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Diversity in insect axis formation: two *orthodenticle* genes and *hunchback* act in anterior patterning and influence dorsoventral organization in the honeybee (*Apis mellifera*)

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SUMMARY

Axis formation is a key step in development, but studies indicate that genes involved in insect axis formation are relatively fast evolving. Orthodenticle genes have conserved roles, often with *hunchback*, in maternal anterior patterning in several insect species. We show that two orthodenticle genes, *otd1* and *otd2*, and hunchback act as maternal anterior patterning genes in the honeybee (*Apis mellifera*) but, unlike other insects, act to pattern the majority of the anteroposterior axis. These genes regulate the expression domains of anterior, central and posterior gap genes and may directly regulate the anterior gap gene *giant*. We show *otd1* and *hunchback* also influence dorsoventral patterning by regulating *zerknült* (*zen*) as they do in *Tribolium*, but that *zen* does not regulate the expression of honeybee gap genes. This suggests that interactions between anteroposterior and dorsal-ventral patterning are ancestral in holometabolous insects. Honeybee axis formation, and the function of the conserved anterior patterning gene *orthodenticle,* displays unique characters that indicate that, even when conserved genes pattern the axis, their regulatory interactions differ within orders of insects, consistent with relatively fast evolution in axis formation pathways.

KEY WORDS: Axis formation, Developmental hourglass, Anterior patterning, Dorsoventral patterning, Extra-embryonic membranes, Segmentation

INTRODUCTION

The genes that control axis formation in the fruit fly *Drosophila melanogaster* are often missing from the genomes of other insects (Dearden et al., 2006; The Honey Bee Genome Sequencing Consortium, 2006; Richards et al., 2008; The International Aphid Genomics Consortium, 2010) implying that axis formation is a relatively fast evolving pathway. Although variation exists in axis formation, some molecules, such as those encoded by *orthodenticle* (Otd) genes, seem to have conserved roles (Schröder, 2003; Lynch et al., 2006; Schinko et al., 2008; Lemke and Schmidt-Ott, 2009; Kotkamp et al., 2010; Nakamura et al., 2010).

Otd genes have a long evolutionary history; patterning the anterior in vertebrates (Mercier et al., 1995; Pannese et al., 1995; Ang et al., 1996) and invertebrates (Chuang et al., 1996; Stornaiuolo et al., 1998; Wada and Saiga, 1999; Nederbragt et al., 2002). Insect genomes often have two Otd genes, *otd1* and *otd2*. The function of *otd1* has been examined in a small group of insects including some Diptera (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Royet and Finkelstein, 1995; Lemke and Schmidt-Ott, 2009), the beetle *Tribolium castaneum* (Schröder, 2003; Kotkamp et al., 2010), the jewel wasp *Nasonia vitripennis* (Lynch et al., 2006) and the cricket *Gryllus bimaculatus* (Nakamura et al., 2010). In these insects knockdown of *otd1* leads to anterior defects consistent with a role in anterior patterning. In *Drosophila*, a single Otd gene is present in the genome, *ocelliless* (*oc*), an *otd1* ortholog, that acts in head patterning although not early in development (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Wieschaus et al., 1992). In *Drosophila*, this early anterior patterning role has been taken by bicoid, a hox3-derived transcription factor whose DNA-binding domain has evolved to be much like Otd (Finkelstein et al., 1990; Mercier et al., 1995; Klein and Li, 1999). It is suggested that changes in Bicoid protein sequence have led to it 'taking over' the anterior patterning role by regulating genes ancestrally downstream of *oc* (Dearden and Akam, 1999; Stauber et al., 1999; Brown et al., 2001; Stauber et al., 2002).

In comparison, *otd2* has been less characterized. In *Tribolium* and *Nasonia, otd2* is expressed late in embryogenesis, with no probable role in anterior patterning (Lynch et al., 2006; Schinko et al., 2008). *otd2* is missing from the *Drosophila* genome and has not been examined in *Gryllus*. The pea aphid (*Acyrthosiphon pisum*) has only one Otd gene, *otd2* (Huang et al., 2010; Shigenobu et al., 2010; The International Aphid Genomics Consortium, 2010), which is not expressed early in anterior regions (Huang et al., 2010).

Alongside *otd1*, *hunchback* (*hb*) has been implicated in anterior patterning in a number of insects (Wolff et al., 1995; Patel et al., 2001; Mito et al., 2005). In *Nasonia*, *Nv-hb* acts with *Nv-otd1* in anterior patterning (Lynch et al., 2006). In *Drosophila*, *hb* is a direct target of Bicoid, and patterns the anterior (Simpson-Brose et al., 1994). In *Tribolium*, *hunchback* (*Tc-hb*) has been reported to act with *otd1* to regulate anterior patterning (Schröder, 2003), but recent evidence indicates that *Tc-hb* actually regulates trunk specification, with anterior segments being transformed to abdominal fates in an RNAi knockdown (Marques-Souza et al., 2008). *Tc-hb* knockdown also causes expansion of trunk Hox gene expression towards the anterior, and reduction in the expression of the thoracic gap gene *Krüppel* (Marques-Souza et al., 2008). This is inconsistent with *Tc-hb* acting to pattern the anterior; indeed *Tc-hb* knockdown does not cause loss of the anterior expression of a zygotic anterior gap gene, *giant* (*Tc-gt*). Trunk patterning may be a

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more ancestral function of *hb* than anterior patterning as it is also seen in widely diverged insects such as *Oncopeltus* and *Gryllus* (Liu and Kaufman, 2004; Mito et al., 2005).

Although *otd1* genes are a common element in insect anterior patterning, their actions differ, implying the interactions between otd1 proteins and their targets differ between species. In *Tribolium*, RNA interference (RNAi) targeting maternal and zygotic *Tc-otd1* partially mimics the *bicoid* mutant phenotype in *Drosophila* (Schröder, 2003). Double knockdown of *Tc-otd1* and *Tc-hb* leads to loss of anterior structures (Schröder, 2003), but whereas the defects produced suggest an anteroposterior patterning role, knockout of *Tc-otd1* causes only minor anteriorward shifts in gap gene expression. Indeed, the anteroposterior phenotype of *Tc-otd1* knockdown in *Tribolium* can be recapitulated by double knockdown of two dorsoventral patterning genes, *zen* (*Tc-zen*) and *Short on gastrulation* (*Tc-Sog*) (Kotkamp et al., 2010). The defects in dorsoventral patterning appear to cause loss of pattern from the anterior.

In *Nasonia*, *orthodenticle-1* (*Nv-otd1*) and *hunchback* (*Nv-hb*) together pattern the anterior of the embryo (Lynch et al., 2006). *Nv-otd1* RNA is localized to the anterior and posterior poles of the oocyte (Lynch et al., 2006) and patterns both ends of the embryo. RNAi against *Nv-otd1* gives both anterior and posterior patterning defects, but when in combination with RNAi against *Nv-hb*, the anterior defects are more severe. Consistent with this, *Nv-otd1* is required for *giant* (*Nv-gt*) expression (Lynch et al., 2006) the key anterior gap gene in this species (Brent et al., 2007).

The diversity in the regulation of axis formation in holometabolous insects is consistent with previous findings that, in insects at least, genes in pathways that act early in development are less conserved than later ones (Dearden et al., 2006; Wilson et al., 2010), and with recent molecular evidence for a 'developmental hourglass' model of development (Hazkani-Covo et al., 2005; Cruickshank and Wade, 2008; Kalinka et al., 2010). Diversity in genes involved in axis formation indicates that these pathways are fast evolving. Such relatively fast evolution might also be detected in changes in the regulatory interactions of conserved genes involved in axis formation.

