Mapping and cloning of *MOC1*

MOC1 was mapped primarily with Simple Sequence Length Polymorphism (SSLP) and Restriction Fragment Length Polymorphism (RFLP) markers[25], using 280 F_2 mutant plants. The *MOC1* locus was further placed within a 20-kb region between the markers 17-3 and 12-2 by using 2,010 F_2 mutant plants and newly developed molecular markers (Supplementary Table 1). The candidate gene was amplified from both the *moc1* and wild-type genomic DNA using primers MOC1F1 and MOC1R3 (5′-TCGTTGTAGTAGCTCT GGTG-3′ and 5′-CTAACTAGAGATCGAGTAGC-3′), and the PCR products were sequenced directly.

Complementation test

A 3.2-kb genomic DNA fragment containing the entire *MOC1* coding region, the 1,534-bp upstream sequence and the 316-bp downstream sequence, was inserted into the binary vector pCAMBIA1300 to generate a transformation plasmid pC8247. A control plasmid, pC8247S, containing a 3′ truncated *MOC1* gene that encodes the first 283 amino-acid residues, was also constructed. The two binary plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 and the *moc1* mutant was transformed as reported previously[26]. Fifteen independent transgenic lines were obtained for pC8247 and four for pC8247S, respectively. Each transgenic line included 10–100 sibling plants.

Subcellular localization

MOC1–GFP fusion was made by replacing the CaMV35SΩ region with a 2.9-kb DNA fragment that contained the entire *MOC1* coding region and 1.5-kb upstream sequence in the CaMV35SΩ-sGFP (S65T)-NOS-3′ cassette vector[27]. The construct was sequenced to verify the in-frame fusion and no nucleotide mutations. The *MOC1–GFP* fusion gene was subcloned into the binary vector pCAMBIA1300 and then transformed into wild-type rice plants. The tip of the transgenic rice plant stem was sectioned longitudinally, stained for 30 min with 2 μg ml^{-1} propidium iodide (PI) (in 30 mM 2-(N-morpholino)-ethanesulphonic acid and 100 mM mannitol, pH 5.9), and visualized with a confocal microscope (Bio-Rad MRC 1024).

Histology and *in situ* hybridization

Shoot apexes of rice seedlings at the four-leaf stage were fixed with formalin–acetic acid–alcohol (FAA) fixative solution at 4 °C overnight followed by dehydration steps and then embedded in paraffin (Paraplast Plus, Sigma). The tissues were sliced into 7–10-μm sections with a microtome (Leica RM2145), affixed to microscope slides, and stained with Safranin O and Fast Green (Fisher). Sections were observed under bright-field through a microscope (Leica DMR) and photographed using a Micro Color charge-coupled device (CCD) camera (Apogee Instruments).

RNA *in situ* hybridization was performed as described previously[28]. The 3′ ends of *MOC1*, *OsTB1* and *OSH1* were subcloned into pBluescript SK(+) vector and used as templates to generate sense and antisense RNA probes. Digoxigenin-labelled RNA probes were prepared using a DIG Northern Starter Kit (Cat. No. 2039672, Roche) according to the manufacturer's instructions. The slides were observed under bright-field and epifluorescence at the same time with a microscope (Leica DMR) and photographed using a 3CCD colour video camera (DXC-390P, Sony).

RNA extraction and analysis

Total RNA extraction was performed as described previously[29]. The first-strand cDNA was synthesized from total RNA and used as RT–PCR templates. RT–PCR primers for *OSH1* were 5′-GAGATTGATGCACATGGTGTG-3′ and 5′-ATTAGCAGCAGCAAGAGTAGC-3′, for *OsTB1* were 5′-AAGTTCTTCGCGCTCCAGGA-3′ and 5′-AACGCCATGATCACGT CGCT-3′, and for actin were 5′-TCATGAAGATCCTGACGGAG-3′ and 5′-AACAGCTCC TCTTGGCTTAG-3′. Rapid amplification of cDNA ends (RACE)–PCR was performed with the 5′/3′ RACE Kit (Roche) according to the manufacturer's instructions.

