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Tribolium Hox genes repress antennal development in the gnathos and trunk

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Abstract

Evidence from Drosophila suggests that Hox genes not only specify regional identity, but have the additional function of repressing antennal development within their normal domains. This is dramatically demonstrated by a series of Hox mutants in the red flour beetle, *Tribolium castaneum*, and is likely an ancient function of Hox genes in insetcs.

Hox genes encode transcription factors that regulate developmental fate along the anterior–posterior axis of virtually all metazoans (Carroll, 1995). In addition, Hox genes repress anterior development in posterior body regions. This latter function has been revealed by analyzing the effects of loss-of-function mutations and ectopically expressed transgenes in distantly related model organisms such as flies and mice. These studies have revealed a functional hierarchy among Hox genes in which posterior genes predominate over more anterior ones. This relationship has been termed posterior prevalence in vertebrate systems (Duboule and Morata, 1994) and phenotypic suppression in the fruitfly, *Drosophila melanogaster* (Gonzalez-Reyes and Morata, 1990; Lewis, 1978).

In insects, Hox genes also repress antennal appendage identity. In Drosophila, *sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) all repress antennal identity when ectopically expressed in the developing eye-antennal disk (see Yao et al., 1999). Instead, ectopic legs develop in place of the normal antennae, due to the ability of the Hox genes to repress antenna-specifying genes such as *homothorax* (*hth*) and *extradenticle* (*exd*) (Casares and Mann, 1998; Yao et al., 1999). There is limited evidence, however, that Hox genes repress antennal development in their normal expression domains in gnathal and trunk segments. $Antp^{-}$ clones in the T2 leg disk (Struhl, 1981) and *proboscipedia*$^{-}$, Scr^{-} clones in the labial disk assume antennal identity (Percival-Smith et al., 1997). Since most Hox gene mutations cause larval lethality, mitotic clones must be used to assay gene function during adult development. Interestingly, there are no corresponding embryonic transformations. Although Drosophila embryos are limbless, they do display sense organs, which are homologous to the embryonic limbs of less derived insects. In the absence of Hox genes, however, the cuticle of the embryonic trunk and gnathal segments displays prothoracic-like denticle belts and sclerotic plates (Sato et al., 1985; Struhl, 1983). These structures develop due to the function of *teashirt* (*tsh*) (Röder et al., 1992) and *empty spiracles* (Macías and Morata, 1996), respectively. Removal of *tsh* in addition to the Hox genes enhances the anterior transformation such that additional anterior head skeleton is deposited (Röder et al., 1992), but ectopic antennal sense organs are still not observed. Thus, the default appendage state for each segment in the absence of Hox genes is not clear from Drosophila studies.

RNA interference (RNAi) experiments in the milkweed bug, *Oncopeltus fasciatus*, provide additional data to support the hypothesis that a limb generated in the absence of Hox genes takes on antennal identity. In the milkweed bug, depletion of *Dfd* mRNA by RNAi, or

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similar removal of both *Scr* and *pb* mRNA, yields homeotic antennae in the mandibular and labial segments, respectively (Hughes and Kaufman, 2000). However, it is still not clear whether this model is applicable to all segments.

The red flour beetle, *Tribolium castaneum*, is an ideal organism in which to address this issue. In contrast to the limbless maggot of Drosophila, Tribolium larvae sport well-developed appendages. The head displays antennae and mandibles, as well as maxillary and labial palps. Walking legs develop on the three thoracic segments while the abdomen is limbless. The pleuropodia, glands located on the first abdominal segment, appear to be modified legs (Lewis et al., 2000). Furthermore, in contrast to most non-Drosophilid insects, Tribolium offers facile genetics (Brown et al., 2000; Shippy et al., 2000a,b). Among its attributes are small size, ease of culture, short generation time (about one month), and long life (over a year). Numerous mutant alleles are available and germline transformation is now feasible (Berghammer et al., 1999). The Tribolium Hox genes are located in a single cluster that is the equivalent of the two Drosophila complexes (Beeman, 1987). They are expressed in similar segmental domains to their Drosophila orthologs and mutations in orthologous genes affect similar body regions (summarized below and in Fig. 1; also see Denell et al., 1996 for a review).

By removing combinations of Hox genes from Tribolium embryos, we have created a series of phenotypes that strongly support the hypothesis that default appendage fate in the absence of Hox genes is antenna. The wild type Tribolium larva has a single pair of antennae (Fig. 2A). In *TcDfd* mutants, the mandibles (which are now lacking any Hox gene expression) are homeotically transformed to antennae (Fig. 2B and (Brown et al., 2000)). In the *mxp TcDfd* double mutant, both the mandibular and maxillary segments lack Hox gene expression (except for a small amount of *Cx* in the posterior maxillary compartment) and ectopic antennae develop in both segments (Fig. 2C). In larvae lacking *TcDfd* and *Cx* mRNA (produced by RNAi) (Fig. 2D), the appendages on all three gnathal segments are transformed to antennae. The effect on the labial segment is not surprising because *Cx* is required for *mxp* expression in the labial appendages (DeCamillis et al., 2002). However, the identity of the maxillary appendages is not affected in the absence of either *TcDfd* or *Cx* alone [although a basal lobe (endite) is missing in *TcDfd* mutants]. Thus, removal of both *TcDfd* and *Cx* has a synergistic effect on maxillary appendage identity. The most likely explanation of this observation is that *mxp* function in the maxillary appendage requires the presence of either *TcDfd* or *Cx*. In Drosophila, both *Dfd* and *Scr* positively regulate *pb* expression{7595}. We are currently assaying whether *mxp* transcription is altered in *TcDfd Cx* RNAi embryos. Larvae heterozygous for

