

# Do *teashirt* family genes specify trunk identity? Insights from the single *tiptop/teashirt* homolog of *Tribolium castaneum*

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**Abstract** The *Drosophila teashirt* gene acts in concert with the homeotic selector (Hox) genes to specify trunk (thorax and abdomen) identity. There has been speculation that this trunk-specifying function might be very ancient, dating back to the common ancestor of insects and vertebrates. However, other evidence suggests that the role of *teashirt* in trunk identity is not well conserved even within the Insecta. To address this issue, we have analyzed the function of *Tc-tiotsh*, the lone *teashirt* family member in the red flour beetle, *Tribolium castaneum*. Although *Tc-tiotsh* is important for aspects of both embryonic and imaginal development including some trunk features, we find no evidence that it acts as a trunk identity gene. We discuss this finding in the context of recent insights into the evolution and function of the *Drosophila teashirt* family genes.

**Keywords** *Tribolium* · Teashirt · Tiptop · Trunk · Homeotic

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## Introduction

Homeotic genes are responsible for defining regional identity, and mutations in these genes often transform one body part into another. The best characterized homeotic genes are the Hox genes, which have been identified in representatives of most animal phyla (reviewed by Martindale 2005) and have been extensively studied in several organisms. However, some non-Hox genes have also been classified as homeotic genes based on their mutant phenotypes. One such gene encodes the zinc finger transcription factor Teashirt (Fasano et al. 1991). Loss-of-function (LOF) *teashirt* (*tsh*) mutations are reported to cause partial transformation of the larval prothorax to a labial identity due to ectopic expression of the Hox gene *Sex combs reduced* (Fasano et al. 1991; Roder et al. 1992). The trunk (thoracic and abdominal) segments of *tsh*<sup>-</sup> larvae are shorter than normal and the denticle belts (a trunk-specific feature) are severely disorganized. Moreover, patches of sclerotized cuticle, which have been interpreted as head structures, form between the remaining denticle belts (Fasano et al. 1991). Conversely, ectopic expression of *tsh* results in the development of denticle belts in the head (de Zulueta et al. 1994). On the basis of these phenotypes, *tsh* has been suggested to function as a specifier of trunk vs head identity (Roder et al. 1992).

*tsh* is believed to act in parallel with the trunk Hox genes (*Scr*, *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*)) to determine segmental identity (de Zulueta et al. 1994; Roder et al. 1992). When all of these Hox genes are inactivated, trunk segments develop with what has been interpreted as a mixed head/trunk identity. That is, sclerotized tissue develops in the posterior compartment of each trunk segment, whereas denticle belts (a trunk-specific feature) still develop in the anterior compartment (Sato et al. 1985;

Struhl 1983). When *tsh* function is also eliminated, the trunk segments are more severely affected in that denticle belts disappear and additional head-like cuticle is observed (Roder et al. 1992).

Recent data suggests that *tsh* may play an even greater role in trunk development than was previously recognized. In the absence of *tsh* function, its paralog *tiptop* (*tio*) is ectopically expressed in the trunk region (Laugier et al. 2005). When *tio* and *tsh* functions are both absent, the trunk denticles are even more severely affected than in mutants that lack only *tsh*, suggesting that *tio* can partially substitute for *tsh* and reduce the effects of *tsh* mutations.

Robertson and coworkers (2004) proposed the existence of a system of zinc-finger proteins that divides the *Drosophila* embryo into sections and limits the functional domains of Hox genes. They suggest that within this system *tsh* specifies the trunk region, while *disconnected* (*disco*) specifies the gnathal region. In the absence of *tsh*, *disco* (as well as its paralog *disco-related*) is ectopically expressed in the trunk, and there is some evidence that ectopic *disco* expression is responsible for at least some features of *tsh* mutants (e.g., loss of denticles and abnormal sensory neurons) that are thought to represent a switch to head/gnathal identity (Robertson et al. 2004).