MATERIALS AND METHODS

Isolation of honeybee genes

Am-otd2 was amplified from honeybee cDNA using the following oligonucleotides primers: CCCTACGGCGCCCTCAAGAC, amotx25′; TCCCGGGGTGGTGGCGACTA, amotx23′. *Am-hb* was amplified from honeybee cDNA using: CACGGCAGGATGGGAGTA, amhbRNA5′; GATCTGGCAATATGGAGGAAAAAG, amhbRNA3′ Cloning of other honeybee genes has been described previously (Osborne and Dearden, 2005b; Osborne and Dearden, 2005a; Dearden et al., 2006; Wilson and Dearden, 2009; Wilson et al., 2010).

Phylogenetic analysis

ClustalX (Thompson et al., 1994) alignments of full-length protein sequences were analyzed using MrBayes (Ronquist and Huelsenbeck, 2003) under the WAG model (Whelan and Goldman, 2001).

In situ hybridization

In situ hybridization was carried out as described previously (Osborne and Dearden, 2005b; Dearden et al., 2010).

RNAi knockdown in honeybee embryos

RNAi was performed as described previously (Wilson and Dearden, 2009; Dearden et al., 2010). Injected embryos were incubated at 35°C until the desired stage of development (24 hours for stage 4; 30-35 hours for stages 5-6). Stage 9 (65 hours) and later embryos were mounted in oil, or fixed and DAPI stained, and then photographed using an Olympus BX61 microscope. Hatched larvae were mounted in oil and photographed using a Leica dissecting microscope and digital camera. In the case of stained embryos at least 50 examples of each phenotype were examined and representative examples photographed.

Drosophila transgenesis, immunohistochemistry and in situ hybridization

Genomic regions around *Am-gt* were cloned into pCaSpeR-hs43-*lacZ* or pHpelican and used to transform w^{1118} *Drosophila* (Rubin and Spradling, 1982). Transgenic lines were crossed to mutants for *caudal* (*cad*), *hb*, *zerknült* (*zen*) and Otd (cad^{3}, hb^{b1}, zen^{2}, $oc^{otd\text{-}XC86}$). *Drosophila* in situ hybridization was carried out using established protocols (Patel, 1994).

Identification of *cis*-regulatory motifs

ClusterDraw (Papatsenko, 2007) was used to identify binding-site clusters for transcription factors in the *giant* locus of *A. mellifera* and *D. melangaster*. Binding site motifs used are available at http://line.bioinfolab.net/webgate/submit.cgi.

RESULTS

Identification of honeybee Otd and *hb* orthologs

Blast searches (Altschul et al., 1990) identified two predicted honeybee genes with similarity to *Drosophila* Otd: *GB16866* and *GB11566*. Phylogenetic analysis (Ronquist and Huelsenbeck, 2003) of aligned orthodenticle and vertebrate Otx protein sequences indicate *GB16866* clusters with a clade of *orthodenticle-1* sequences and *GB11566* clusters with *orthodenticle-2* sequences (see Fig. S1A in the supplementary material). We designate *GB16866* as *Am-orthodenticle-1* (*Am-otd1*) and *GB11566* as *Am-orthodenticle-2* (*Am-otd2*).

Blast searches of the honeybee genome identified one predicted gene, *GB19977*, with similarity to *Drosophila hb*. Phylogenetic analysis of aligned hunchback protein sequences indicates *GB19977* clusters with other insect hunchback proteins (see Fig. S1B in the supplementary material). We designate *GB19977*, *Am-hunchback* (*Am-hb*).

Expression of *Am-otd1*, *Am-otd2* and *Am-hb*

To determine whether *orthodenticle* and *hunchback* orthologs in the honeybee are expressed in patterns consistent with anterior specification we examined RNA expression using in situ hybridization. *Am-otd1* is expressed by nurse cells of the honeybee queen ovary and accumulates throughout the cytoplasm of the oocyte at all stages (Fig. 1A). Maternal RNA is enriched in the anterior of the syncytial blastoderm embryo within a few hours of the egg being laid (Fig. 1B; stage 1) where it is associated with energid cytoplasm. *Am-otd1* RNA becomes enriched in the anterior half of the embryo with highest concentration at the anterior pole (Fig. 1C; stage 2). Expression at the pole is reduced by stage 4 (Fig. 1D), after which it is upregulated in a triangular anterior domain and weakly at the posterior pole (stage 5; Fig. 1E). Posterior expression vanishes by stage 6, when faint expression is seen on the boundaries of the gastrulation furrow (Fig. 1F). At stage 9 *Am-otd1* RNA is detected in the CNS (Fig. 1G).

Am-otd2 RNA is expressed by posterior nurse cells (Fig. 1H) and is present in the oocyte (Fig. 1I,J) and freshly laid egg (Fig. 1K). *Am-otd2* expression fades in an anterior to posterior sequence after egg laying (Fig. 1K,L). At stage 4, *Am-otd2* RNA expression appears in cells at the anterior and posterior poles (Fig. 1M). Anterior expression resolves into a triangular domain, similar to *Am-otd1*. A cap of cells at the posterior expresses *Am-otd2* at stages 5 and 6 and faint expression is seen at the anterior end of the edges of the gastrulation furrow (Fig. 1N,O). At stages 8 and 9, *Am-otd2* RNA is limited to the developing CNS (Fig. 1P,Q).

