LETTERS

Localized maternal *orthodenticle* patterns anterior and posterior in the long germ wasp *Nasonia*

Jeremy A. Lynch[1], Ava E. Brent[1], David S. Leaf[2], Mary Anne Pultz[2] & Claude Desplan[1]

The Bicoid (Bcd) gradient in *Drosophila* has long been a model for the action of a morphogen in establishing embryonic polarity[1]. However, it is now clear that *bcd* is a unique feature of higher Diptera[2,3]. An evolutionarily ancient gene, *orthodenticle* (*otd*), has a *bcd*-like role in the beetle *Tribolium*[4]. Unlike the Bcd gradient, which arises by diffusion of protein from an anteriorly localized messenger RNA[1,5], the *Tribolium* Otd gradient forms by translational repression of *otd* mRNA by a posteriorly localized factor. These differences in gradient formation are correlated with differences in modes of embryonic patterning. *Drosophila* uses long germ embryogenesis, where the embryo derives from the entire anterior–posterior axis, and all segments are patterned at the blastoderm stage, before gastrulation. In contrast, *Tribolium* undergoes short germ embryogenesis: the embryo arises from cells in the posterior of the egg, and only anterior segments are patterned at the blastoderm stage, with the remaining segments arising after gastrulation from a growth zone. Here we describe the role of *otd* in the long germband embryo of the wasp *Nasonia vitripennis*. We show that *Nasonia otd* maternal mRNA is localized at both poles of the embryo, and resulting protein gradients pattern both poles. Thus, localized *Nasonia otd* has two major roles that allow long germ development. It activates anterior targets at the anterior of the egg in a manner reminiscent of the Bcd gradient, and it is required for pre-gastrulation expression of posterior gap genes.

otd had been proposed as an ancestral anterior patterning gene in insects for two major reasons. First, it is highly conserved among animals and has an anterior patterning role in most phyla in which its function has been tested. Second, although it is quite distantly related to *bicoid*, Otd protein has a lysine at position 50 of its homeodomain that gives it the same DNA binding specificity as Bcd[6].

Because a *Tribolium*-like system that forms an Otd gradient for patterning anterior structures based on a posteriorly localized source of translational repression would become increasingly inefficient as the germ rudiment extends more anteriorly, we sought to understand whether *otd* is a conserved anterior patterning factor in long germ insects that lack *bcd*.

To address this question, we examined the expression and function of *otd1* in the wasp *Nasonia*, which undergoes long germ development that seems to be morphologically similar to that of *Drosophila*, although early development takes longer[7,8]. The *Nasonia otd1* gene is orthologous to *Tribolium otd1*, which has an early role in axis formation[4]. There is a second *otd* gene (*otd2*) in both species that is only expressed later in development[9] (J.A.L. and C.D., manuscript in preparation).

We find that *Nasonia otd1* is expressed maternally in a surprising pattern. In early ovarian follicles, *otd1* mRNA is expressed in the nurse cells, and unexpectedly accumulates at the posterior of the oocyte (Fig. 1a). In later follicles, *otd1* mRNA remains localized at the posterior of the oocyte but also begins accumulating at the anterior pole (Fig. 1b).

In pre-pole cell embryos, *otd1* mRNA is seen tightly localized at the anterior pole, whereas posteriorly localized mRNA becomes associated with the oosome, a structure that is thought to be an equivalent of germ plasm. As seen in Fig. 1c, this oosome-associated mRNA can migrate some distance from the posterior extremity, but returns to the pole just before the pole cells begin to form. After pole cell formation and nuclear migration to the surface of the embryo, *otd1* mRNA remains in a bi-polar expression pattern, but appears to