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The genes *orthodenticle* and *hunchback* substitute for *bicoid* in the beetle *Tribolium*

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In *Drosophila*, the morphogen Bicoid organizes anterior patterning in a concentration-dependent manner by activating the transcription of target genes such as *orthodenticle* (*otd*)[1] and *hunchback* (*hb*), and by repressing the translation of *caudal*[2,3]. Homologues of the *bicoid* gene have not been isolated in any organism apart from the higher Dipterans[4–7]. In fact, head and thorax formation in other insects is poorly understood. To elucidate this process in a short-germband insect, I analysed

the function of the conserved genes *orthodenticle-1* (*otd-1*) and *hb* in the flour beetle *Tribolium castaneum*. Here I show that, in contrast to *Drosophila*, *Tribolium otd-1* messenger RNA is maternally inherited by the embryo. Reduction of *Tribolium otd-1* levels by RNA interference (RNAi) results in headless embryos. This shows that *otd-1* is required for anterior patterning in *Tribolium*. As in *Drosophila*, *Tribolium hb* specifies posterior gnathal and thoracic segments. The head, thorax and the anterior abdomen fail to develop in *otd-1/hb* double-RNAi embryos. This phenotype is similar to that of strong *bicoid* mutants in *Drosophila*. I propose that *otd-1* and *hb* are part of an ancestral anterior patterning system.

In *Drosophila*, *bicoid* is the central gene in the anterior patterning system. Embryos that do not express Bicoid protein fail to develop a head or thorax, and form a second telson at the anterior pole instead. Binding specificity of Bicoid to its DNA[1,2] and RNA[8,9] targets is mediated by a lysine (K) at position 50 of its homeodomain (K_{50}HD). Despite its pivotal role in defining anterior pattern in *Drosophila*, *bicoid* orthologues have been isolated only from closely related higher Dipterans[4–6]. In *Drosophila* and other higher Dipterans[7], a *bicoid* orthologue is located in the *Hox* gene complex close to the *zerknüllt/Hox3* locus—between *proboscipedia/Hox2* and *Deformed/Hox4*. A *bicoid* orthologue is not present in this genomic interval in the beetle *Tribolium*[7]. This finding corroborates the hypothesis that *bicoid* evolved recently—probably through the divergence of a *Hox3* paralogue during evolution of the higher Dipterans[6,10,11]—and is not part of an ancient anterior system common to more basal insects.

How is anterior patterning in the embryo organized in the absence of *bicoid*? In *Drosophila bicoid* mutants, high levels of *bicoid*-independent zygotic *hb* are able to direct the formation of a partial thorax[12]. However, since *hb* cannot induce formation of head structures on its own, the conserved protein Otd, which like *Bicoid* contains a K_{50} homeodomain, has been considered to be an ancestral head determinant[12]. This hypothesis has been tested in an insect that develops as a short-germband embryo—and therefore shows a more general type of embryogenesis[13].

First, I analysed the function of *otd-1* in the beetle *Tribolium*. In *Drosophila*, *otd* acts as a zygotic head gap gene that specifies the ocular and the antennal segments[14]. Of the two *otd* paralogues—*otd-1* and *otd-2*—known to exist in *Tribolium*, only *otd-1* is expressed during early blastoderm formation[15]. Like its orthologue in *Drosophila*, zygotic *otd-1* in *Tribolium* is expressed in an anterior position during early embryogenesis. This indicates that *otd-1* might specify similar head segments in both species[15]. In contrast to *Drosophila*, *Tribolium otd-1* mRNA is provided maternally to the developing embryo. *Tribolium otd-1* mRNA is initially distributed uniformly in the egg, and is present at the beginning of blastoderm formation (Fig. 1a–d). During blastoderm stages, *otd-1* mRNA and protein recede first from the posterior (Fig. 1i–l) and later also from the anterior pole, resulting in a belt-like domain in the prospective head region (Fig. 1e). Dorsal *otd-1* mRNA then begins to disappear (Fig. 1f), leading to a ventrally located head stripe.