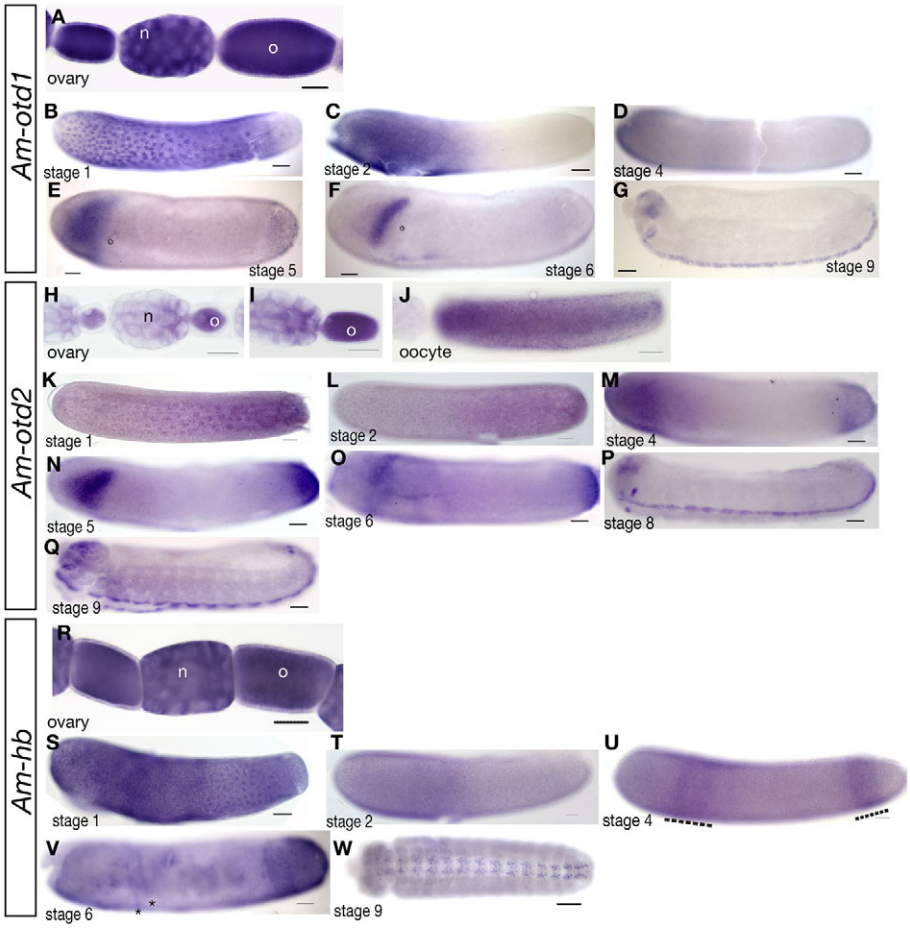

Fig. 1. Expression of *Am-otd1, Am-otd2* and *Am-hb* in honeybee queen ovaries and embryos. Embryos and ovaries are orientated with anterior left and dorsal up, unless otherwise stated. (A) Maternal expression of *Am-otd1*. *Am-otd1* mRNA is detected in nurse cells (n) and maturing oocytes (o). (**A-G**) *Am-otd1* RNA is associated with energids in stage 1 embryos (A) and is detected in the anterior of stage 2 embryos (C). This expression domain becomes weaker (D) until stage 5 (E) where *Am-otd1* is detected in a triangular anterior domain and at the posterior pole. By stage 6 (F) the anterior domain narrows, weak expression occurs at the edges of the gastrulation furrow and posterior expression is not visible. From stage 9 (G) *Am-otd1* RNA is present in CNS cells. (**H**,**I**,**J**) Queen ovary expression of *Am-otd2*. *Am-otd2* RNA is present in posterior nurse cells (n) and throughout the oocyte (o). (**K-Q**) In stage 1 embryos (K), *Am-otd2* RNA is enriched around energids in the posterior, where RNA remains, though loses association with energids by stage 2 (L) and is no longer detected after cellularization. By stage 4 (M), *Am-otd2* is expressed in an anterior domain and posterior cap that remains through stages 5 (N) and 6 (O). By stage 8 (P) and 9 (Q), *Am-otd2* is expressed in the CNS. (**R**) Queen ovary expression of *Am-hb*. *Am-hb* RNA is detected in nurse cells (n) and oocytes (o). (**S-W**) In stages 1 (S) and 2 (T) embryos *Am-hb* RNA is present throughout the embryo. RNA is enriched in the anterior and in a posterior stripe of cells by stage 4 (dotted lines, U). By stage 6 (V), only a posterior cap remains, which fades and is replaced (at stage 9; W) with expression in the CNS. Scale bars: 100 μm.

Am-hb RNA is present throughout maturing oocytes and nurse cells (Fig. 1R). It is ubiquitous in early embryos and comes to be associated with nuclei, with stronger staining in the anterior two-thirds of the embryo (Fig. 1S,T). By stage 2, *Am-hb* RNA is enriched in the anterior of the embryo (Fig. 1T). By stage 4 it becomes enriched in a broad anterior stripe and a weaker posterior one (Fig. 1U). This posterior expression later (stage 6) expands to a cap of cells, but weak *Am-hb* expression is detected throughout the embryo (Fig. 1V). In later stages *Am-hb* is expressed in the CNS (Fig. 1W).

RNAi knockdown of *Am-otd1, Am-otd2* and *Am-hb*

If *Am-otd1, Am-otd2* and *Am-hb* act to pattern the anterior, then removing their function using RNAi early in development should lead to defects in anterior patterning.

Am-otd1 knockdown produces two phenotypic classes. Compared with controls (Fig. 2A), mildly affected larvae (Fig. 2B) show anterior defects, although not loss of segments, whereas the majority of larvae (Table 1) are more severely affected, lacking head, thoracic and abdominal segments (Fig. 2C). In anterior regions, compared with controls (Fig. 2D), mildly affected embryos at stage 9 retain all segments but have dorsoventrally duplicated gnathal segments (Fig. 2E), whereas severely affected individuals have no anterior segments (Fig. 2F). In situ hybridization for *e30* RNA, a honeybee ortholog of *engrailed* (Walldorf et al., 1989; Dearden et al., 2006) allows better definition of segments (Fig. 2F). In mildly affected larvae, *e30* stripes have an unusual orientation being focused around a dorsal thoracic region (Fig. 2H), which is a phenotype associated with gnathal duplications (Fig. 2E) and ectopic anterior tracheal pits (circled in Fig. 2I). Severely affected embryos stained for *e30* RNA have staining in only the most posterior abdominal segments, indicating extensive loss of pattern from the anterior (Fig. 2J).

Knockdown of *Am-otd2* by RNAi also produces mild and severe phenotypes. In mildly affected larvae the labrum is lost and head structures, resembling mandibles or maxillae, are shifted

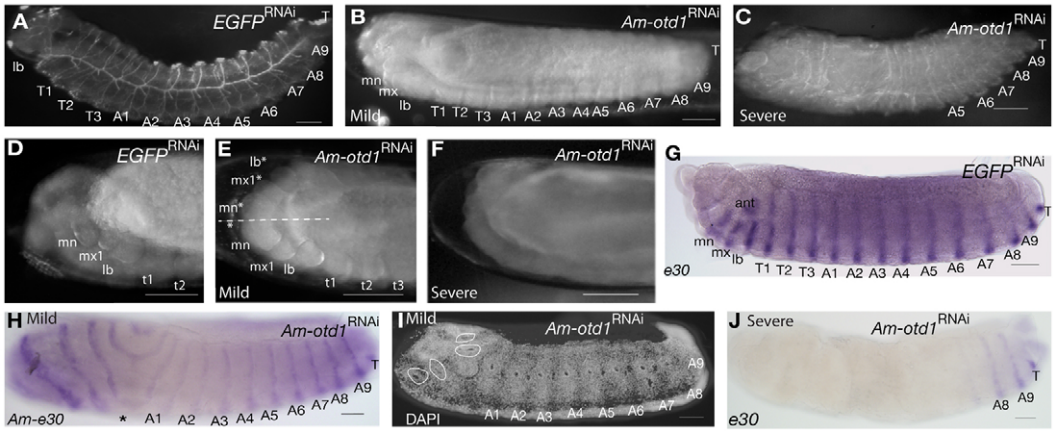

Fig. 2. RNAi-mediated knockdown of *Am-otd1*. Embryos and larvae are oriented with anterior left, dorsal up. (**A**) *EGFP* RNAi control larva. (**B**) Mildly affected larva injected as an embryo with *Am-otd1* RNAi, showing loss of anterior structures. (**C**) Severely affected larva with loss of anterior segments, thoracic and A1-A6 abdominal segments. (**D-F**) Head regions of control injected (D) and *Am-otd1* RNAi (E,F) stage 9 embryos. In mild *Am-otd1* RNAi knockdown a dorsoventral mirror-image duplication of anterior structures occurs (E). Normal gnathal limbs are labeled (mn, mandible; mx1, maxilla; lb, labium; T1, 2, 3; thoracic limb buds) duplicated gnathal appendages, identified by examination of their morphology, are labeled mn*, mx1*, lb*. The line of symmetry for the duplication is marked with a dotted line. The region from which the anterior-most structures are missing is marked with an asterisk. In severe cases (F) anterior structures and gnathal and thoracic limb buds are missing. (**G**) *e30* RNA expression in a *EGFP* RNAi stage 9 embryo. (**H**). *e30* RNA staining of a mildly-affected *Am-otd1* RNAi stage 9 embryo showing disruption of the *e30* pattern in anterior regions. (**I**) DAPI-stained embryo similar to H; ectopic tracheal pits are circled. (**J**) *e30* RNA staining of a severely affected *Am-otd1* RNAi stage 9 embryo. Scale bars: 100 μm. Ant, antennal segment; T, Telson.

toward the anterior (Fig. 3A). In mildly affected embryos anterior stripes of cells expressing *e30* RNA are shifted to more anterior positions (Fig. 3B). In severe phenotypes, all anterior segments are missing and posterior segments appear twice-normal size (Fig. 3C), implying fused or expanded segments. The presence of the telson suggest that these are fused segments rather than expanded ones with segments missing at the posterior. *e30* RNA staining reveals the loss of every second stripe of *e30* throughout the abdomen, and loss or reduction of anterior stripes (Fig. 3D). We examined this unusual phenotype in *Am-otd2* knockdown embryos for expression of *Am-paired* (*Am-prd*) and *Am-even-skipped* (*Am-eve*), orthologs of *Drosophila* pair rule genes (Osborne and Dearden, 2005a; Wilson et al., 2010). *Am-prd* RNA is expressed after stage 6 in 15 stripes of cells (Fig. 3E) (Osborne and Dearden, 2005a). In *Am-otd2* RNAi-injected embryos, 10 faint stripes of *Am-prd* RNA are detected (Fig. 3F). *Am-eve* RNA expression normally occurs in six broad stripes of cells across the embryo that split into two in an anterior to posterior progression (Fig. 3G) (Wilson et al., 2010). In *Am-otd2* RNAi embryos, the most anterior and posterior stripes of *Am-eve* expression appear normal and split, but central abdominal stripes have weaker expression and do not split (Fig. 3H).