Manfroid et al. (2004) speculated that the trunk-specifying function of *tsh* family members might be conserved in vertebrates, as *tsh* genes from mice are expressed in the trunk region and can also rescue the phenotype of *Drosophila tsh* mutants when ectopically expressed. However, such conjecture requires a rather large phylogenetic leap, as it is not clear that the role of *tsh* in trunk specification is conserved even in other insects. *tsh* family genes of the firebrat, *Thermobia domestica*, and the milkweed bug, *Oncopeltus fasciatus*, are expressed predominantly in the gnathos and thorax, arguing against a function in trunk specification (Herke et al. 2005; Peterson et al. 1999). Furthermore, when Herke et al. (2005) knocked down the function of the *Oncopeltus tsh* family gene by RNA interference (RNAi), they observed transformations of distal leg to antenna, but did not report any changes in trunk identity. Finally, our previous studies suggest that genetic specification of trunk identity in the red flour beetle, *Tribolium castaneum*, differs significantly from that in *Drosophila*. Removal of trunk Hox genes alone is sufficient to produce dramatic transformation of trunk to head identity (Brown et al. 2002). Antennae develop on all gnathal and trunk segments of embryos homozygous for a deficiency that removes the *Tribolium* orthologs of *Deformed* (*Dfd*), *Scr*, *Antp*, *Ubx*, and *abd-A* (Stuart et al. 1991). Thus, there is apparently no need to invoke the function of another gene for trunk identity. Peterson et al. (1999) predicted that either the *Tribolium tsh* gene has no function in trunk specification or that the expression of *tsh* is controlled by

Hox genes. Taken together, these observations suggest that the trunk-specifying role of *tsh* observed in *Drosophila* might not be conserved in other insects.

To better understand the evolution of *tsh* family gene function, we cloned and characterized a *tsh* homolog from *Tribolium*. This gene, *Tc-tiotsh*, is the sole member of this family in the *Tribolium* genome. It has a broad embryonic expression domain, encompassing not only the trunk region but much of the head as well. RNAi experiments revealed that *Tc-tiotsh* functions in several embryonic and imaginal tissues, but they did not suggest a conserved function in trunk specification. We discuss these results and reexamine the classification of *tsh* as a trunk-specifying gene in *Drosophila*.

## Materials and methods

### cDNA cloning

A genomic fragment of the *Tc-tiotsh* gene was amplified using degenerate primers based on the conserved amino acid motifs VWLGKG and CKVCDK (first round) and ILKCMW and CKVCDK (second round). Additional, overlapping fragments were identified by RACE, using the 5' and 3' RACE Systems (GibcoBRL), by polymerase chain reaction (PCR) screening a *Tribolium castaneum* cDNA library as described previously (Nie et al. 2001), or by inverse PCR (Ochman et al. 1988). To confirm that these overlapping fragments represent a contiguous sequence, primers were designed near the ends of the putative contig, and reverse transcriptase (RT)-PCR was performed using total embryonic RNA (0–30 h of development) as template. The resulting PCR product was cloned and sequenced (GenBank accession number AF356647).

### Sequence analysis

The nucleotide contig for *Tc-tiotsh* was assembled using the Seqman module of Lasergene (DNASTAR). To identify putative *tsh* family members in other genomes, we performed tBLASTn searches using *Drosophila melanogaster* Tsh and Tio as query sequences. Multiple alignments were created with the AlignX module of Vector NTI 8 (InforMax). Minor manual adjustments were performed to maximize the alignments. The final output of the multiple alignment was created using BOXSHADE (available at [http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Phylogenetic analyses were performed using MEGA version 3.1 (Kumar et al. 2004) or TreePuzzle (<http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html>), and TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>) was used to visualize the output.

### In situ hybridization and immunostaining of embryos

A digoxigenin-labeled fragment of *Tc-tiotsh* was hybridized to whole-mount wild-type (Georgia-1) embryos as previously described (Brown et al. 1994b). Embryos were secondarily immunostained with either  $\alpha$ -Invected antibody (4D9) (Patel et al. 1989) or with  $\alpha$ -Even-skipped antibody (2B8) (Patel et al. 1994), which cross-react with Tc-Engrailed and Tc-Even-skipped, respectively. *Tc-spalt* (*Tc-sal*) expression was assayed using the probe described by Tomoyasu et al. (2005).

