Implications of the *Tribolium Deformed* mutant phenotype for the evolution of Hox gene function

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Among insects, the genetic regulation of regional identities in the postoral head or gnathal segments (mandibular, maxillary, and labial) is best understood in the fly *Drosophila melanogaster*. In part, normal gnathal development depends on *Deformed (Dfd)* and *Sex combs reduced (Scr)*, genes in the split *Drosophila* homeotic complex. The gnathal segments of *Dfd* and *Scr* mutant larvae are abnormal but not homeotically transformed. In the red flour beetle, *Tribolium castaneum*, we have isolated loss-of-function mutations of the *Deformed* ortholog. Mutant larvae display a strong transformation of mandibular appendages to antennae. The maxillary appendages, normally composed of an endite and a telopodite, develop only the telopodite in mutant larvae. We previously reported that mutations in the beetle *Scr* and *Antennapedia* orthologs cause the labial and thoracic appendages, respectively, to be transformed to antennae. Moreover, a deficiency of most of the beetle homeotic complex causes all gnathal (as well as thoracic and abdominal) segments to develop antennae. These and other observations are consistent with the hypothesis that ancestral insect homeotic gene functions have been modified considerably during the evolution of the highly specialized maggot head. One of the ancestral homeobox genes that arose close to the root of the Eumetazoa appears to have given rise to *Dfd, Scr*, and the Antennapedia homeobox-class homeotic genes. Evidence from both *Tribolium* and *Drosophila* suggests that this ancestral gene served to repress anterior development as well as confer a trunk-specific identity.

Scientists were originally attracted to the study of *Drosophila* homeotic genes by striking adult mutant phenotypes in which one body region is developmentally replaced by another. We now understand that these genes specify region-specific developmental pathways by encoding homeodomain-bearing transcription factors that control the expression of downstream target genes. In his classic studies of the bithorax complex (BXC), Lewis (1) emphasized that BXC functions are necessary to confer progressively more posterior identities in the metathorax (T3) and abdomen. In the absence of BXC activity, these regions display reiterations of a developmental "ground state" later identified as parasegment (PS)4 (posterior first thoracic segment/anterior second thoracic segment, or T1p/T2a) (2). The identity of PS4 in turn depends on the expression of homeotic genes in the Antennapedia complex. If *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*) functions are eliminated in addition to those of the BXC, the reiterated unit includes an abnormal T1a and ill-defined head structures (3, 4).

For technical reasons, no one has examined the development of a *Drosophila* embryo lacking all homeotic complex (HOMC) functions. One HOMC gene, *proboscipedia* (*pb*), is completely dispensable for normal embryogenesis. Normal development of the mandible requires *Deformed* (*Dfd*) and *cap "n" collar* (*cnc*) (a gene not located in the homeotic complex), whereas *Dfd* is important for the maxilla and *Scr* for the labium. Mutations in *Dfd* and *Scr* do not result in overt homeotic transformations of larval gnathal segments, which retain a gnathal character but display a loss of segment-specific features (5).

During early embryogenesis, *Drosophila* has a normal complement of head segments, albeit somewhat reorganized with respect to the linear arrangement typical of most insects (6). However, these segments involute through the stomodeum to elaborate internal structures, and only vestigial portions of the gnathal segments remain external. It is likely that the evolution of the maggot head was accompanied by changes in the genetic control of developmental commitments.

We have been characterizing the HOMC of the red flour beetle, an insect that allows the possibility of sophisticated genetic analysis. *Tribolium* larvae display relatively unspecialized heads with mandibulate mouth parts. *Tribolium* has a *Deformed* ortholog that is located in the HOMC (7) and expressed in an embryonic pattern essentially identical to that in *Drosophila* (8). We will refer to this beetle gene as *Tc Deformed* (*TcDfd*). Here we describe the isolation and mutant phenotypes of *TcDfd* variants and discuss their implications for the evolution of Hox gene function.

Materials and Methods

Genetics. The genetic variants used in this study are summarized in Table 1.

Mutagenesis. Males homozygous for *tar*, a recessive viable mutation tightly linked to the HOMC (9), were mutagenized with 0.04 M ethyl methanesulfonate as previously described (10). The *tar* chromosome was recently isogenized by using a balancer in the region of the HOMC and was shown to be lethal free. Mutagenized males were mated (1:2) with A^{Es}/Ey females. After 3 days, the males were removed, and females were transferred weekly to fresh flour. Individual Es progeny were mated to $mxp^{Stbd}/Df(HOMC)$ beetles and their progeny screened for non-Es non-Stbd animals. The absence of this class indicated the presence of a lethal mutation in the region uncovered by the deficiency. Stocks of putative mutants were established by intermating the Es non-Stbd siblings of the absent class. Eight mutants were recovered in a screen of 1,600 F_1 individuals, one of which ($TcDfd^1$) is described here.