In *Tribolium*, RNAi has been shown to phenocopy gene disruption[16]. To investigate the function of *otd-1* during early pattern formation in *Tribolium*, I injected double-stranded (ds) *otd-1* RNA into eggs (*otd-1*^{eRNAi}) or female pupae (*otd-1*^{pRNAi}) (ref. 17). By applying pRNAi, I specifically aimed to knock down maternally supplied *otd-1* RNA and to avoid injection artefacts. Of the larval cuticles analysed, 91% of *otd*-1^{eRNAi} and 100% of *otd-1*^{pRNAi} developed defects in anterior patterning (Fig. 2a and Table 1). In the most extreme cases, the embryos lacked all head structures (Fig. 3e), indicating that *Tribolium otd-1* is required for head formation. Depending on severity, these cuticles can be grouped into classes I–VI—from weakly to strongly affected larvae (Figs 2c–h and 3c, d, e and Table 1). The range of cuticle defects is reminiscent of weak and intermediate *bicoid* mutants in *Drosophila*[18]. Moreover, head defects are detectable before cuticle formation in embryos, as determined by an analysis of Engrailed expression (see Fig. 4b and Supplementary Information). This shows that *Tribolium otd-1* is required for anterior patterning early during embryogenesis.

A significantly greater number of the more strongly affected class III and IV embryos was observed after pRNAi, compared with eRNAi (Fig. 2a and Table 1). The pRNAi depletes most of the maternal and zygotic *otd-1* mRNA, whereas eRNAi seems to deplete only zygotic *otd-1* mRNA. So, *otd-1* seems to be required to define the presumptive head region in *Tribolium*. Furthermore, maternal *otd-1* displays functional characteristics of a coordinate gene, as *bicoid* does in *Drosophila*, rather than of a canonical head gap gene. In *Tribolium* and *Drosophila*, zygotic *otd* seems to act as a head gap gene.

otd-1 is also involved in the formation of the anterior-most region of the egg—the extraembryonic serosa. In *otd-1*^{pRNAi} embryos, the nuclei of the serosa are irregularly arranged or, in more severe cases, reduced in number (see Supplementary Information). *hb*, which is strongly expressed in the serosa of the wild-type embryos[19], is also still detectable in the nuclei of the serosa in *otd-1*^{pRNAi} embryos. *otd-1* is therefore not required to activate *hb* expression in the

Table 1 **Phenotypes obtained in the *otd-1*, *hb* and *otd-1/hb* RNAi experiments**

***otd-1* RNAi cuticles**

		Weak to strong head defects				Headless ϕ		
	WT	I	II	III	IV	V	VI	*Dll* ϕ
Experiment	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)
otd-1 eRNAi ($n = 100$)	9 (9)	1 (1)	45 (45)	25 (25)	10 (10)	6 (6)	4 (4)	-
Control: H_2O injection into eggs ($n = 74$)	96 (71)	3 (2*)	-	1 (1)	-	-	-	-
otd-1 pRNAi ($n = 111$)	-	-	8 (9)	52 (58)	35 (39)	3 (3)	2 (2)	-
Control: H_2O injection into pupae ($n = 32$)	100 (32)	-	-	-	-	-	-	-
Control: *Dll* pRNAi ($n = 43$)	-	-	-	-	-	-	-	100 (43)

***hb* pRNAi cuticles**

	WT	Weak ϕ	Gap ϕ	8 AS	7 AS	6 AS
Experiment	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)
hb pRNAi ($n = 168$)	11 (19)	39 (65)	50 (84)	67 (56)	21 (18)	12 (10)

***otd-1/hb* pRNAi embryos stained for Engrailed expression**

	WT	*hb* ϕ	Headless ϕ	6 AS	5 AS	4 AS	3 AS	2 AS
Experiment	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)
otd-1/hb pRNAi ($n = 61$)	33 (20)	1.5 (1)	65.5 (40)	2.5 (1)	12.5 (5)	35 (14)	30 (12)	20 (8)

The *otd-1*^{RNAi} phenotype is variable. The cuticles obtained are grouped into classes I–VI (also shown in Fig. 2c–h). Class V and VI show the headless phenotype (headless ϕ). In the water-injected control embryos, two (*) without eyes but with intact antennae were also grouped as class I cuticles. For the *Distal-less* phenotype (*Dll* ϕ), see also ref. 17. The *hb*^{pRNAi} gap phenotype (see Fig. 3f) was observed in 50% of the analysed cuticles. Weaker phenotypes (weak ϕ) have also been obtained and will be described elsewhere. The number of abdominal segments (AS) varies in embryos that display the *hb*^{pRNAi} gap phenotype. Presumably, the abdominal segments A8 and A7 are missing in embryos with only six abdominal segments, since *hb* is also expressed in the primordia of these segments[19]. Cuticles with fewer abdominal segments were not found. *otd-1/hb*^{pRNAi} embryos were fixed and immunohistochemically stained for the expression of Engrailed. 65.5% of the analysed cuticles have the headless phenotype (headless ϕ). The number of abdominal segments varies in these headless embryos from two to six.

serosa. Nevertheless, *otd-1* could assist other factors in activating *hb* expression, such as *zerknüllt*, which is also expressed in this tissue[20].

