and *Apis* the pathway is not involved in *tll* expression. In both these species no orthologue of the Torso receptor is encoded in the genome (Dearden et al., 2006 and Supplemental Figure 2). If Coleoptera are the deepest branching group of Holometabola, then loss of the torso pathway is a derived character. If Hymenoptera are the deepest branch then *tll* regulation may have been captured by the torso pathway in Coleoptera and Diptera. Studies of *tll* regulation in hemimetabolous insects, such as the pea aphid *Acyrthosiphon pisum*, will determine which of these scenarios is more probable. It is interesting to point out in this context that pea aphids do appear to have a Torso orthologue in their genomes, implying more components of the *Drosophila* terminal pathway may be present in this insect (Shigenobu et al., in press) than in the Hymenoptera.

The evolution of early events in development

It has been hypothesized, with some supporting evidence, that the earliest-occurring events in a developmental process have evolved most recently (Davidson, 2006; Wilkins, 2002) and should therefore be the most variable when compared between species. This idea would appear problematic for cases such as axis formation, where fast evolving initial events in a pathway must still produce a stable output for the rest of development to be built upon. In this paper we have shown that placement of *tll* RNA in the posterior of the holometabolous insect embryo is a key early event in axis formation, but comes about through a diversity of mechanisms. Indeed within the two species of Hymenoptera studied, two mechanisms occur, implying that more diversity in this pathway may yet exist in this group.

The key outcome of these processes is the posterior expression cap of *tll*, and later the anterior expression domain. Stabilizing selection must be acting to maintain these expression domains, but the way in which this expression is achieved is not under such stabilizing selection, and are thus able to change over long evolutionary periods. To understand how conservation of expression but not of regulation occurs we need to determine both the ancestral state of this process and the steps in its evolution into the diverse mechanisms we see in insects today.

Acknowledgments

We thank James Smith and Elizabeth Duncan for critical reading of the manuscript. We would also like to thank E.C. Dearden and D.J. Smith for their input during this project. The anti-even-skipped monoclonal antibody developed by Nipam Patel was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This research was supported by a Royal Society of New Zealand Marsden Grant (UOO0401) to PKD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.002.

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