Reversion. Ag^{Pin}/mxp^{Stm} Cx^5 males were subjected to γ irradiation (6,000 rads), allowed to recover for 2 days, and mated to wild-type females. The males were removed after 4 days and the F_1 progeny visually screened for revertants. Putative Ag^{Pin}

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Abbreviations: BXC, bithorax complex; T3, metathorax; PS, parasegment; HOMC, homeotic complex.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AAB39355 (TcDfd) and AF243042 ($TcDfd^1$)].

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Table 1. Alleles described in this work

Allele name	Symbol	Description	Reference
maxillopedia^{Stuboid}	*mxp*^{Stbd}	Dominant; short antennae; recessive lethal; crossover suppressor	10
maxillopedia^{Stumpy}	*mcp*^{Stm}	Dominant; fused antennae; recessive lethal; crossover suppressor	10
Cephalothorax^{5}	*Cx*^{5}	Haploinsufficient; T1→T2; induced on a *mxp*^{Stm} chromosome recessive lethal; crossover suppressor	10
Eyeless	*Ey*	Dominant; eye facets missing; recessive lethal; crossover suppressor	10
Abdominal$^{Extra\ Sclerite}$	*A*^{Es}	Dominant; A2→A3; recessive lethal; crossover suppressor	10
Deficiency (*HOMC*)	*Df* (*HOMC*)	Homeotic genes *TcDfd, Cx, ptl, Utx,* and *A* deleted; recessive lethal; MN-A8→AN	7
tar	*tar*	Recessive; prothoracic quinone glands darkened	9
Antennagalia	*Ag*	Dominant; maxillary galea on base of antennae; recessive lethal; gamma irradiation induced	7
Ag^{Pinhead}	*Ag*^{Pin}	Dominant; reduced head capsule, tightly linked to *Ag*; recessive lethal, gamma irradiation induced	This work
TcDeformed^{1}	*TcDfd*^{1}	Recessive lethal; EMS induced	This work
TcDeformed^{2}	*TcDfd*^{2}	Gamma irradiation induced revertant of *Ag*^{Pin}, recessive lethal	This work

The allele name and symbol are listed. The dominant or haploinsufficient phenotype associated with each allele is listed first. The recessive phenotype is listed second. The first five alleles are dominant markers on balancer chromosomes.

revertants were mated to $A^{Es/Ey}$ beetles to allow secondary mutations to segregate and to establish balanced stocks. Four revertants of the *Ag*^{Pin} phenotype were recovered in a screen of 9,250 F_1 individuals, one of which (*TcDfd*^{2}) is described here.

Complementation. Balanced stocks of *TcDfd*^{1}, *TcDfd*^{2}, and *Df*(*HOMC*) were crossed *inter se* to assess complementation with respect to viability. The two new variants were also tested and shown to complement mutations at all of the other homeotic genes in the HOMC for which variants are available (data not shown).

Molecular Analysis of *TcDfd*^{1}. Primers were designed to amplify the first exon of *TcDfd* (Fig. 1*a*). The fragment amplified from a single homozygous mutant larva was gel purified and sequenced with internal primers.

Microscopy. Larval cuticles were prepared and documented as previously described (11). *In situ* hybridization to *TcDfd* mRNA and immunohistochemical analysis were performed as previously described (12). The anti-*Drosophila* Dll antibody was the kind gift of G. Panganiban (University of Wisconsin–Madison, Madison, WI).