The fact that the *otd-1* ^{RNAi} phenotype only incompletely mimics the *Drosophila bicoid* mutant phenotype implies that *otd-1* is only a partial functional equivalent of *bicoid* in *Tribolium*. I disrupted *hb* expression by pRNAi to determine whether this would result in a phenotype overlapping with that of *otd-1* ^{RNAi}. Disruption of *hb* mRNA resulted in a lack of the maxillary, the labial and all three thoracic segments in 50% of the analysed embryos (see Fig. 3f, Table 1 and Supplementary Information), whereas the anterior-most head segments all developed normally. *Drosophila* embryos that lack maternally derived and zygotic *hb* have the same phenotype[21] (E. A. Wimmer and C. Desplan, personal communication). This indicates that, in *Drosophila* and *Tribolium*, *hb* acts as a canonical head gap gene. In the wasp *Nasonia*, the *hb* mutant phenotype is more severe, in that the complete head (except for the anterior-most labrum) and the thorax are missing[22]. So, the function of *hb* as a gap gene has been conserved throughout evolution.

The head defects observed in the *otd-1* ^{RNAi} experiments indicate that *otd-1* and *hb* are both involved in regulating the development of gnathal segments. To evaluate the extent of their overlapping functions, embryos were generated in which both *otd-1* and *hb* expression were disrupted by pRNAi. These embryos were called