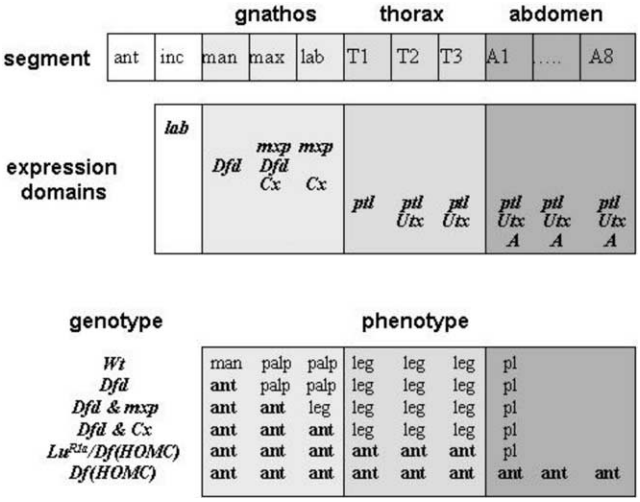

Fig. 1. Expression domains and mutant phenotypes of Hox genes in Tribolium. Segments are diagramed from anterior (left) to posterior (right) in the top panel: ant, antennal; inc, intercalary; man, mandibular; max, maxillary; lab, labial; T1, prothorax; T2, mesothorax; T3, metathorax; A1, first abdominal; A8, eighth abdominal. The A2–A7 segments are denoted by dots between A1 and A8. The gnathal, thoracic, and abdominal tagma are progressively darker shades of gray, respectively. Tribolium Hox gene domains are shown in the middle panel. See text for details. Genotypes and the corresponding mutant phenotypes are listed in the bottom panel: man, mandible; ant, antenna; pl, pleuropodia. Lu^{R1} is a revertant of *Lucifer* (*Lu*) that removes all of the genes in Tribolium homeotic complex downstream of *A*. The deficiency *Df*(*HOMC*) removes the genes between *TcDfd* and *A* inclusive. In Tribolium, *Tclabial* expression is limited to the intercalary segment (Nie et al., 2001). *TcDeformed* (*TcDfd*) is expressed in the mandibular and maxillary segments (Brown et al., 1999) and mutations in *TcDfd* affect these segments (Brown et al., 2000). *Maxillopedia* (*mxp*), the Tribolium ortholog of *proboscipedia* (*pb*) (Shippy et al., 2000a,b), is expressed in the maxillary and labial palps and was originally identified by mutations that transform these appendages to legs (Beeman et al., 1989; Shippy et al., 2000a,b). *Cephalothorax* (*Cx*), the ortholog of *Scr*, is expressed in the labial and first thoracic segments, as well as the mesoderm of the developing legs and the posterior compartment of the maxillary segment (Curtis et al., 2001). Mutations in *Cx* affect the labium and first thoracic segments. *Prothoraxless* (*ptl*), the ortholog of *Antp*, is expressed throughout the thorax (unpublished data) and mutations affect the thoracic legs {4839}. *Ultrathorax* (*Utx*), the ortholog of *Ubx*, is expressed in the second and third thoracic segments, and throughout the abdomen, while mutations in *Utx* affect the third thoracic and first abdominal segment. Expression of *abdominal* (*A*), the ortholog of *abd-A*, begins in the posterior compartment of the first abdominal segment (A1p) and extends posteriorly through the length of the abdomen. Mutations in *A* affect abdominal segments A1–A8 {4526}. Finally, *TcAbd-B* is expressed in the postabdomen (He and Brown, unpublished data) and mutations in the probable ortholog, *extra urigomphi* (*eu*), affect A9 and A10.

two deficiencies ($Df(HOMC)/Lu^{R1}$) lack *TcDfd*, *Cx*, *ptl*, and *Utx*. In these individuals, the gnathal and thoracic appendages are all transformed to antennae (Fig. 2E). In *Df*(*HOMC*) homozygotes which additionally lack *A*, all segments (including those of the normally limbless abdomen) bear antennae (Fig. 2F).