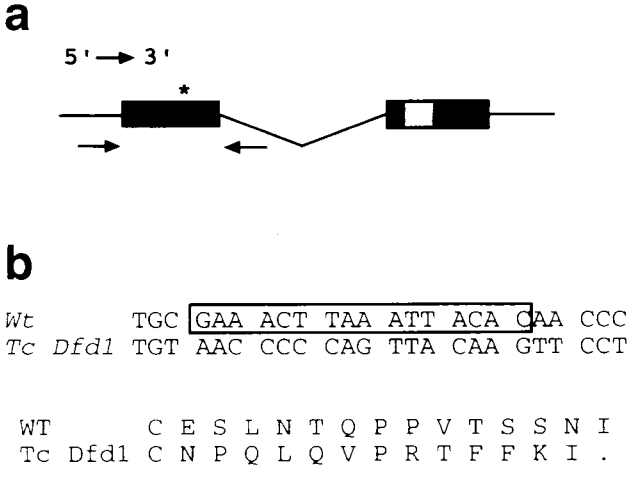

Fig. 1. Molecular analysis of *TcDfd*^{1}. (*a*) Diagram of the *TcDfd* transcription unit. Coding regions are shown as solid boxes, whereas the homeobox in the second exon is hatched. The arrows below exon one denote the primers used to amplify that region. The asterisk marks the location of the molecular lesion. (*b*) Comparison of wild-type (wt) and *TcDfd*^{1} DNA and amino acid sequences. The 16 bases deleted in *TcDfd*^{1} are boxed. The shift in DNA reading frame results in a protein truncated after the next 13 residues.

Results

We had previously molecularly cloned and characterized *TcDfd* and determined that the gene lies within a region of the *Tribolium* HOMC deleted by *Df*(*HOMC*) (7). We have isolated two new mutations at the *TcDfd* locus. One, *TcDfd*^{1}, was induced

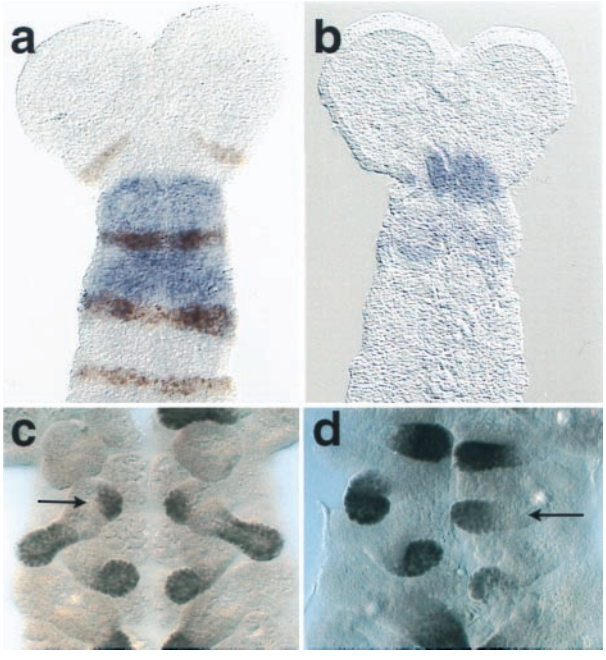

Fig. 2. *TcDfd* and Dll expression in wild-type and *TcDfd*^{1} mutant embryos. (*a*) *TcDfd* is expressed throughout the mandibular (arrow) and maxillary segments of wild-type embryos. Engrailed (En) expression marks the posterior compartment of each segment. [Reproduced with permission from ref. 8 (Copyright 1999, Springer)]. (*b*) *TcDfd* expression in mutant embryos is severely reduced. (*c*) Dll is expressed in each limb tip of wild-type embryos except the mandibular. In addition, it is expressed in the developing endite (arrow) of the maxillary appendage. (*d*) In mutants, Dll is expressed in the transformed mandibular appendage. However, expression normally associated with the developing maxillary endite is missing, and no endite forms.

by ethylmethanesulfonate and isolated by its failure to complement *Df*(*HOMC*) for viability. The second, *TcDfd*^{2}, was isolated as a γ ray-induced revertant of the dominant variant *Antenna-galea-pinhead* (*Ag*^{pin}). The original *Ag* lesion causes a galea (a maxillary structure) to appear on the base of the antenna (7), whereas the derivative *pinhead* variant additionally causes a reduction of the head capsule. *TcDfd*^{1} gives an identical lethal mutant phenotype (see below) in homozygous and hemizygous condition. This phenotype is shared by *TcDfd*^{1}/*TcDfd*^{2} and *TcDfd*^{2}/*Df*(*HOMC*) individuals, suggesting that *TcDfd*^{1} and *TcDfd*^{2} are loss-of-function (probably null) mutations at the gene. Although *Ag*^{pin}/*Df*(*HOMC*) larvae are normal, *Ag*^{pin} homozygotes die as early embryonic lethals. Moreover, the *Ag*^{pin} derivative *TcDfd*^{2} gives early embryonic lethality when homozygous and when heterozygous with *Ag*^{pin}. Because *Ag*^{pin} is associated with crossover suppression, it is likely that it and *TcDfd*^{2} share a recessive lethal breakpoint outside of the limits of the deficiency. These data suggest *Ag*^{pin} is a gain-of-function variant that retains normal *TcDfd* function, and that the *TcDfd*^{2} revertant was generated by inactivation of the gene.