### RNA interference (RNAi)

dsRNA of two different, non-overlapping fragments of *Tc-tiotsh* was synthesized using the PCR template method described by Kennerdell and Carthew (2000). Briefly, primers were chosen to amplify a fragment of *Tc-tiotsh* (bases 1358–1786 or 1848–2581 of GenBank sequence AF356647) and the T7 promoter sequence (TAATACGACTCACTATAGG) was added to the 5' end of each primer. The resulting primers were used to amplify template for RNA synthesis. The resulting product was purified and 1  $\mu$ g used as template for RNA synthesis using the Megascript T7 High Yield Transcription kit (Ambion).

For embryonic RNAi, 0–2 h eggs were collected from wild-type beetles (GA-1) and injected with dsRNA (2–4  $\mu$ g/ $\mu$ l) as previously described by Brown et al. (1999). After several days, hatched larvae and unhatched eggs were treated with lactic acid/ethanol. Additional larvae were documented by SEM as described by Curtis et al. (2001).

Injection into non-virgin adult females was performed by manually opening the elytra and wings of ether-anesthetized beetles and injecting 2  $\mu$ g/ $\mu$ l dsRNA into the dorsal abdomen. Larval RNAi was performed as described by Tomoyasu and Denell (2004).

## Results

### *Tribolium* has a single *teashirt* family gene

We cloned a member of the *tsh* gene family from the red flour beetle, *Tribolium castaneum* (see [Materials and methods](#) for details). Comparison of the cDNA sequence to the recently completed *Tribolium* genome sequence revealed three exons distributed over about 90 kb (Fig. 1). Most of this distance can be attributed to an approximately 86-kb intron between the first and second exons.

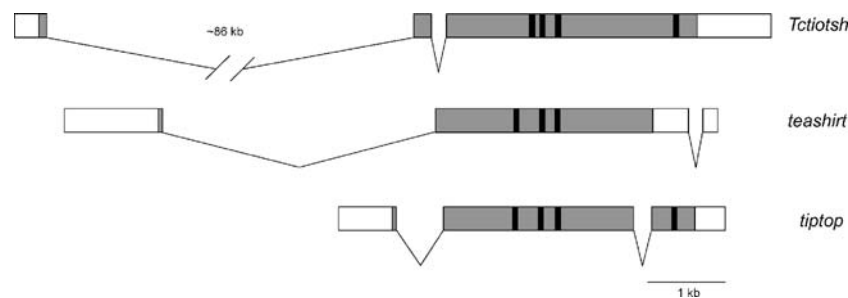
Only three full-length Tsh family proteins have been described from insects: the *Drosophila melanogaster* paralogs Tsh and Tio, and the single member of the Tsh family in *Anopheles gambiae*, Ag-TioTsh (Fasano et al.

1991; Laugier et al. 2005). In addition, small fragments of *tsh* family genes have been amplified from several other insects (Herke et al. 2005; Peterson et al. 1999). Two conclusions have been drawn from these limited data. First, the two *Drosophila* genes seem to be the result of a recent duplication in the *Drosophila* lineage. Second, all of the Tsh family members from other insects appear to be more similar in sequence to Tio than to Tsh. In this paper, we present data that support and greatly strengthen these conclusions.

We performed a tBLASTn search of several recently sequenced insect genomes for *tsh* family members (Table 1). The *Tribolium castaneum* gene that we cloned is the only member of the *tsh* family present in the genome sequence of this insect. Likewise, the *Apis mellifera* (honeybee) genome contains only a single member of this gene family. In contrast, all of the additional *Drosophila* species we searched (*D. pseudoobscura*, *D. simulans*, *D. yakuba* and *D. virilis*) have orthologs of both *tio* and *tsh*. Consistent with the situation in *D. melanogaster* (Laugier et al. 2005), *tio* and *tsh* are closely linked (less than 250 kb apart) in all of the *Drosophila* species examined, suggesting that these genes likely arose from a relatively recent tandem duplication event.