Both *Am-otd1* and *Am-otd2* are expressed in posterior domains from stage 4 (Fig. 1E,N), similar to *Nv-otd1* expression in *Nasonia*, but no posterior defects are seen with RNAi knockdown (Figs 2 and 3). To determine whether these genes are acting redundantly we carried out double RNAi knockdown. Even severely affected double-knockdown embryos, with extensive loss of anterior structures and fusion of abdominal segments, have a segmented posterior region as shown by *e30* RNA (Fig. 3I) and DAPI staining (Fig. 3J). Despite expression in posterior regions neither *orthodenticle* gene appears to contribute to posterior segmentation.

RNAi knockdown of *Am-hb* causes the majority of embryos to die before stage 9, before they develop the required morphology to distinguish affected segments. More mildly affected embryos show a range of phenotypes, from a lack of head and gnathal segments (Fig. 4A), to loss of anterior segments all the way to the posterior abdomen (Fig. 4B). Staining these embryos at stage 9 for *e30* RNA, and comparing them with controls (Fig. 4C) indicates mildly affected embryos have defective anterior segments, fusions of T3 to A1, and loss of A8 and A9 (Fig. 4D), whereas severely affected embryos have only two or three of the most posterior segments (Fig. 4E).

Table 1. Larval, or stage 9 embryo, RNAi phenotype frequency

RNAi target	Mild-moderate phenotype	Severe phenotype
Am-otd1	19 (19%) with loss of brain only 16 (16%) with loss of brain and duplication of head structures	64 (65%) with no anterior development, loss or fusion of abdominal segments and terminal segments normal)
Am-otd2	48 (70%) with loss of brain structure and normal segments	21 (30%) with loss of anterior structures and fusion of segments, but normal terminal structures
Am-hb	15 (25%) with anterior structures missing, ten segments remaining and normal terminal structure	45 (75%) with only three or four segments remaining and terminal segment
Am-otd1+ Am-otd2	15 (35%) with loss of brain and duplication of head structures	27 (64%) with loss anterior structures and segments, loss of abdominal segments or fusion of some segments

Table refers to injected embryos allowed to hatch or that die at late stage 9.

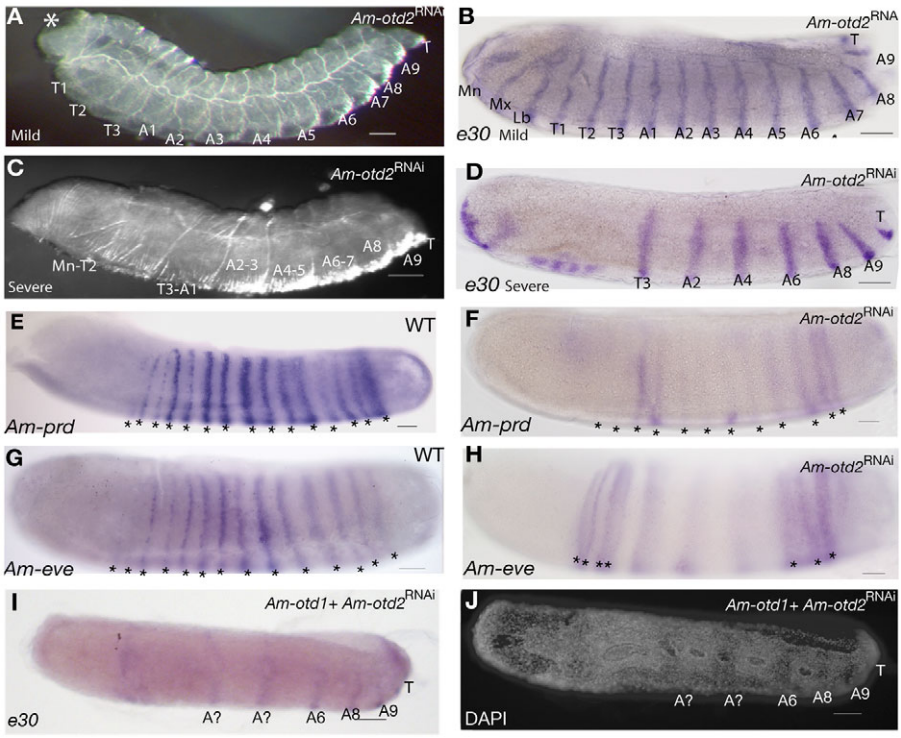

Fig. 3. RNAi knockdown of *Am-otd2*. Embryos and larvae are oriented with anterior left, dorsal up. (**A**) Mild *Am-otd2* RNAi phenotype, showing minor defects in the head (asterisk). (**B**) *e30* RNA-stained stage 5 mildly affected *Am-otd2* RNAi embryo. *e30* staining reveals that all segments are present but anterior stripes are disrupted. (**C**) Severely affected larva derived from embryonic *Am-otd2* RNAi injection, with fused segments and loss of anterior pattern. (**D**) *e30* RNA-stained, severely affected, *Am-otd2* RNAi embryo. Every second *e30*-expressing stripe of cells in abdominal regions of the embryo is missing and anterior stripes are reduced or absent. (**E**) Expression of *Am-prd* in a wild-type early stage 6 embryo. *Am-prd* RNA is expressed in 15 stripes of cells at this stage (asterisks). (**F**) *Am-otd2* RNAi stage 6 embryo stained for *Am-prd* RNA showing reduction in expression in all *Am-prd* stripes (asterisks). (**G**) Expression of *Am-eve* RNA at stage 6 when *Am-eve* is expressed in 14 stripes of cells around the embryo (asterisks). These stripes derive from the splitting of 7 primary stripes. (**H**) *Am-otd2* RNAi stage 6 embryo stained for *Am-eve* RNA showing reduction of *Am-eve* RNA in abdominal stripes of cells as well as failure of primary stripes to split. (**I**,**J**) Double RNAi knockdown of *Am-otd1* and *Am-otd2*. Only posterior segments remain as revealed by staining for *e30* RNA (I) and DAPI (J). Scale bars: 100 μm.

RNAi knockdown of *Am-otd1*, *Am-otd2* and *Am-hb* all indicate roles for these genes in maternal patterning of anterior regions of the embryo, but the defects in severe cases are extensive and stretch into the abdominal segments.

Regulation of gap gene expression by anterior patterning genes

If *Am-otd1*, *Am-otd2* and *Am-hb* act as maternal anterior patterning genes they should regulate the expression of gap genes. Knockdown of these genes should lead to changes in the expression levels and boundaries of gap genes. We examined the effect of knockdown on *Am-Krüppel (Am-Kr), Am-caudal (Am-cad)* and *Am-giant* (*Am-gt*) expression; genes previously shown to act as gap genes in the honeybee (Wilson et al., 2010).

In control-injected embryos, *Am-Kr* is expressed in a broad central domain at stage 5 where it acts to pattern thoracic and anterior abdominal segments (Fig. 5A). At stage 6 this domain splits into three stripes (asterisks in Fig. 5B). At this time a posterior cap and an anterior domain of expression are detected (Fig. 5B) (Wilson et al., 2010). *Am-cad* is required for abdominal patterning in the honeybee and is expressed from the central to the posterior regions (Fig. 5C) (Wilson et al., 2010). *Am-gt* RNA is expressed in a broad anterior domain and posterior stripe by stage 3. Later, *Am-gt* RNA expression is lost from the anterior dorsal region of the embryo (Fig. 5D) (Wilson et al., 2010).