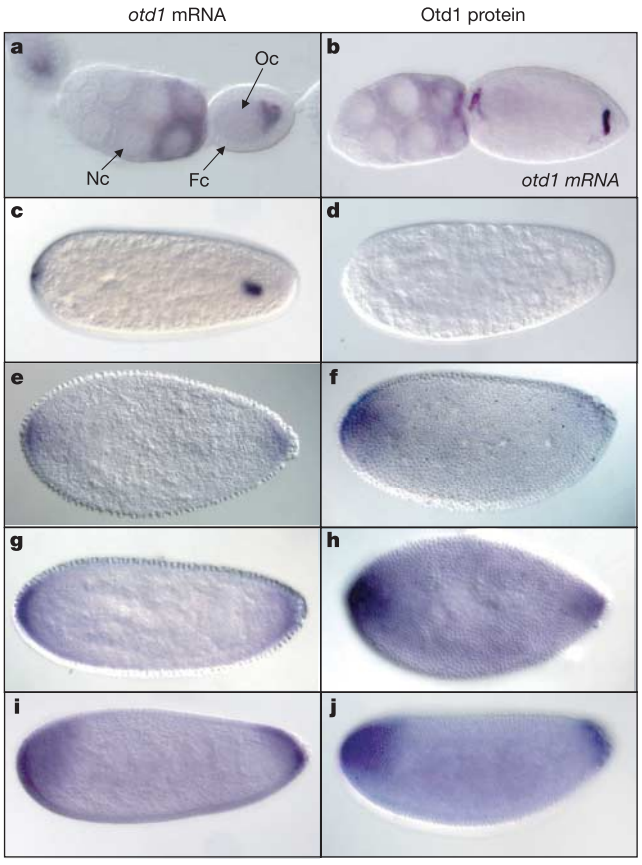

Figure 1 | *otd1* expression and localization. **a**, **b**, Ovarian *otd1* expression in early (**a**) and late (**b**) follicles. Nc, nurse cells; Fc, follicle cells; Oc, oocyte. **c**, **e**, **g**, **i**, Embryonic *otd1* mRNA expression in pre-pole cell (**c**), early (**e**) and late (**g**) syncytial blastoderm and cellular blastoderm (**i**) stages. **d**, **f**, **h**, **j**, Otd1 protein expression in pre-pole cell (**d**), early (**f**) and late (**h**) syncytial blastoderm, and cellular blastoderm (**j**) stages.

[1]Center for Developmental Genetics, NYU Biology, 100 Washington Square East New York, New York 10003, USA. [2]Western Washington University, Bellingham, Washington 98225, USA.

become less tightly localized to the cortex (Fig. 1c, e). Finally, after cellularization of the blastoderm, *otd1* mRNA is seen in cap-like domains at both the anterior and posterior poles (Fig. 1g).

The ovarian and early embryonic expression patterns of *Nasonia otd1* mRNA suggest that gradients of Otd1 protein may form by diffusion from localized sources of mRNA. An anterior Otd1 gradient would indicate possible convergent evolution of maternally localized mRNAs giving rise to gradients of K_{50} homeoproteins in patterning the anterior of both *Drosophila* and *Nasonia*. A posterior Otd1 gradient would show that *Nasonia* has recruited *otd1* to perform a novel posterior patterning role not present in any of its known orthologues. To determine whether Otd1 gradients exist in *Nasonia*, we generated an antibody to *Nasonia* Otd1.

No Otd1 protein is seen before pole cell formation (Fig. 1d), indicating a mechanism of translational repression at this stage. *Nasonia* Otd1 protein is first detected when nuclei begin arriving at the surface of the embryo, just after pole cell formation. Notably, Otd1 is initially only seen at the anterior, where it forms an anterior to posterior gradient (Fig. 1f). A posterior to anterior gradient becomes visible at the posterior pole towards the end of the syncytial blastoderm stage (Fig. 1h). This pattern indicates a second level of translational repression specific to the posterior aspect of *Nasonia otd1* mRNA, and is consistent with the timing of posterior translational repression seen for *Nasonia hunchback* (*hb*)[10]. Interestingly, sequences that are similar to known Nanos response elements (NREs) are found in the 3′ untranslated regions (UTRs) of both *Nasonia otd1* (gCGTTtcgccGcATTGTAcgag) and *hb*[10] (where upper case letters indicate a match with the consensus sequence), indicating that posteriorly localized *nanos* may be responsible for preventing the translation of both mRNAs during early syncytial blastoderm stages. Finally, in the cellular blastoderm, Otd1 protein is seen in the anterior and posterior domain, mimicking the expression of mRNA.

The maternal localization of *otd1* mRNA along with subsequent Otd1 protein gradients are consistent with this gene acting as a morphogen at both poles of the *Nasonia* embryo. If this were indeed the case, when levels of Otd1 are reduced, the posterior borders of anterior Otd1 target genes should be shifted towards the anterior, whereas the anterior borders of posterior targets should be shifted posteriorly. To test the function of *otd1*, we adapted to *Nasonia* the parental RNA interference (pRNAi) technique first developed in *Tribolium*[11]. We examined the expression of Otd1 in the offspring of *otd1* RNAi-injected mothers and observed varying amounts of reduction (see Supplementary Information), which correlates with variations in resulting phenotypes and effects on the expression patterns of the potential target genes *empty spiracles* (*ems*), *giant* (*gt*) and *hb*.

ems requires high levels of Bcd for expression in *Drosophila*[12]. *Nasonia ems* is expressed in a very similar pattern to that of *Drosophila* (Fig. 2a). As a very anterior target of Otd1, it should be extremely sensitive to a reduction in Otd1 levels. Indeed, when *otd1* is knocked down, most embryos lose *ems* expression entirely, with only a few exhibiting reduced and anteriorly shifted expression (Fig. 2b, c).