As the *Tribolium* gene is related to both *tio* and *tsh*, we have named it *Tc-tiotsh*, after the convention established by Laugier et al. (2005) for the single *Anopheles* gene. We compared the predicted Tc-TioTsh protein to the other insect Tsh family members for which full-length predicted proteins are available. (Although, we were able to identify a *tsh* family gene in *Apis*, the 5' portion of the transcription unit was apparently missing from the genome sequence.) Like *tio* and *Ag-tiotsh* (Laugier et al. 2005), *Tc-tiotsh* encodes four zinc fingers (Fig. 1), three of which are clustered near the middle of the protein (Fig. 2a), and one that is located near the C terminus (Fig. 2b). Besides the zinc finger regions, several additional short regions of sequence identity (shaded in Fig. 2 and data not shown) are present in all of the full-length predicted proteins. The three dipteran proteins are highly similar at their N termini, while Tio, Ag-TioTsh, and Tc-TioTsh share a C-terminal motif (in addition to the fourth zinc finger) that is absent in Tsh. Regions shared between the beetle and dipteran proteins have been conserved over a long phylogenetic distance, suggesting that they likely have important functions.

To better understand the evolutionary history of the *tsh* gene family, we have performed phylogenetic analyses using conserved portions of the zinc-finger region (underlined in Fig. 2) from the full-length insect Tsh family proteins that are currently available (*Drosophila*, *Tribolium*, and *Anopheles*). Both maximum likelihood (Fig. 3) and neighbor-joining (data not shown) methods produce trees in



**Fig. 1** Gene structure of the *Tc-tiotsh* gene and its *Drosophila* homologs. Exons are denoted by boxes and introns by thin lines. The coding region is shaded gray and zinc finger-encoding motifs are shown in black

which the two *Drosophila* proteins cluster together, suggesting these genes were produced by duplication after the divergence of the mosquito and fly lineages. The branch lengths of the *Drosophila* proteins are much longer than those of *Anopheles* and *Tribolium*, suggesting rapid divergence of both genes after duplication.

The mouse and human genomes each have three *tsh* family members (Caubit et al. 2000; Manfroid et al. 2004), which are much more similar to one another than they are to any of the insect proteins. Interestingly, vertebrate Tsh family proteins share an N-terminal motif (MPRRKQ) with the *Tribolium* protein (Fig. 2c), which is distinct from the N-terminal motif shared by the dipteran family members (Laugier et al. 2005). The conservation of this sequence in an insect and vertebrates suggests it has an important function and that it was probably present in the common ancestor of protostomes and deuterostomes.

Taken together, these comparisons of *tsh* family members indicate that the ancestor of protostomes and deuterostomes had a single *tsh* family gene. We were unable to identify any *tsh* family members in the genomes of two non-bilateria metazoans: the placozoan *Trichoplax adhaerens* (<http://genome.jgi-psf.org/Triad1/Triad1.home.html>) and the cnidarian *Nematostella vectensis* (Putnam et al. 2007), suggesting this gene family may have arisen after the bilateria/non-bilateria split. Within the vertebrate lineage, duplication events (perhaps in association with whole genome duplications) produced at least three

paralogs. A duplication in the *Drosophila* lineage (after divergence from the mosquito lineage) resulted in the *tsh* and *tio* paralogs.

*Tc-tiotsh* embryonic expression is not limited to the trunk

We examined the embryonic expression pattern of *Tc-tiotsh* (Fig. 4) by in situ hybridization, using the posterior compartment marker Engrailed (Brown et al. 1994a; Patel et al. 1989) to determine the stage of embryonic development (segments are added sequentially) and provide a reference for the register of *Tc-tiotsh* expression. *Tc-tiotsh* expression develops in a dynamic, pair rule-like manner (Fig. S1 and S2), but eventually encompasses not only the trunk segments (thorax and abdomen), but also the gnathal segments and the labrum and intercalary segment in the procephalon (Fig. 4a). *Tc-tiotsh* expression in the thorax and the first abdominal segment appears somewhat stronger than in other regions (Fig. 4b). As appendages develop, *Tc-tiotsh* is expressed in the proximal portion of each leg (Fig. 4d).

After germ-band elongation, *Tc-tiotsh* is expressed in the posterior region of the embryo. Initially, expression is seen in a patch on either side of the ventral midline (Fig. 4c), and later in six buds that extend from the invaginating hindgut (Fig. 4e). These structures are the primordia of the Malpighian tubules; they also express *Tc-dachsund* (Prpic et al. 2001) and *Tc-Cut* (data not shown).