We have demonstrated that *TcDfd*^{1} is associated with a lesion at the molecularly defined *TcDfd* gene. Genomic DNA from a *TcDfd*^{1} homozygous larva was used as a template to amplify the coding portion of the 5′ exon and a short portion of the adjacent intron of the *TcDfd* gene by using PCR (Fig. 1*a*). Sequencing revealed that, compared with wild type, there is a C→T transition and an adjacent 16-bp deletion (Fig. 1*b*). These changes result in a translational frameshift and, 13 codons downstream, a translational stop N-terminal to the homeodomain. This result further supports the likelihood that *TcDfd*^{1} is a null mutation. *In situ* hybridization of wild-type embryos with a probe from *TcDfd* reveals that the gene is transcribed in the mandibular and maxillary segments (Fig. 2*a*) (8). When *TcDfd*^{1} homozygous embryos are treated similarly, they display a dramatic reduction in signal intensity (Fig. 2*b*), suggesting that the deletion results in reduced transcript stability. Finally, the apparent *TcDfd* null phenotype was phenocopied by RNA interference experiments in which double-stranded RNA molecules complementary to a *TcDfd* cDNA were injected into young wild-type embryos (11).

Fig. 3*a* shows a ventral view of a wild-type first instar *Tribolium* larva. The maxillary appendage differs from those of the mandibular and labial segments in having two lobes: a slightly larger distal branch (telopodite) and a ventrally oriented branch (endite) (see Fig. 3 *a* and *c*). The labial appendages have only a telopodite that closely resembles those of the maxillary segment, albeit somewhat smaller. Although the labial appendage primordia are initially more widely separated, they move together at the ventral midline and somewhat anteriorly such that they are nested between the maxillary appendages in the first instar larva. The mandibular appendages are stout toothed structures that are gnathobasic: they represent only a proximal coxopodite derived from the body wall (13). As such, they resemble the mandibular primordia of *Drosophila* and other arthropods in lacking Distal-less (Dll) expression (Fig. 2*c*) typical of the more distal telopodite of most arthropod appendages (14, 15). During appendage development in the maxillary segment, Dll is expressed in the primordia of both the endite and telopodite, whereas there is a single domain of Dll expression associated with each labial appendage (Fig. 2*c*).

In *Tribolium*, individuals homozygous (Fig. 3*b*) or hemizygous (Fig. 3 *d* and *e*) for *TcDfd* mutations display a strong transformation of the mandibular appendages to antennae. This trans-