Knockdown of *Am-otd1* results in loss of anterior *Am-Kr* expression (arrowhead in Fig. 5E,F) and loss or reduction in expression in the central domain (asterisks in Fig. 5E,F). The anterior border of *Am-cad* expression is shifted posteriorly (arrowheads in Fig. 5C,G) with expression being lost from central regions (Fig. 5G). *Am-gt* expression in *Am-otd1* knockdown embryos is missing from the anterior, and ectopic expression appears in central regions of the embryo (Fig. 5H arrow).

In the majority of *Am-otd2* knockdown embryos, expression of *Am-Kr* and *Am-cad* is normal. In a small proportion (13/45) of injected embryos, however, the central domain of *Am-Kr* expression domain shifts slightly toward the posterior (Fig. 5I) and, in stage 6, the central domain fails to split into stripes and anterior expression is lost (Fig. 5J). In a small proportion of embryos (9/25) weak *Am-cad* expression extends toward the anterior from the normal central domain and expression is reduced in the posterior half of the embryo (Fig. 5K). In many *Am-otd2* RNAi embryos, *Am-gt* expression is present at the anterior but fails to be downregulated in the anterior-dorsal region (corresponding to the position of late *Am-otd2* expression; arrow in Fig. 5L), and ectopic expression continues to the trunk regions.

Am-hb RNAi knockdown embryos have a reduction in expression in the central domain of *Am-Kr* expression (Fig. 5M) at stage 4 and a loss of anterior (arrowhead) and central domain expression at stage 6 (asterisk in Fig. 5N). *Am-cad* RNA expression

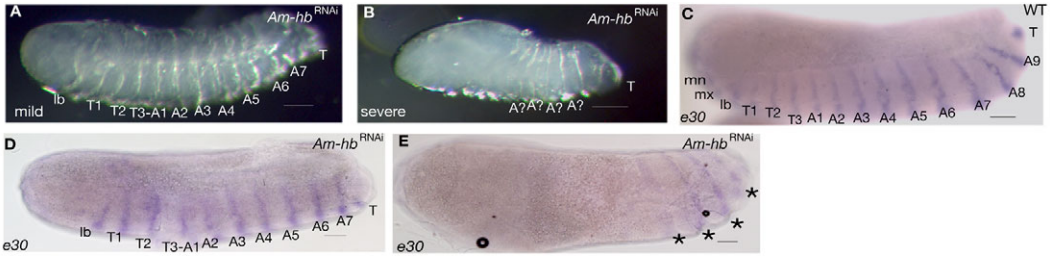

Fig. 4. RNAi knockdown of *Am-hb*. Embryos and larvae are oriented with anterior left, dorsal up. (**A**,**B**) Larvae derived from *Am-hb* RNAi-injected embryos, The phenotypes range from mildly affected with loss of anterior structures, fusion of T3-A1 and loss of A8 (A) to complete loss of anterior, thoracic and abdominal segments and malformation of the posterior terminal (B). (**C-E**) *e30* RNA expression at stage 9 in wild-type (C) and *Am-hb* RNAi (D,E) embryos. In mildly affected embryos (D) stripes of *e30* RNA are missing from the mandibular and first maxilla segments. Posterior stripes are fused (T3-A2) and A8 and A9 posterior stripes are missing. In severely affected embryos (E) *e30* stripes are lost from the majority of the embryo with only a few posterior stripes remaining (asterisks). Black circles are air bubbles. Scale bars: 100 μm.

is reduced through central regions of the embryo, remaining at the posterior, where it expands to the very posterior terminus (Fig. 5O). The anterior domain of *Am-gt* is lost in *Am-hb* RNAi embryos (arrowhead in Fig. 5P).

Zygotic gap-gene domains that regulate patterning along the honeybee embryo are strongly affected by *Am-otd1* and *Am-hb* knockdown, and to a lesser extent by *Am-otd2*.

Am-otd1 and *Am-hb* are required for correct extra-embryonic membrane patterning

In *Tribolium*, *Tc-otd1* regulates the expression of *Tc-zen* and *Tc-sog* (Kotkamp et al., 2010). To determine whether a similar linkage exists in the honeybee we examined the expression of honeybee *zen* [*Am-zen* (Osborne and Dearden, 2005b; Dearden et al., 2006)] in *Am-otd1* RNAi embryos. Honeybee *sog* is not expressed in early embryogenesis (M.J.W. and P.K.D., unpublished data). *Am-zen* RNA is expressed in a dorsal domain that extends from the anterior, along the dorsal surface, to the posterior (stage 4, Fig. 6A,B) (Osborne and Dearden, 2005b; Dearden et al., 2006). In *Am-otd1* RNAi embryos, *Am-zen* RNA expression is lost from only the anterior and posterior poles (Fig. 6C). We also examined *Am-zen* expression in *Am-hb* RNAi embryos. Knockdown of *Am-hb* results in expansion and disorganization of *Am-zen* expression (Fig. 6D,E) and extra-embryonic membranes (evidenced by the more widely spaced serosa cells; Fig. 6F). *Am-zen* mRNA is lost from the anterior in *Am-hb* RNAi embryos but becomes expressed across central regions and expanded dorsally. Both *Am-otd1* and *Am-hb* thus contribute to the regulation of *Am-zen* and extra-embryonic membrane patterning.

In *Tribolium*, RNAi knockdown of *Tc-otd1* leads to ectopic apoptosis contributing to the anterior patterning defect. To determine whether this also occurs with *Am-otd1* knockdown, we examined RNAi-treated embryos with DAPI and found that cell death is increased in *Am-otd1* embryos in anterior regions (see Fig. S2 in the supplementary material).

To determine whether, as in *Tribolium*, loss of *Am-zen* is responsible for the anteroposterior patterning defects found in *Am-otd1* and *Am-hb* RNAi embryos, we examined the phenotype of *Am-zen* knockdown. If *Am-zen* is responsible for the defects in anterior posterior patterning, then knockdown should lead to anterior segmentation defects and changes in the expression of gap and pair-rule genes.

Am-zen RNAi embryos survive to hatching but have a deformed head in dorsal regions and extended embryo flanks (Fig. 6G), causing them to curve differently to control larvae (Fig. 6H). DAPI staining at stage 9 indicates that the dorsal side of the embryo collapses in mild cases (Fig. 6J), and, in severe cases, the germ rudiment extends all the way to the dorsal side of the embryo (Fig. 6K,L) unlike the control (Fig. 6I). All segments are present in *Am-zen* RNAi embryos (Fig. 6J-L), pair-rule gene expression is normal (Fig. 6M) and *e30* stripes extend further toward the dorsal side of the embryo, indicating that dorsal embryonic tissue extends more dorsally (Fig. 6N).

As *Tribolium zen* knockdown produces defects in anteroposterior patterning we examined the regulation of key anteroposterior patterning genes in *Am-zen* knockdown embryos (Fig. 7) expecting that they would be affected by loss of *Am-zen* expression as in *Tribolium*. In *Am-zen* knockdown embryos, *Am-Kr* and *Am-cad* expression domains are indistinguishable from those in wild-type embryos (Fig. 7A,B). Anterior expression of *Am-tailless* (*Am-tll*) is reduced and shifted anterodorsally (Fig. 7C) compared with wild-type embryos (Fig. 7D). *Am-gt* expression is normally absent from dorsal regions of the embryo (Fig. 7E) but in *Am-zen* knockdown, expression is expanded into cells in anterodorsal regions (Fig. 7F-I). Thus although *Am-zen* is required for patterning of the extra-embryonic membranes, loss of *Am-zen* does not affect segmentation, does not have a significant effect on gap gene expression, and is not responsible for the loss of anterior patterning seen in *Am-otd1* RNAi embryos.