**Table 1** *Teashirt* family genes in sequenced insect genomes

Organism	Order	<i>tsh</i> family genes	Genome Reference
<i>Tribolium castaneum</i>	Coleoptera	1	The Tribolium Genome Sequencing Consortium 2008
<i>Apis mellifera</i>	Hymenoptera	1	The Honeybee Genome Sequencing Consortium 2006
<i>Anopheles gambiae</i>	Diptera	1	Holt et al. 2002
<i>Drosophila simulans</i>	Diptera	2 ( <i>tio</i> and <i>tsh</i> )	Drosophila 12 Genomes Consortium 2007
<i>Drosophila yakuba</i>	Diptera	2 ( <i>tio</i> and <i>tsh</i> )	Drosophila 12 Genomes Consortium 2007
<i>Drosophila pseudoobscura</i>	Diptera	2 ( <i>tio</i> and <i>tsh</i> )	Richards et al. 2005
<i>Drosophila virilis</i>	Diptera	2 ( <i>tio</i> and <i>tsh</i> )	Drosophila 12 Genomes Consortium 2007

tBLASTn searches were used to determine the number of *teashirt* (*tsh*) family genes in several assembled insect genomes. All *Drosophila* species examined had orthologs of both *tsh* and *tiptop* (*tio*), while the non-drosophilid species each contained only one *tsh* family member.



**Fig. 2** Multiple alignments of Teashirt family members. Amino acids identical or similar in at least two of the proteins are shaded *black* or *gray*, respectively. Tc *Tribolium castaneum*; Ag *Anopheles gambiae*; Dm *Drosophila melanogaster*; Mm *Mus musculus*; Dr *Danio rerio*. **a** Multiple alignment of the three clustered zinc fingers. Zinc fingers (ZF) 1–3 are indicated above the sequence. Regions underscored with asterisks were concatenated and subjected to phylogenetic analysis. **b** Multiple alignment of the C-terminal zinc finger region. Zinc finger 4 is delimited above the alignment. Tsh lacks this zinc finger. **c** Distinct N-terminal motifs in dipteran Tsh family members (*upper*) and in *Tribolium* and vertebrates (*lower*)

**a**

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-----ZF1-----
Ag_TioTsh 241 DIYRS..PS.....RHSAWQSHWLNKGADSAKDVLKCVWCKQSFPS
Dm_Tsh    308 VQPTPNQNSSES...GGGSGGGAAGSGAVTKARHNIWQSHWQNG...VASSVFR...CVWCKQSFPT
Dm_Tio    279 SSVGAGVPA...GASSNSGG.....RHSAWQSHWLNKGADTAKDVF...KCVWCKQSFST
Tc_TioTsh 341 DLFRITMANA...PGNSASN.....RHSAWQSHWLNKGAEQAKNV...LKCVWCKQSFPS
*****

-----ZF2-----
Ag_TioTsh 280 LAAMTTHMKETKHC...VNVPPGGGGGGAGGGGGHTQQPIPPPAQGNMNNMGGSGGQCGG
Dm_Tsh    366 LEALTTHMKDSKH...CGVNVPPFGNLPSSNNPQF.QHHHPTPPPPQNHNLRRKHSSGSA...SNH
Dm_Tio    328 LANLTAHMKET...HCGVQIPSPPLPTGGVGT...PSA.....PPPTRLATSASNSACSSSSSS
Tc_TioTsh 390 LAAMTTHMKETA...KHC...VNVVPEPMSQI...LNPQF.....QITS...SNTNTS...TSS...TNS...KNPK
*****

-----ZF3-----
Ag_TioTsh 400 TAHM...QTOHYTNI...ISQEQI...ISWKSGGGANGGAG...GGAGGPASAAAAA...TNSHVS...AVLTC
Dm_Tsh    482 TKHM...QTOHYTNI...ISQEQSI...ISKSGNANAN...SDAKESHNSLSSEBSRT...L...SAVLTC
Dm_Tio    441 TSHM...QTOHYTNI...ISQEQI...ISWKS...GDERP...TNT...VPST...T...AAPS...S...TAPS...V...SAVLTC
Tc_TioTsh 495 TSHM...QTOHYTNI...ISQEQI...ISWR...S...D...K...G...GGGGSG...GNNQPPGGP...SSHVS...AVLTC
*****