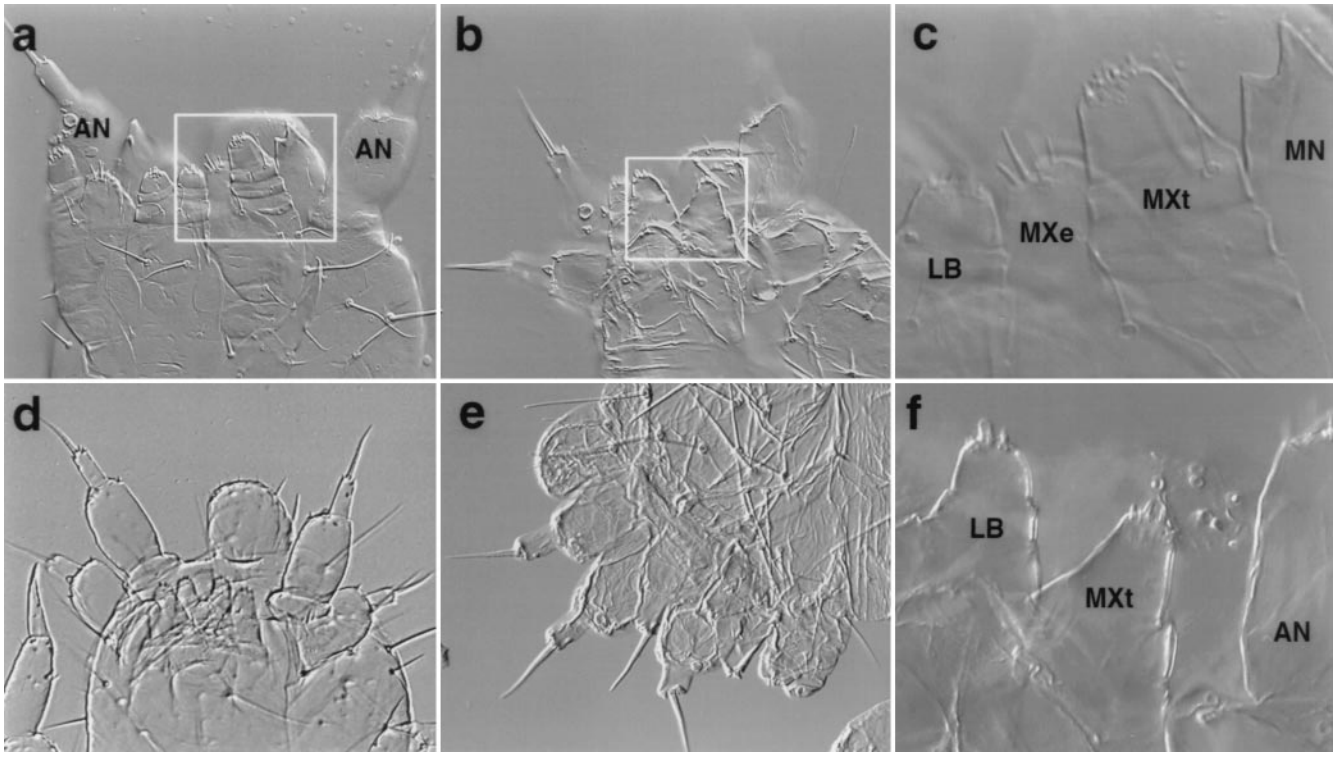

Fig. 3. Wild-type and mutant first instar larval cuticles. (*a*) Ventral view of a wild-type cuticle. (*b*) Ventral view of *TcDfd*^{1} homozygote. The terminal seta of the homeotic antenna on the right is missing. (*c*) The boxed region in *a* photographed at higher magnification. (*d*) Ventral view of TcDfd^1/*Df*(*HOMC*). The homeotic antennae on the mandibular segment are fully transformed. (*e*) Lateral view of *TcDfd*^{2}/*Df*(*HOMC*). Note both normal and homoetic antennae. (*f*) The boxed region in *b* photographed at higher magnification. The maxillary endite is missing, and the maxillary telopodite is reduced in size. The homeotic antenna is out of the plane of focus. AN, antennal; LB, labium; MXe, maxillary endite; MXt, maxillary telopodite; MN, mandible; LR, labrum. Lower magnification = ×200; higher magnification = ×400.

formation is associated with Dll expression in the homeotic mandibular appendages (Fig. 2*d*). In addition, the maxillary appendages lack endites (Fig. 3*f*), a change associated with the loss of the normal ventral domain of Dll expression (Fig. 2*d*). The remaining maxillary telopodites are somewhat smaller, but otherwise appear unchanged. Given the similarity of the maxillary and labial telopodites, it is conceivable that the mutant maxillary appendages have a labial identity. Two observations suggest to us that the identity of the mutant maxillary telopodites is unchanged. First, in normal embryos the developing labial telopodites express both the *pb* and *Scr* orthologs, whereas the maxillary telopodites express the *pb* and *Dfd* orthologs. Expression of the *Scr* ortholog is not significantly altered in *TcDfd* mutants (unpublished observations). Second, in *TcDfd* mutants the maxillary telopodites do not migrate to the midline as is typical of the labium.

Discussion

***Deformed* Function in Beetles and Flies.** The *Deformed* gene of *Drosophila* plays a role during embryogenesis in specifying maxillary identity, but loss of *Dfd* function does not result in overt homeotic transformation of that segment. Some ventral structures of maxillary origin (such as the mouth hooks, ventral organs, and some cirri) are missing and thus are *Dfd* dependent, whereas the features still present are *Dfd* independent (16). Mouth hooks, ventral organs, and cirri appear in the labial and thoracic segments because of ectopic *Dfd* expression, confirming the conclusion that they are *Dfd*-dependent structures. There are two domains of *Dll* expression in the embryonic maxillary segment: a ventral–lateral domain and a dorsal domain. These also represent *Dfd*-dependent and -independent features, respectively, and ventral–lateral *Dll* expression is missing in *Dfd* mutants (17). Moreover, ventral–lateral *Dll* expression is necessary for the development of most cirri.