Regulation of *Am-gt* expression

Our experiments suggest that *Am-otd1*, *Am-otd2* and *Am-hb* regulate *Am-gt* (Fig. 5). To determine whether this regulation is direct and indicative of a role in regulating key anterior gap genes, as expected for their maternal anterior patterning function, we searched for *cis*-regulatory motifs (CRMs) that regulate the expression of *Am-gt*. We searched for clustered transcription-factor binding sites for known regulators of *giant* upstream of *Am-gt* on the assumption that the target binding sites of potential regulators remained the same as their *Drosophila* orthologs, using ClusterDraw (Papatsenko, 2007) (see Fig. S3 in the supplementary material). A 4 kb region (*Am-gt* CRM) indicated in this analysis was cloned upstream of a *lacZ* reporter gene and used to produce transgenic *Drosophila* lines.

lacZ expression driven by the *Am-gt* CRM was examined by in situ hybridization (Fig. 8). Weak expression of *lacZ* transcripts was detected in stage 1 embryos (Fig. 8A), with significant expression appearing by stage 4 in an anterior domain (Fig. 8B,C). At stage 5, *lacZ* is expressed along the dorsal midline in the developing aminoserosa (Fig. 8D). We crossed the *Am-gt* CRM reporter into mutants homozygous for transcription factors that potentially regulate binding to this CRM. In Otd mutants, *lacZ* is absent in the anterior

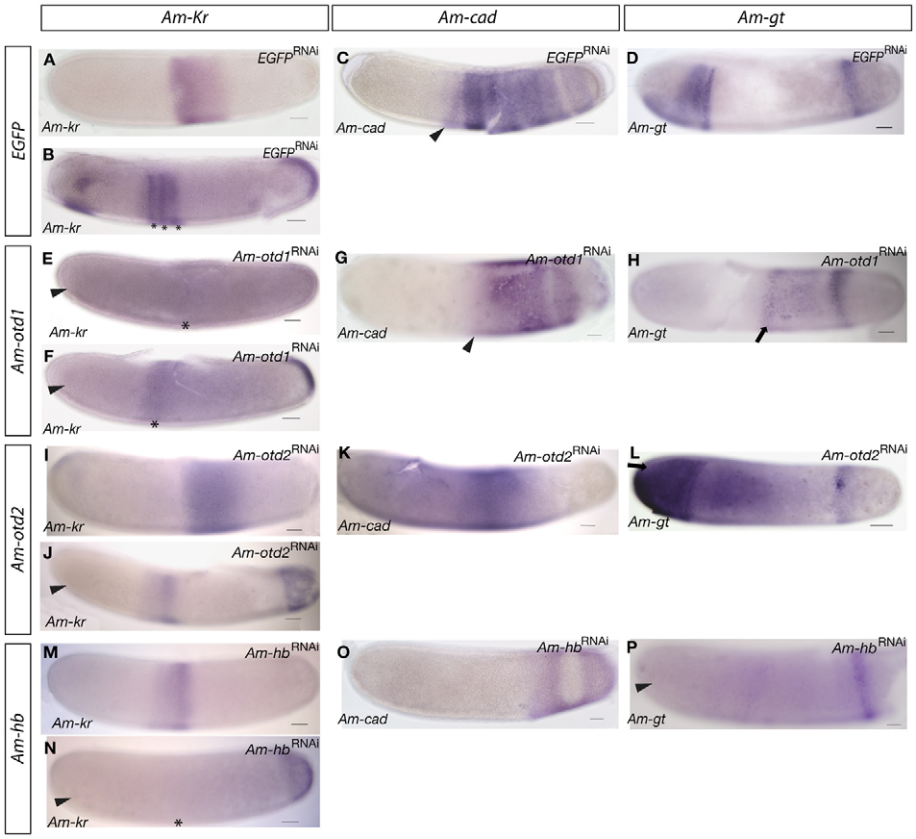

Fig. 5. Altered gap gene expression in RNAi-treated embryos. Embryos are oriented with anterior to left, dorsal up. (**A**,**B**) Expression of *Am-Kr* RNA in stage 5 (A) and stage 6 *EGFP* RNAi control embryos (B). (**C**) Expression of *Am-cad* RNA in a stage 5 *EGFP* RNAi control embryo. (**D**) Expression of *Am-gt* RNA in a stage 5 *EGFP* RNAi control embryo. (**E**,**F**) Expression of *Am-Kr* RNA in *Am-otd* RNAi stage 5 (E) and stage 6 (F) embryos showing reduced expression in central (asterisk) and anterior regions (arrowhead). (**G**) Expression of *Am-cad* RNA in an *Am-otd1* RNAi stage 5 embryo showing a posterior shift in the anterior boundary of *Am-cad* expression. (**H**) *Am-gt* RNA expression in an *Am-otd1* RNAi stage 5 embryo. Anterior *Am-gt* expression is lost, with ectopic expression in trunk regions of the embryo (arrow) and the posterior stripe shifting to the anterior. (**I**,**J**) *Am-Kr* RNA expression in *Am-otd2* RNAi stage 5 (I) and 6 (J) embryos. *Am-Kr* expression is reduced and the central band of expression is slightly shifted towards the posterior (I); no splitting of the central domain into stripes occurs and anterior expression is lost (arrowhead, J). (**K**) *Am-cad* RNA expression in an *Am-otd2* RNAi stage 5 severely affected embryo. Expression extends towards the anterior in severely affected embryos. (**L**) *Am-gt* RNA expression in an *Am-otd2* RNAi stage 5 embryo. RNA is expressed throughout the central regions of the embryo and is detected throughout the anterior head region (arrow). (**M**,**N**) *Am-hb* RNAi stage 5 (M) and stage 6 (N) embryos stained for *Am-Kr* RNA, showing either reduction (M) or loss (N) of both central (asterisk in N) and anterior domains (arrowhead in N) of *Am-Kr* expression. (**O**) *Am-hb* RNAi stage 5 embryo stained for *Am-cad* RNA, showing disruption of the central domain of *Am-cad* and a posterior shift of anterior and posterior boundaries of *Am-cad* expression. (**P**) *Am-hb* RNAi stage 5 embryos stained for *Am-gt* RNA showing loss of the anterior domain (arrowhead). Scale bars: 100 μm.

but remains in the dorsal midline (Fig. 8E). In *hb* mutants *lacZ* expression is reduced through the embryo (Fig. 8F). In *zen* mutants, *lacZ* expression is lost from the aminoserosa (Fig. 8G). In *Drosophila*, *cad* has been implicated in activating the posterior domain of *gt* (Schulz and Tautz, 1995). *Am-gt* CRM expression in *cad* mutants is upregulated throughout the embryo, consistent with a role for *Am-cad* as a repressor of *Am-gt* (Fig. 8H). To test this interaction in the honeybee, RNAi knockdown of *Am-cad* was used in embryos, which resulted in a posterior shift of the posterior border of the anterior *Am-gt* domain, consistent with a role for *Am-cad* in repressing *Am-gt* expression (see Fig. S4 in the supplementary material).

DISCUSSION

Diversity in the roles of Otd genes in insect anterior patterning

We have shown that *Am-otd1*, *Am-otd2* and *Am-hb* have anterior patterning roles in honeybee embryos. We have also shown that *Am-otd1*, *Am-otd2* and *Am-hb* probably directly regulate the expression of the key anterior gap gene *giant*. RNAi knockdown of these genes, especially *Am-otd1* and *Am-hb*, affect the expression domain of every gap gene we have examined, including anterior and more posterior-acting genes. Given this evidence we propose that *Am-otd1* and *Am-otd2* together with *Am-hb* act as maternal anterior patterning genes in the honeybee embryo.

Although this is consistent with the composite anterior patterning system identified in *Nasonia* (Lynch et al., 2006), it is clear that the regulatory interactions, and functions, of these genes in honeybees differ from all other described insects.