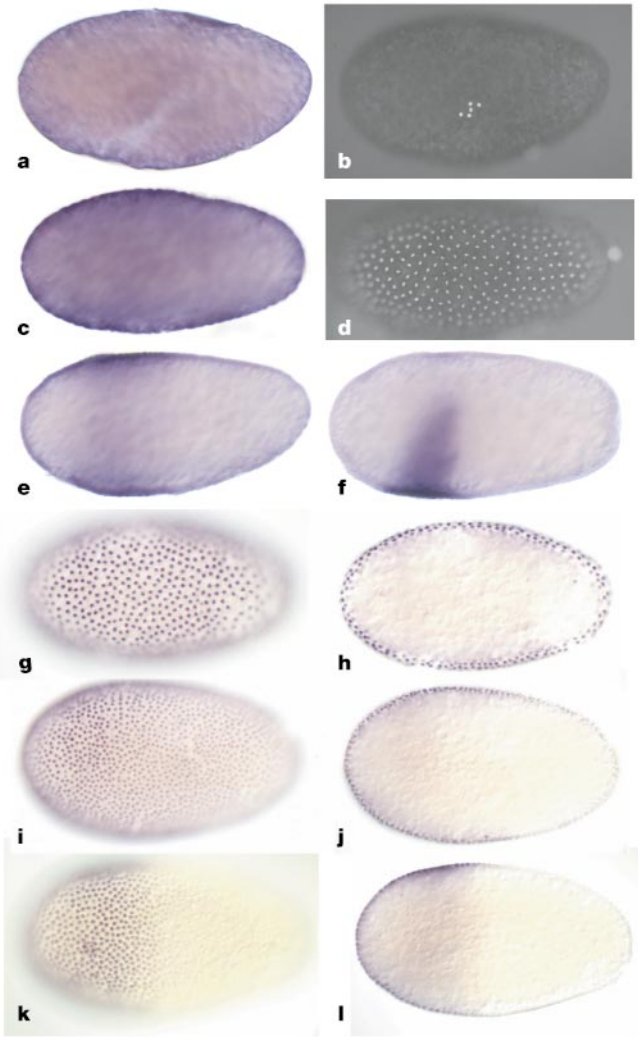

Figure 1 *otd-1* expression during blastoderm formation in *Tribolium*. **a**–**f**, *otd-1* mRNA expression (optical sections; anterior to the left). **a**, Uniform distribution of maternal *otd-1* mRNA in a freshly laid egg. **b**, The same egg as in **a**, but with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining. **c**, *otd-1* mRNA remains evenly distributed during the early stages of blastoderm formation. **d**, The same egg as in **c**, but with DAPI staining. **e**, At a later blastoderm stage, *otd-1* mRNA retracts first from the posterior and then from the anterior egg pole. Presumably, at around this stage, maternal expression starts to overlap with increasing levels of zygotic expression. **f**, Ventral head-specific *otd-1* is expressed in a wedge-like domain only after the beginning of serosa differentiation (anterior to the *otd-1* stripe). At this stage, *otd-1* is probably expressed solely from zygotic mRNA. **g**–**l**, Otd-1 protein expression (**g**, **i**, **k**, surface view; **h**, **j**, **l**, optical section). **g**, **h**, Uniform expression of Otd-1 protein at the beginning of blastoderm formation. **i**, **j**, Otd-1 protein recedes from the posterior pole, leading to a transient gradient that spans the posterior half of the embryo. **k**, **l**, *otd-1* mRNA (as previously described[15]) and protein are expressed as an anterior cap. A short-range gradient is seen in the centre of the egg at the border between expressing and non-expressing cells.

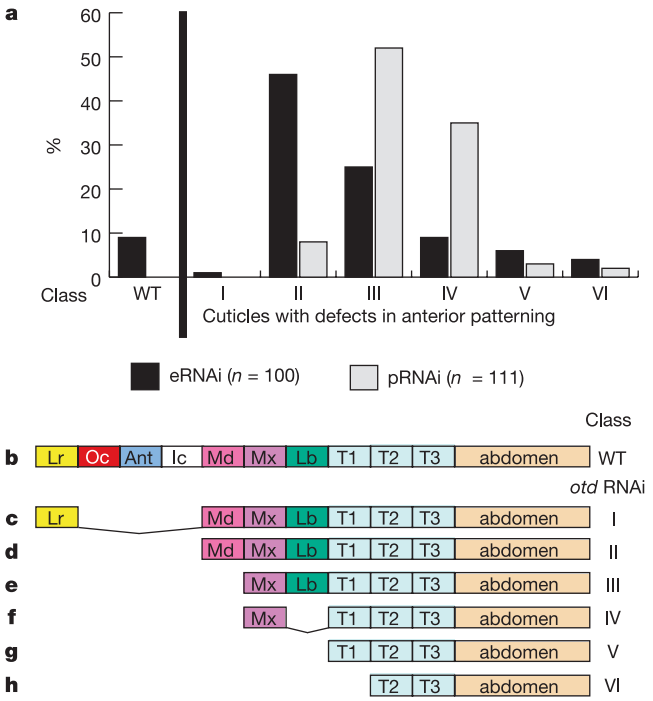

Figure 2 Effect of *otd-1* ^{RNAi} on embryonic development. **a**, Analysis of the cuticle phenotype of *otd*-1^{eRNAi} and *otd-1* ^{pRNAi} larvae. The same batch of dsRNA was used in both experimental series. WT, wild type. **b**, Organization of head and trunk: Lr, labrum; Oc, ocular segment with the eyes; Ant, antennae; Md, mandibles; Mx, maxillae; Lb, labium; T1–3, thoracic segments 1 to 3; abdomen. The intercalary (Ic) segment between the antennal and mandibular segment is not represented in the larval cuticle, but was added for completeness. **c**–**h**, Schematic representation of *otd-1* ^{RNAi} phenocopies. **c**, *otd-1* ^{RNAi} class I: gap phenotype. In *Drosophila*, this phenotype is seen in strong *otd* mutants. According to this phenotype, *Drosophila otd* was classified as a head gap gene[14]. Class I embryos frequently occur in other *otd-1* ^{RNAi} series using different *otd-1* dsRNA preparations. **d**, *otd-1* ^{RNAi} class II: pregnathal head missing. As in class I, it cannot be judged whether the intercalary segment is present. **e**, *otd-1* ^{RNAi} class III: anterior-most gnathal segment (mandible) missing. **f**, *otd-1* ^{RNAi} class IV: maxillary segment fused to thorax. Of the gnathum, the maxilla is the most stable segment with respect to the reduction of *otd-1* function. This indicates that parallel to *otd-1*, other genes are involved in specifying this segment. As judged from the analysis of *otd-1* ^{RNAi} embryos stained for Engrailed expression (see Supplementary Information), the appendage seen is not a transformation of the labium. Rather, the labium is lost, leaving the maxilla in front of the thorax. **g**, *otd-1* ^{RNAi} class V: headless phenotype; thorax and abdomen complete. **h**, *otd-1* ^{RNAi} class VI: headless phenotype; thorax 1 missing; thoracic segment 2 and 3 formed normally; abdomen complete. Defects in thoracic segment 1 are probably not directly related to missing *otd-1* function, but rather the indirect consequence of defects in anterior pattering on more posterior patterning.