Although *Dfd* is expressed broadly in the *Drosophila* mandibular segment, it is not clear that it is important to the establishment of mandibular identity. *Dfd* mutant larvae are subject to disruptions of the organization of the mandible as well as other head segments but appear to have a full complement of mandibular structures except for a sensory papilla (16). McGinnis *et al.* (18) have shown that a protein isoform encoded by the *cap "n" collar* gene selectively interferes with the activation of Dfd response elements in the mandibular segment. In *cnc* mutants, homeotic maxillary structures develop in the mandibular segment. The genetic functions acting to promote mandibular identity in *Drosophila* are not entirely clear.

We report here the isolation of two mutations of the *Tribolium Deformed* ortholog *TcDfd*. Their phenotypes in hemizygous vs. homozygous condition and the demonstration that one of them encodes a protein that is truncated N-terminal to the homeodomain indicate that they are both nulls or near nulls. Loss of *TcDfd* function results in a strong homeotic transformation of the mandibular appendages to antennae. Clearly, unlike in flies, *TcDfd* is important for repressing anterior development in the mandibular segment. It is not clear whether *TcDfd* also acts to promote mandibular identity in that segment. The role of *TcDfd* in the beetle maxilla appears to parallel that of its fly ortholog. Beetles resemble flies in having two domains of embryonic maxillary Distal-less expression: one dorsal and one ventral. In each insect, dorsal expression is *Dfd*-independent, whereas ventral expression is *Dfd* dependent. Further, mutation in each insect results in the loss of structures in the ventral region (the endite in the case of *Tribolium*). Jürgens *et al.* (6) suggested that the cirri, ventral organ, and mouth hooks are all homologous to structures (lacinia or galea) forming parts of the maxillary endite of some other insects. It appears that the *Dfd* orthologs of both insects act to promote ventral maxillary identity. The observation that Ag^{pin}, an apparent *TcDfd* gain-of-function mutation, causes ectopic appearance of the galea in adults further supports the conclusion that the gene promotes ventral maxillary identity in beetles, as it does in flies.

Evolving Roles of the Antennapedia Complex (ANTC) Genes. The *Drosophila* ANTC includes five homeotic selector genes. Probable null mutant phenotypes have now been identified for *Tribolium* orthologs of four of these genes [to date no *Tc labial* (*Tclab*) variant has been isolated]. In each case, the beetle larval mutant phenotype differs significantly from that described in flies (ref. 19 and this work). Most significantly, mutants of the *Antp, Scr*, and *Dfd* orthologs include transformations of thoracic, labial, and mandibular appendages, respectively, to antennae [it is likely that the ancestral *pb* gene function became highly derived before the origin of the insects (5)]. Moreover, in homozygous condition a deficiency of most of the complex results in an antennal transformation of all gnathal, thoracic, and abdominal segments (7). Given that anterior development in *Tribolium* embryos is far less derived than that in *Drosophila*, it is likely that the functions of the beetle homeotic genes are more ancestral. We suggest the following model based on the information to date. The first eumetazoans had a complex of three genes (20, 21). Conventional thought considers these to represent head, trunk, and tail genes, although it seems likely that anterior-most development did not depend on the Hox genes. Duplication and divergence of the central gene ultimately gave rise to *Dfd*, *Scr*, and the Antp-class genes (*Antp*, *Ubx*, and *abd-A*) before the arthropod radiation (22). We propose that the ancestral trunk gene and its derivatives played two roles: suppression of genes resulting in anterior development and (probably in an evolutionarily labile fashion) determination of specialized trunk segment features. As noted, the beetle *Antp, Scr*, and *Dfd* orthologs all perform the former function in at least part of their expression domains [some evidence suggests that *Drosophila* homeotic genes play similar embryonic roles (3, 4, 23)]. On the other hand, it appears that the *Tribolium Scr* and *Ubx* orthologs no longer have this function in the thorax. That is, loss of function of the *Antp* ortholog is sufficient to transform the thoracic segments to antenna despite the normal expression of the *Scr* and *Ubx* orthologs in the anterior and posterior thorax, respectively (ref. 24 and unpublished observations). No data from flies or other arthropods speak to a possible ancestral role of *lab* in repressing anterior development in the intercalary segment; further speculation awaits the isolation of a *Tclab* mutation.

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