Similar to *Drosophila*, the gap gene *gt* has two domains of expression in *Nasonia* (Fig. 2c). In most cases, when *otd1* is knocked down both *gt* stripes are shifted towards their respective poles (Fig. 2e); in the most strongly affected embryos, expression is lost at the anterior, whereas some residual expression is still seen at the posterior pole (Fig. 2f).

The gap gene *hb* responds to low levels of Bcd[1,5,13] in flies and shows a broad anterior expression domain, as well as a posterior stripe[14]. *Nasonia hb* shows a similar pattern in the late blastoderm stages (Fig. 2g)[10]. In *otd1* RNAi embryos, the anterior *hb* domain shows a clear, although modest, anterior shift of its posterior boundary of expression (Fig. 2h, i). The degree to which *hb* is resilient to this

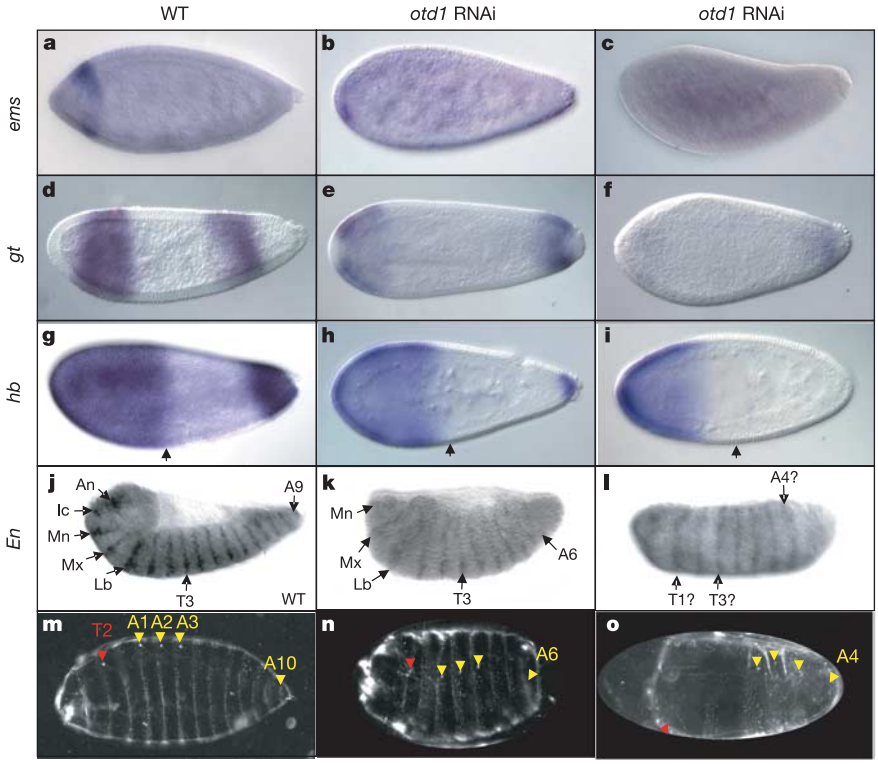

Figure 2 | Effects of *otd1* RNAi. **a–c**, *ems* expression in wild-type and *otd1* RNAi embryos. **d–f**, *gt* expression. **g–i**, *hb* expression (arrows indicate 50% egg length). **j–l**, Engrailed protein expression. The head segments have characteristic shapes. A, abdominal; An, antennal; Ic, intercalary; Lb, labial; Mn, mandibular; Mx, maxillary; T, thoracic. **m–o**, Cuticles of wild-type and *otd1* RNAi larvae. In wild-type larvae (**m**), mouthparts are visible anteriorly, and large spiracles are visible on the second thoracic (red arrow) and on the first three abdominal segments (yellow arrows). The left panel shows the wild-type pattern, whereas the middle panel shows moderate *otd1* RNAi phenotypes and the right panel severe *otd1* RNAi phenotypes.

knockdown is surprising, although it has been suggested that anterior zygotic *hb* can be activated by maternal *hb*[10]. Notably, although one aspect of zygotic *hb* expression is Bcd-dependent in *Drosophila*, this expression can be made dispensable as high levels of maternal *hb* can activate the zygotic function of *hb* required for thorax formation[15].