otd-1/hb^{pRNAi} double phenocopies. Forty out of 61 (65.5%) of these embryos developed a headless phenotype (Table 1), indicating that *otd-1* and *hb* function together to regulate head development.

Analysis of the Engrailed expression pattern in the *otd-1/hb*^{pRNAi} embryos revealed that only two to six abdominal segments of normal polarity develop in *otd-1/hb*^{pRNAi} embryos (see Figs 3g and 4d, Table 1 and Supplementary Information). This shows that *otd-1* and *hb* not only direct the development of head and thorax, but also act synergistically during the segmentation process of the anterior abdomen—possibly by regulating posterior gap and/or homeotic genes. A synergistic relationship between *bicoid* and *hb* has been described previously in *Drosophila*[10], and might represent an evolutionary principle to confer robustness to the segmentation process. In *Tribolium*, *Otd-1* and *Hb* might substitute for the function of *Bicoid* as a transcriptional activator in *Drosophila*.

In *Drosophila*, *caudal* is translationally repressed in the anterior half of the egg by Bicoid[2,3]. Since no ectopic posterior structures form at the anterior pole of *otd-1/hb*^{pRNAi} embryos, Otd-1 might not serve as a translational repressor in *Tribolium*, as Bicoid does in *Drosophila*. In *Drosophila*, the RNA-binding activity of Bicoid—and therefore its ability to inhibit *caudal* translation—requires the presence of an arginine at residue 54 of its homeodomain 23. This amino acid is replaced by an alanine in the homologous position of *Tribolium Otd-1*, so its RNA- but not DNA-binding specificity might be lost. A repressor of *Tribolium caudal* is therefore likely to exist in the beetle, and serves as a further component of the anterior development system. Such a repressor has yet to be identified, but different mechanisms of *caudal* regulation have been described in other systems[24,25]. So, during Dipteran evolution, the function of Bicoid seems to have replaced the function of maternally inherited *otd-1*, and that of a repressor of *caudal*.

The expression of *otd-1* (Fig. 1) might be controlled early in embryogenesis by repression, and during later stages by autoactivation. Maternal *otd-1* mRNA translation could be repressed at the posterior pole, in a manner similar to that of maternal *hb* in *Drosophila*. In *Drosophila*[26], and similarly in the grasshopper *Schistocerca*[27], *hb* expression is repressed by the Pumilio/Nanos complex. A predicted Nanos-response element (NRE), identified by the sequence TgGTTGTattatAATTGTAggTA (position 1429–1451, capital letters indicating identity to the NRE of *hb* in *Drosophila* and *Musca*[28]), is present in the 3′ untranslated region of *Tribolium otd-1*. Although no orthologue of *pumilio* or *nanos* has been isolated from *Tribolium*, their function in *Tribolium* has already been implicated by the downregulation of maternal *hb* at the posterior pole of the blastoderm embryo[19]. Maternal *otd-1* could serve to activate the expression of zygotic *otd-1* only in the