The effects of knockdown of either *Am-otd1* or *Am-hb* are more extensive than in any other insect reported. Severely affected larvae from embryonic *Am-otd1* RNAi have anterior defects stretching all the way to eighth abdominal segment, and similarly *Am-hb* RNAi embryos retain only the three to four most-posterior segments. *Nv-otd1* knockdown in *Nasonia* leads to loss of the anterior and gnathal regions, as well as defects in the most posterior segments. *Nasonia hb* knockdown produces, at most, anterior, gnathal and

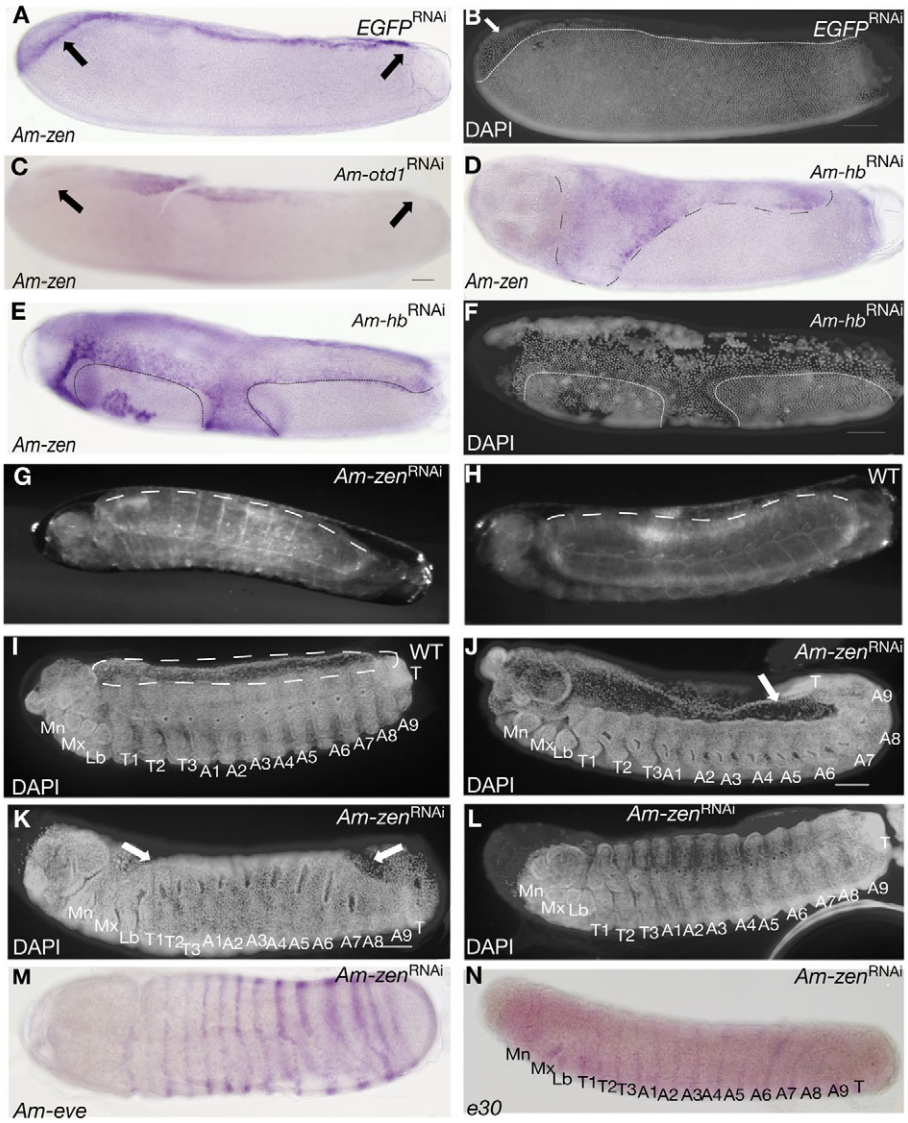

Fig. 6. *Am-otd1* and *Am-hb* regulate *Am-zen* expression and extra-embryonic membrane patterning. Embryos and larvae are oriented with anterior left, dorsal up. (**A**) Expression of *Am-zen* RNA in a stage 5 *EGFP* RNAi control embryo. *Am-zen* is expressed dorsally, with expansion in its domain at the anterior (arrow) corresponding to developing serosa and amnion, as determined by DAPI staining (**B**, arrow; nuclei are less densely packed in extra-embryonic membranes). (**C**) *Am-zen* RNA expression in a *Am-otd1* RNAi embryo. *Am-zen* RNA is reduced in the dorsal anterior and posterior, but unaffected in trunk regions. (**D**,**E**) *Am-zen* expression in an *Am-hb* RNAi stage 5 embryo is shifted from anterior into central regions. (**F**) DAPI staining of a stage 5 *Am-hb* RNAi embryo showing expansion of the extra-embryonic membrane. (**G**,**H**) *Am-zen* RNAi (G) and control larvae (H). Injected larvae have a more pronounced dorsal surface causing them to curve outwards (dashed line, G.) compared with controls (H). (**I-L**) DAPI stained control (I) and *Am-zen* RNAi embryos (J-L) showing the range of phenotypes produced. All segments are present in *Am-zen* RNAi embryos but the amnion is reduced (arrow, J) or missing (K), and segments extend to the dorsal edge of the embryo (K,L). (**M**,**N**) Stage 9 *Am-zen* RNAi embryos stained for *Am-eve* (M) and *e30* (N) RNA showing no loss of segments. Scale bars: 100 μm.

thoracic defects. RNAi against both genes gives broader defects, though rarely as extensive as individual knockdown in honeybee (Lynch et al., 2006). Mutants of the presumed replacement for *otd1* in *Drosophila*, *bicoid*, also only affect anterior patterning of the anterior abdominal segments (Struhl et al., 1989). In crickets RNAi knockdown of *Gb-otd1* leads to loss of anterior head regions (Nakamura et al., 2010). In honeybees definition of the anterior seems a crucial event in patterning the entire body, and in these embryos, anterior patterning defines most of the abdominal segments. This unusual extent of the influence of anterior patterning must be related to the unusual function of honeybee *caudal*, which regulates abdominal fate (Wilson et al., 2010) and is activated by *Am-otd1*. Loss of *Am-otd1* and *Am-otd2* causes loss of *Am-cad* leading to abdominal defects. This does not occur in *Nasonia*, as in this species *Nv-otd1* represses, rather than activates, *Nv-cad* (Olesnicky et al., 2006). Repression of caudal by anterior patterning molecules is common in insects (Rivera-Pomar et al., 1996; Schulz et al., 1998; Wolff et al., 1998; Moreno and Morata, 1999). The regulation of *cad* by Otd genes appears to have changed from an ancestral repression mechanism to activation in the lineage leading to honeybees.

Am-otd2 has acquired unique functions in honeybee embryogenesis. It is maternally expressed and required for both anterior patterning and segmental patterning in abdominal regions. In *Tribolium, Nasonia* and *Acrythosiphon*, *otd2* is reported as being expressed late in embryogenesis with no possible role in axis formation (Yuebing et al., 1996; Lynch et al., 2006; Schinko et al., 2008; Huang et al., 2010). In honeybee *Am-otd2* regulates known gap genes and affects the expression of pair-rule genes leading to defects in segmentation. In contrast to *Am-otd1*, *Am-otd2* appears to act as a repressor, restricting expression of *Am-gt and Am-cad*.

Some of the activity of both honeybee Otd genes and *hb* appear to be transduced through *giant*. In honeybees, as in *Nasonia*, *giant* is a key regulator of anterior fate required for head and thorax formation (Brent et al., 2007; Olesnicky and Desplan, 2007; Wilson et al., 2010). The *Am-gt* CRM we have identified has predicted binding sites for both hb and Otd, and removing these proteins in *Drosophila* embryos changes the expression of a reporter gene driven by the honeybee giant CRM. Both *Am-otd1*, *Am-otd2* and *Am-hb* knockdown affect *Am-gt* expression, indicating this CRM construct accurately reflects the regulation of *Am-gt*, and regulation by *Am-otd1*, *Am-otd2* and *Am-hb* is likely to be direct.