In contrast to the modest effects on anterior *hb* expression, knocking down *otd1* expression has a marked effect on the posterior domain of *hb*. In *Drosophila*, this stripe is activated by *tailless*[16], whereas in *Tribolium* it is not expressed until the latest stages of germband extension[17]. In *otd1* knocked down embryos, the anterior border of this stripe is either shifted to the extreme posterior pole of the embryo (Fig. 2h), or completely lost (Fig. 2i).

Knockdown of *otd1* results—presumably as a consequence of changes in expression of its targets—in the loss of both anterior and posterior segments: defects of varying severity are seen in both the expression of Engrailed protein (Fig. 2j–l and Table 1) and larval cuticle structures (Fig. 2m–o and Table 1). At the anterior end, Engrailed stripes and their corresponding cuticular segments are lost in an anterior to posterior progression, with RNAi phenotypes ranging from the loss of only the antennal stripe to the complete lack of head segments. At the posterior, Engrailed stripes and denticle belts appear to be lost in a posterior to anterior progression.

Because *hb* has been shown to cooperate with *bcd* in *Drosophila* and with *otd* in *Tribolium*[4,18], we performed double knockdown of *otd1* and *hb* (Table 1) to see whether a similar interaction exists in *Nasonia*. In the most severe cases, the entire anterior is lost, including several anterior abdominal segments. This is more severe than the combination of the most severe phenotypes seen by individual knockdown of either *otd1* or *hb*, or in the zygotic null *Nasonia hb* allele ($hb^{headless}$)[7,8,10] (Table 1; see also Supplementary Information), and the entire range of phenotypes is more severe. This indicates that *Nasonia otd1* and *hb* cooperate in anterior patterning. Although *hb* is also expressed and functions at the posterior, as in *Drosophila*, no increase in the severity or frequency of severe phenotypes is observed at the posterior in the double knockdown. The lack of synergy between Otd1 and Hb at the posterior may allow different functions for the anterior and posterior Otd1 gradients: Otd1 acts with Hb at the anterior, whereas it might act alone or in combination with a different factor (for example, Caudal; Fig. 3c) at the posterior to activate distinct sets of target genes.

These results show that *otd1* has broad roles in patterning both the anterior and posterior of the long germ *Nasonia* embryo. This

Table 1 | Effects of *otd1* RNAi on segmentation and synergy of *otd1* and *hb*

RNAi treatment	Remaining Engrailed head stripes (%)*						
	WT	Four	Three	Two	One	None	*n*
otd1 RNAi	0	2	41	38	12	7	85
RNAi treatment	Cuticle phenotypes (%)*						
	WT	I	II	III	IV	V	*n*
otd1 RNAi	0	91	7	2	0	0	120
hb RNAi	0	44	28	22	5	1	79
hb otd1 RNAi	0	22	15	25	23	16	109
RNAi treatment	Remaining posterior abdominal segments (%)†						
	WT	≥Nine	Eight	Seven	Six	Five	*n*
otd1 RNAi	0	11	27	33	22	7	107
hb RNAi	0	34	29	37	0	0	79
hb otd1 RNAi	0	16	33	27	13	11	100

*Anterior defects caused by RNAi for *otd1*, *hb* and a combination of the two. Anterior *otd1* defects were quantified by examining the expression of Engrailed using the antibody 4D9 (ref. 26) to take advantage of the easily discernible shapes of the five head stripes[7] (see also Fig. 2). The cuticle phenotypes are divided into five categories: I, weak head defects; II, no head structures; III, headless with thoracic defects; IV, no head or thorax; V, defects extending into anterior abdomen.
†Posterior RNAi phenotypes. There are ten abdominal denticle belts on the wild-type cuticle. The ≥nine abdominal segment class includes embryos with the normal number of denticle belts but defects in spacing.