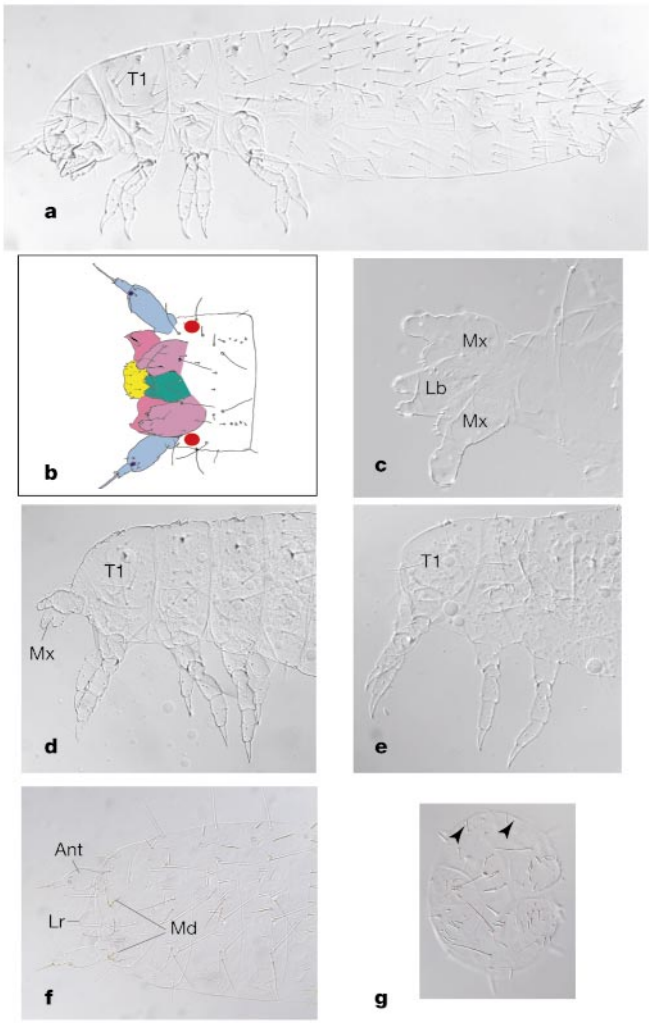

Figure 3 Cuticle preparations of wild-type and RNAi embryos. **a**, Wild-type cuticle of a first-instar larva. The thorax bears three pairs of larval legs. **b**, Cuticle of a wild-type head (camera lucida drawing of a first-instar larva). The appendages of each head segment are colour-coded, according to Fig. 2b. The eyes, which can be recognized before embedding, were added. **c**, *otd-1*^{RNAi} class III cuticle: compare to Fig. 4b. **d**, *otd-1*^{RNAi} class IV cuticle. **e**, *otd-1*^{RNAi} class V cuticle: headless phenotype. **f**, *hb*^{RNAi} cuticle. **g**, *otd-1/hb*^{RNAi} cuticle. Two segments are represented by two tracheal openings (arrowheads).

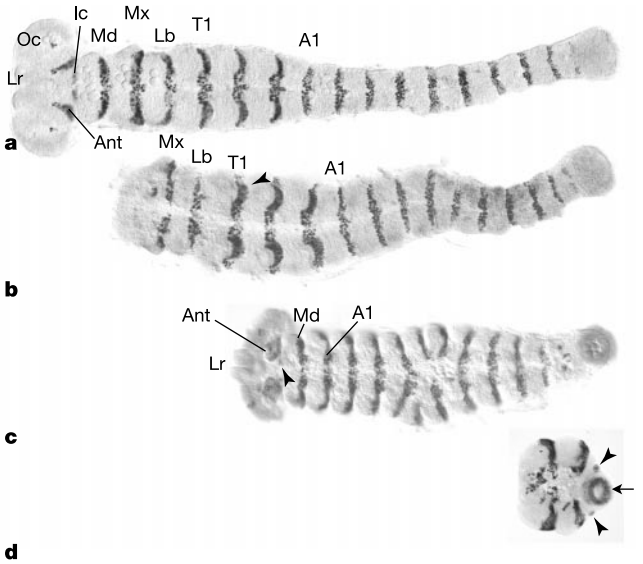

Figure 4 Engrailed staining of wild-type and RNAi embryos. *Engrailed* expression is seen in the posterior segmental compartment, and corresponds to the following segments: Lr, labrum, Ant, antenna; Oc: ocular segment; Ic, intercalary segment; Md, mandible; Mx, maxilla; Lb, labium; T1, thoracic segment 1; A1, abdominal segment 1. **a**, Wild-type embryo. The appendages of the gnathal segments and the thorax start to bulge out (anterior points to the left). **b**, *otd-1*^{pRNAi} embryo (class III). The gnathal and thoracic appendages are elongated (arrowhead points to the growing leg in T1). The maxillary segment can be identified by its drumstick-like appearance. Engrailed-positive spots, anterior to the maxillary *Engrailed* stripe, mark parts of a segment that is not properly organized. **c**, *hb*^{pRNAi} embryo. Identity of the palps, anterior to abdominal segment 1 (A1), as the mandibles is inferred from an analysis of the cuticle (Fig. 3f). Segmentation defects in the central abdomen are frequently seen. The intercalary segment is represented by a few Engrailed-positive cells posterior to the antennae (arrowhead). **d**, *otd-1/hb*^{pRNAi} embryo: extreme phenotype with two abdominal *Engrailed* stripes of normal polarity. Anlagen for the hindgut (arrow) and the malpighian tubules (arrowheads) develop only at the posterior end.