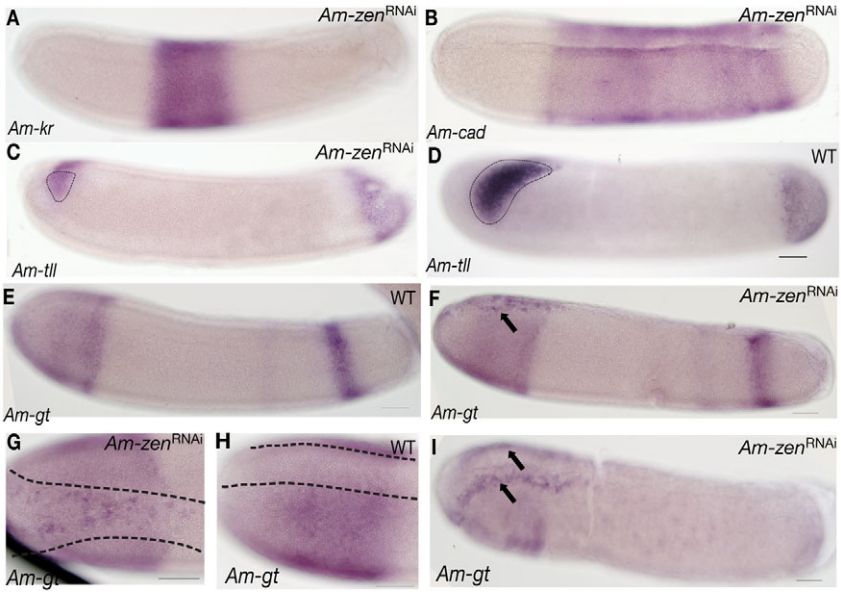

Fig. 7. Gap gene expression in *Am-zen* RNAi embryos. (**A**) Expression of *Am-Kr* is normal in *Am-zen* RNAi embryos (stage 4). (**B**) *Am-cad* expression is similar to that in wild type (cf. Fig. 5I) in *Am-zen* RNAi embryos. Expression of *Am-tll* expression is shifted anteriorly and reduced in *Am-zen* RNAi embryos (**C**) compared with controls (stage 5; **D**). Posterior expression is unaffected. (**E**) Wild-type expression of *Am-gt* (**F**). Expression of *Am-gt* is similar to that in wild type in *Am-zen* RNAi embryos at stage 4, however, ectopic expression occurs in anterodorsal cells (arrow, F). (**G**) Dorsal view of the anterior of an *Am-zen* RNAi embryo stained for *Am-gt* showing ectopic expression compared with controls (**H**). Ectopic expression persists into stage 7 (**I**). Scale bars: 100 μm.

Finally, in *Nasonia*, *Nv-otd1* is required for both anterior and posterior patterning, and the RNA is tethered to a posterior organelle, the oosome, in oocytes (Lynch et al., 2006). In honeybees both *Am-otd1* and *Am-otd2* are expressed in posterior regions, but no evidence of RNAi disruption of segmentation can be seen in the posterior, though these genes have later roles in this region, as shown by the loss of *Am-zen* expression in the posterior of *Am-otd1* knockdown embryos.

Regulation of dorsoventral gene expression by *orthodenticle* is an ancestral character

In *Tribolium*, anteroposterior patterning defects caused by *Tc-otd1* knockdown appear to be due to interactions with dorsoventral patterning (Kotkamp et al., 2010). *Tc-otd1* activates *zen* and *sog*, and these genes specify anterior fate. This regulatory linkage has not been investigated in *Nasonia*, and is not present in *Drosophila*, suggesting that it might be a quirk of *Tribolium* biology, perhaps related to the anterior placement of extra-embryonic membranes in *Tribolium* embryos (Falciani et al., 1996).

However, we show that some of the regulatory linkages identified in *Tribolium* are also present in the honeybee. *Am-otd1* regulates *zen*, and *zen* expression regulates *Am-gt*, as shown by our *Am-gt* CRM experiments. *Zen* is the ancestor of *bcd* (Stauber et al., 1999), but it is not *bcd* that is regulating our CRM fragment because *lacZ* is not expressed early enough, nor in a concentration-dependent way. This linkage between Otd genes and *zen* may be intact in some Diptera because in the cyclorrhaphan fly *Episyrphus*, *otd1* overexpression causes both reduction of the serosa and *zen* expression (Lemke and Schmidt-Ott, 2009).

The dorsoventral duplication of gnathal segments seen in mild *Am-otd1* knockdown is probably related to this dorsoventral patterning effect. The duplication is not, however, due solely to loss of *Am-zen*, because although the embryo extends more dorsally in *Am-zen* knockdown, it does not have the gnathal duplications seen in *Am-otd1* knockdown. We suggest that mild knockdown of *Am-otd1* may disrupt the identity of the anterior regions, perhaps due to loss of the highest concentrations of an *Am-otd1* gradient, leading to the uncovering of a anterodorsal patterning centre as suggested by *Am-e30* staining in Fig. 2H. Loss of *Am-zen* expression as a result of *Am-otd1* knockdown, leads to ectopic dorsal expansion of the embryo, which now, because of the absence of *Am-otd*, takes up a ventral, gnathal fate.

We show that the cross-talk between anteroposterior and dorsoventral patterning, seen in *Tribolium*, is conserved in honeybee. Honeybees are hymenoptera and considered to be the most basal branch of holometabolous insects (Krauss et al., 2005; Savard et al., 2006; Zdobnov and Bork, 2007), indicating this is an ancestral character in holometabolous insects. It seems that Otd

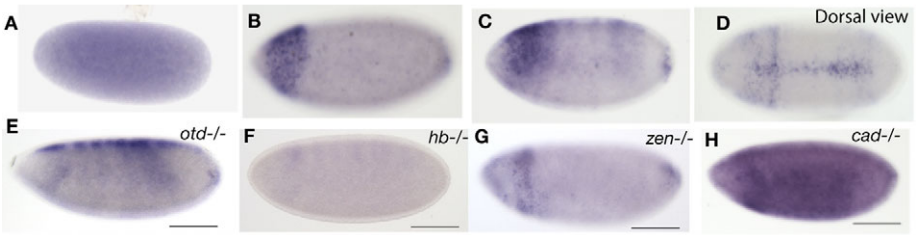

Fig. 8. Expression and regulation of *Am-gt* CRM in *Drosophila* embryos. (**A-D**) *Am-gt* CRM embryos stained for *lacZ* RNA. Embryos are oriented anterior left, dorsal up, unless otherwise stated. (A) Weak background expression is seen in stage 1 embryos. (B,C) Stage 5 embryos display an anterior domain of expression. (D) Dorsal view of a stage 6 embryo showing expression in a dorsal domain. (**E**) Expression of *Am-gt* CRM in a homozygous $otd^{-/-}$ embryo. Expression in the anterior domain is reduced. (**F**). Expression in a $hb^{-/-}$ embryo showing loss of expression. (**G**) *lacZ* reporter expression in a $zen^{-/-}$ embryo showing loss of dorsal expression. (**H**) Expression in a $cad^{-/-}$ mutant embryo with ubiquitous ectopic expression. Scale bars: 100 μm.

genes in the ancestor of holometabolous insects regulated both anteroposterior and dorsoventral systems, but in *Tribolium*, the contribution of *otd1* to anteroposterior patterning has been reduced.

The evolution of anterior patterning and buffering

The regulation of anterior fate in the honeybee is similar to anterior patterning in *Tribolium*, *Nasonia* and the honeybee, but the honeybee system is distinctly different. These differences are related to both the nature and strength of the regulatory interactions between anterior patterning molecules. We have shown that some of these changes are caused by functional changes in the regulatory regions driving expression in these genes.

These findings indicate that, as in *Nasonia*, Otd genes act, with *hb*, as maternal anterior patterning genes in the honeybee, but that the details of this regulation differs between these species. This diversity indicates that anteroposterior axis formation is an evolutionarily labile pathway, despite being required as the foundation of later, highly conserved, patterning. An important challenge for the future is to understand how changes in this regulatory network are buffered to produce the conserved gene expression outputs of both segmentation and dorsoventral patterning.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.067926/-/DC1

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