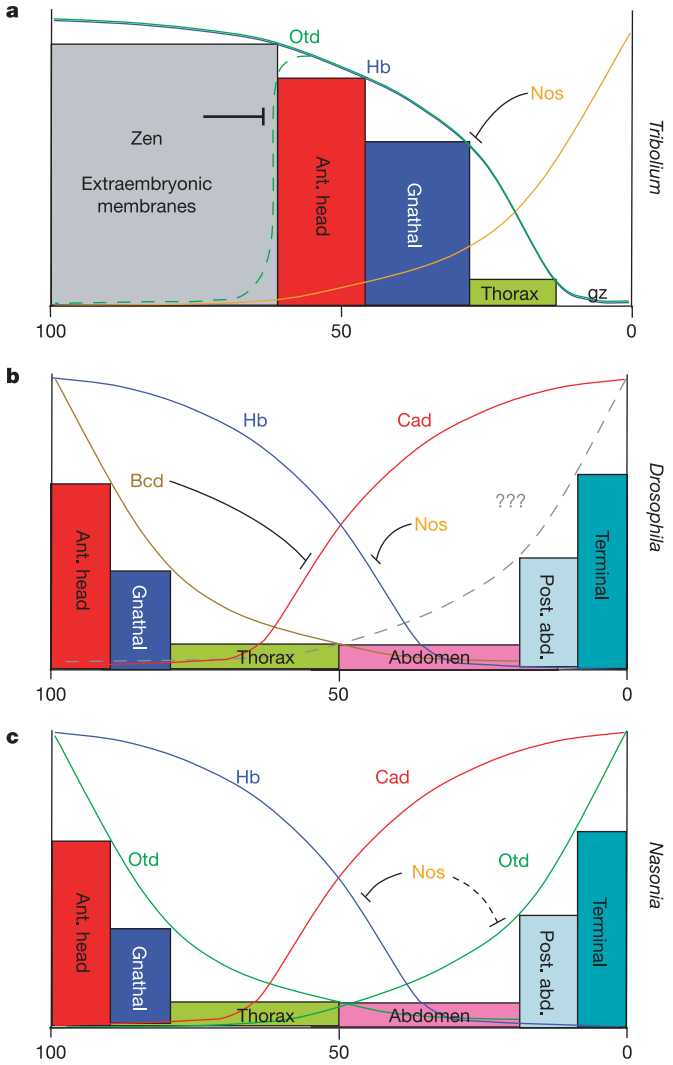

Figure 3 | Models for patterning the blastoderm of insect model systems. **a**, Schematic representation of patterning of the blastoderm stage of a short germ insect, based on what is known in *Tribolium*. In *Tribolium*, Otd and Hb gradients result from translational repression of ubiquitous mRNAs by a posteriorly localized factor, most likely Nanos (both of the genes have NREs in their 3′ UTRs)[4,17]. Genes responsible for patterning the head and thorax probably respond to these factors in a concentration-specific manner, much as they do to Bcd in *Drosophila*. The remainder of the embryo is patterned in the growth zone. *Tribolium* Zerknullt (Zen) represses embryonic fates in the anterior, and specifies the extraembryonic membranes[27]. gz, growth zone. **b**, Summary of patterning in *Drosophila*. In this long germ embryo, anterior fates are established at the anterior pole as a result of a gradient of Bcd formed by diffusion of protein translated from a localized source of mRNA. It is not clear how the posterior genes are activated, but it may involve cooperation between Bcd and Cad, and possibly an additional posterior factor, such as Tailless. **c**, Schematic representation of the long germ blastoderm of *Nasonia*. The localization of *otd1* mRNA at the anterior pole of the egg probably results in a steep gradient of protein. This allows efficient patterning of the head and thorax in the anterior half of the egg, in synergy with Hb, which is restricted from the posterior pole, probably by Nanos (Nanos also seems to delay the formation of the posterior Otd1 gradient, represented by the dashed line). *Nasonia otd1* is also localized to the posterior pole, which provides posterior positional information that is interpreted by downstream targets, perhaps by acting with Cad. Nos, Nanos.

provides insight into how a long germ type of patterning might have arisen from a short germ embryo. Major changes in patterning mechanisms at both the anterior and posterior ends of the embryo are required to pattern a long germ embryo. At the anterior, the egg fate map must be shifted so that the anterior (that is, head and thoracic) segments are patterned near the anterior pole. In addition, there must be a source of patterning information that is sufficiently strong to allow relatively tight spacing of these segments, in order to allow enough room at the posterior to pattern the abdomen. A system such as that found in *Tribolium* (Fig. 3a) does not seem compatible with this because the diffusion gradient arising from a posteriorly localized patterning centre will be shallowest at the anterior, where it would carry the least patterning information. *Nasonia* solves this by localizing *otd1* mRNA at the anterior pole, which results in a protein gradient with the most patterning information at the anterior of the embryo. This allows genes for which the orthologues are expressed more posteriorly in *Tribolium* to be activated close to the anterior pole in *Nasonia* (Fig. 3c). This is similar to the strategy used in *Drosophila* with localized *bcd* transcript (Fig. 3b).