embryonic region, at the border of the serosa. The zygotic expression could then be maintained by an autoregulatory loop. To test this possibility, *otd-1*-binding sites in its regulatory region will have to be identified, and the effects of disrupting this site will have to be tested *in vitro* and *in vivo*.

As previously proposed[11,29], *bicoid* might have evolved from a duplicated *zerknüllt*-like gene, which was converted by mutation into a gene that codes for a K_{50}HD-containing protein—thus adopting the same DNA-binding specificity as Otd-1. During evolution of the higher Diptera, *otd* expression came under the regulation of the maternal gene *bicoid*, so that maternal Otd-1 function was not longer required. Thus, *otd* was restricted to become a gap gene in insects derived from this lineage. □

Methods

GenBank accession number AJ223627 (*Tribolium castaneum* mRNA for Orthodenticle-1 protein) was used in this study.

Antibody production and staining

A polyclonal antibody was produced by immunizing rabbits with bacterially expressed *Tribolium* Orthodenticle-1 protein (Eurogentec). The coding region of the protein used in immunization corresponds to position 760–1474 of the mRNA[15], which was cloned into the *Pvu*II site of the PRSET A vector (Invitrogen). Immunohistochemistry for Otd-1 and Engrailed (using the 4D9 antibody) expression, was carried out as described previously[15]. *In situ* hybridizations were performed according to ref. 19; the embryos shown in Fig. 1a–f are from the same immunohistochemical reaction and were processed identically.

RNA interference

dsRNA was produced as described[30] using the Maxi-Script Kit (Ambion). The *otd-1* dsRNA was produced from a complementary-DNA deletion clone (ΔPst), representing the 3′ part of the RNA (position 1215–1991)[15], which does not contain the homeobox. The cDNA clone that contains the complete open reading frame, and another deletion construct, ΔXho, which represents the 5′ part of the RNA (position 1–918)[15], included the homeobox and were used as controls (not shown). *Tribolium hb* dsRNA was produced from a 2.4-kilobase cDNA clone[19]. Eggs were collected at intervals of 2 h and were microinjected using a standard injection apparatus. At the time of injection, embryos were at an early stage of nuclear division. For parental RNAi, female pupae were immobilized with heptane glue in a Petri dish lid, and injected by free hand under a stereomicroscope, using a glass capillary held by a needle holder connected to an air-filled syringe. dsRNA (500 ng μl^{-1}) was injected to produce *otd-1*^{RNAi} or *hb*^{RNAi} embryos, and equal amounts of both dsRNA preparations (1 $\mu g\,\mu l^{-1}$) were injected to produce *otd-1/hb*^{RNAi} embryos. *Distal-less* dsRNA (900 base pairs) was injected at a concentration of 1 $\mu g\,\mu l^{-1}$ as a control. Only the appendage-specific phenotype[17] was obtained in all of the analysed cuticles ($n = 43$), irrespective of the higher number of molecules injected.

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The BMP antagonist noggin regulates cranial suture fusion

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During skull development, the cranial connective tissue framework undergoes intramembranous ossification to form skull bones (calvaria). As the calvarial bones advance to envelop the brain, fibrous sutures form between the calvarial plates[1]. Expansion of the brain is coupled with calvarial growth through a series of tissue interactions within the cranial suture complex[2]. Craniosynostosis, or premature cranial suture fusion, results in an abnormal skull shape, blindness and mental retardation[3]. Recent studies have demonstrated that gain-of-function mutations in fibroblast growth factor receptors (*fgfr*) are associated with syndromic forms of craniosynostosis[4,5]. Noggin, an antagonist of bone morphogenetic proteins (BMPs), is required for embryonic neural tube, somites and skeleton patterning[6–8]. Here we show that *noggin* is expressed postnatally in the suture mesenchyme of patent, but not fusing, cranial sutures, and that *noggin* expression is suppressed by FGF2 and syndromic *fgfr*