These Tribolium larval phenotypes clearly demonstrate that, in the absence of Hox genes, the default

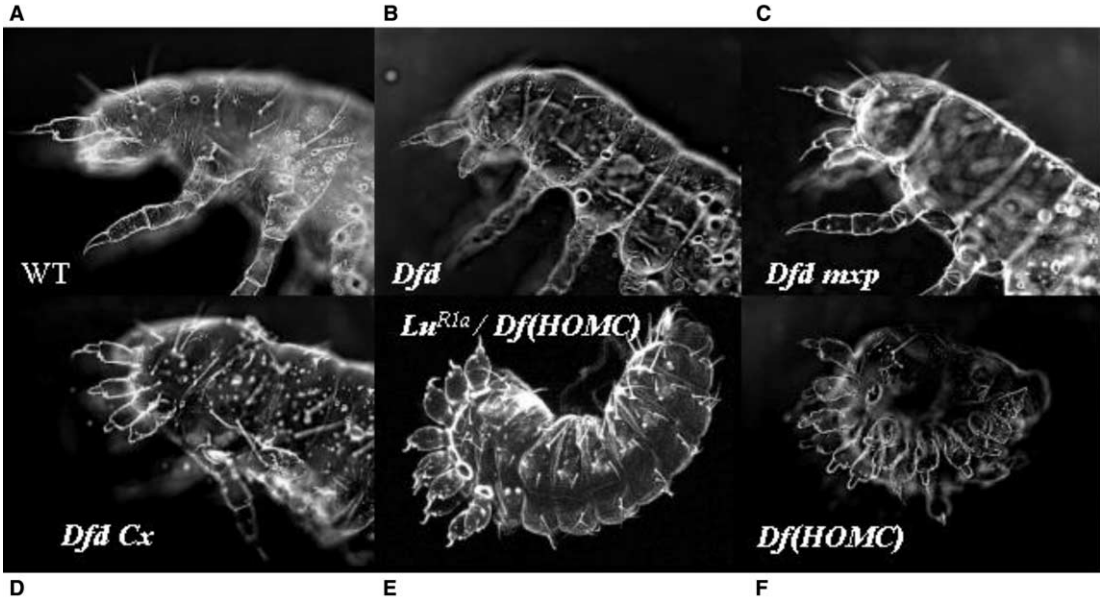

Fig. 2. Wild type and mutant Tribolium larvae. Normal antennae and maxillary palps, as well as thoracic legs, are visible on the wild type (WT) specimen (A). In an individual lacking *TcDfd* function (B), the mandibles develop as homeotic antennae. In a *TcDfd mxp* double mutant (C), the mandibles and maxillary palps are transformed to antennae. When *TcDfd* and *Cx* are depleted by RNAi (D), all the gnathal appendages are transformed to antennae. The gnathal and thoracic appendages are transformed to antennae when the region containing the *TcDfd* through *Utx* genes is removed (E). The additional removal of the *A* gene results in the development of antennae on all segments (F). Lateral views of first larval instar cuticle preps are shown. The $Lu^{R1}/Df(HOMC)$ heterozygote is shown in confocal view. The rest are DIC images that were inverted using Photoshop software (Adobe).

appendage identity for each segment is antenna. Since our results are consistent with the limited data available from Drosophila and Oncopeltus, it seems likely that defaulting to antennal identity in the absence of Hox genes is ancestral to these three species. However, antennae do not represent the appendage ground state. In Drosophila, removal of *hth* or *exd* function from antennal disks results in the formation of leg structures (Casares and Mann, 1998), suggesting that multiple levels of specification regulate appendage identity. Consistent with this hypothesis, eliminating *hth* and Hox gene expression from leg disks results in transformations similar to those seen when *hth* is removed from antennal disks (Casares and Mann, 2001). Interestingly, this putative ground state appendage is not a normal leg. Rather, it consists of only two segments, a morphologically normal tarsus and a rudimentary segment that seems to represent fusion of the four proximal segments. An alternative ground state has been suggested by Duncan et al. (1998). They describe a limb with mixed leg and antennal identity that develops from the antennal disk in the absence of *spineless* (*ss*). Perhaps uncovering the true appendage ground state will require analysis of limb development in the absence of *hth*, *ss*, Hox, and possibly other genes.

Analysis of limb patterning in other insects will be required to determine the extent to which these multiple levels of specification are conserved. We have shown that Hox genes repress antennal development in the gnathal and trunk segments of Tribolium. However, it remains to be demonstrated whether the mechanisms by which Hox genes alter appendage identity (including repression of *hth* expression) are conserved. Recently, it has been shown that ectopic expression of the Tribolium ortholog of *fushi tarazu* (*Tcftz*) (a divergent Hox gene) in Drosophila eye-antennal imaginal disks can cause transformation of antenna to leg (Lohr et al., 2001). A reduction in *hth* expression was observed in disks expressing *Tcftz*, suggesting that at least one of the underlying molecular mechanisms is conserved. Ectopic expression of Hox genes in Tribolium embryos and analysis of the resulting larval phenotypes as well as any changes in gene expression should lend further insight into this matter. Furthermore, it is now possible to examine the expression of *ss* and *hth* orthologs and assess their function by RNAi in non-model insects to determine whether multiple levels of specification are evolutionarily conserved in patterning limbs.

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