Posterior patterning poses another problem for long germ embryos. In short germ insects, genes patterning posterior segments (such as the posterior domains of *gt* and *hb*) are expressed after gastrulation in a 'growth zone', whereas long germ embryos must express them at the blastoderm stage, before gastrulation. Again, *Nasonia* solves this problem by localizing *otd1* mRNA, this time at the posterior pole. The posterior gradient of Otd1 seems to be required for proper expression of posterior gap genes before gastrulation (Fig. 3c). This is a divergent strategy compared to that used in *Drosophila*, where neither *bcd* nor *otd* mRNA is localized at the posterior, and it is not clear which gene(s) provides morphogenetic information at the posterior. It may be that Bcd function extends posteriorly, because it acts with Caudal to activate posterior *knirps*[19] and *hairy* stripe seven[20]. Alternatively, or in addition, the terminal system might act through the induction of *tailless*, which has been shown to have some characteristics of a morphogen at the posterior[16].

Although our results give insights into mechanisms of developmental evolutionary change, it will be of interest to understand whether what is seen in *Nasonia* is part of a larger pattern. For example, both *Nasonia* and *Drosophila* use the localization of mRNA encoding K_{50} homeoprotein (Otd1 or Bcd) to pattern the anterior of the egg. Is this a general strategy for long germband patterning? On the other hand, there is no clear parallel between the posterior patterning systems of *Nasonia* and *Drosophila*. Is either strategy more common in other long germband embryos? To address this, the mechanisms used in other insects that have evolved long germband embryos must be elucidated. A number of key long germband taxa, including flies that lack *bcd*[3], a mosquito[21], moths[22] and a beetle[23] have been used as laboratory organisms, and the application of further gene expression and functional analyses to these organisms should allow the placement of the *Nasonia* patterning system in a larger context.

METHODS

Nasonia orthologues of *otd1*, *ems* and *gt* were cloned using the following strategy: orthologues of these genes were identified in GenBank, and aligned using the MEGALIGN program in the DNAstar package. Degenerate primers corresponding to conserved regions were designed using the CODEHOP algorithm[24]. Sequences obtained by degenerate polymerase chain reaction (PCR) were extended in the 5′ and 3′ directions by RACE–PCR using the SMART RACE kit (Clontech).

For single RNAi, a $1\,\mu g\,\mu l^{-1}$ solution of double stranded (ds)RNA was injected into young female pupae, and the embryos laid by the injected mothers were examined. For double RNAi experiments, dsRNA for both genes were at final concentration of $0.75\,\mu g\,\mu l^{-1}$. Negative controls of distilled water and GFP dsRNA caused no defects.

Embryos for *in situ* hybridization and antibody experiments were fixed in formaldehyde-saturated heptane for 30 min, affixed to double-sided tape and the vitelline membranes removed by hand under PBS.

Digoxigenin-labelled probes were generated using run-off transcription, using either T7 or SP6 RNA polymerase, of cloned fragments of the genes analysed in this work. *In situ* hybridization was done using standard protocols[25].

The affinity purified *Nasonia* Otd1 antibody was raised against the peptide CSPPRAKEAPGPNSP (Sigma-Genosys), used at a concentration of 1:100, and detected using an alkaline phosphatase conjugated anti-rabbit secondary antibody at 1:500 dilution. The monoclonal Engrailed antibody 4D9 was a gift from N. Patel, used at 1:10 dilution, and was detected using an alkaline phosphatase conjugated anti-mouse secondary antibody at 1:500 dilution. Cuticles were incubated at 65 °C overnight in 90% lactic acid/10% ethanol under a coverslip and photographed using dark-field microscopy.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.A.L. and C.D. conceived and designed the experiments. J.A.L. performed the experiments and generated data for the figures. A.E.B. cloned *Nasonia giant* and provided the probe. M.A.P. and D.S.L. cloned *Nasonia hunchback*, provided valuable reagents and were instrumental in initiating the project. J.A.L. and C.D. wrote the paper.

Author Information The sequences reported here are deposited in GenBank under the following accession numbers: *otd1*, AY684810; *ems*, AY684808; *giant*, DQ250085. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.D. (cd38@nyu.edu).