-----ZF4-----
Ag_TioTsh 459 KVC...DQAF...SSLKELSNHMVKN...AHYKEHIMRSIT...ESGRR...RQTR...EKRK...KSLPVRK
Dm_Tsh    536 KVC...DKAF...NSL...GDL...SNHMAKNNHY...E...P...L...C...SAGARKRPAP...K...KREK...SLPVRK
Dm_Tio    501 KVC...DQAF...GSLKELSTHMAQKSHYK...E...SP...A...S...P...A...GTGNPKRGRONR...NEKR...KSLPVRK
Tc_TioTsh 551 KVC...DQAF...SSLKELSNHMVKN...SHYKEHIMRSIT...ESGRR...RQTR...EKRK...KSLPVRK
*****

-----ZF4-----
Ag_TioTsh 928 ...G...TIK...SYCDT...IFG...SKGAYRHHL...SKAHFVSDV...IPDPKVPARV...VAPCPLPLP...AAPS...R
Dm_Tsh    954 ...L...SPRAETKRETESFALFSLERAKRIFNI...FVFSAL...PSPT...
Dm_Tio    919 AGD...SS...I...IK...SYCDT...PFASKGAYRHHL...SKVHFVKDAGEDSP...RLKSPAVQSP...RSMPL...L...AS...P...R...R
Tc_TioTsh 1051 ...D...S...IM...K...CAFCDTP...F...ISKGAYRHHL...SKMHFVKD...V...IPDP...V...ALK...SA...Q...G...SS...S...SSE...V...L...K

Ag_TioTsh 984 SRR...AHR...P...P...R...H...R...T...K...S...P...P...A...S...Q...S...P...F...D...E...S...P...H...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...
Dm_Tsh    979 S...A...S...R...S...P...A...T...G...S...Q...Q...P...P...S...P...T...I...S...P...Y...D...E...S...P...Q...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...N...A
Tc_TioTsh 1108 GTAV...P...P...P...S...G...S...G...P...C...S...T...G...S...K...S...P...P...V...A...G...F...E...S...P...H...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...Y...V

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**b**

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-----ZF4-----
Ag_TioTsh 928 ...G...TIK...SYCDT...IFG...SKGAYRHHL...SKAHFVSDV...IPDPKVPARV...VAPCPLPLP...AAPS...R
Dm_Tsh    954 ...L...SPRAETKRETESFALFSLERAKRIFNI...FVFSAL...PSPT...
Dm_Tio    919 AGD...SS...I...IK...SYCDT...PFASKGAYRHHL...SKVHFVKDAGEDSP...RLKSPAVQSP...RSMPL...L...AS...P...R...R
Tc_TioTsh 1051 ...D...S...IM...K...CAFCDTP...F...ISKGAYRHHL...SKMHFVKD...V...IPDP...V...ALK...SA...Q...G...SS...S...SSE...V...L...K

Ag_TioTsh 984 SRR...AHR...P...P...R...H...R...T...K...S...P...P...A...S...Q...S...P...F...D...E...S...P...H...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...
Dm_Tsh    979 S...A...S...R...S...P...A...T...G...S...Q...Q...P...P...S...P...T...I...S...P...Y...D...E...S...P...Q...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...N...A
Tc_TioTsh 1108 GTAV...P...P...P...S...G...S...G...P...C...S...T...G...S...K...S...P...P...V...A...G...F...E...S...P...H...S...K...F...L...K...Y...T...E...L...A...K...Q...L...S...S...K...Y...V

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**c**

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Ag_TioTsh 1 MLHEAVMLEIYRQAL
Dm_Teashirt 1 MLHEALMLEIYRQAL
Dm_Tiptop 1 MLHEAVMLEIYRQAL

Mm_tsh1 10 MPRRKQAPRR
Mm_tsh2 1 MPRRKQAPKR
Mm_tsh3 1 MPRRKQAPRR
Dr_tsh-like 1 MPRRKQAPKR
Tc_Tiptop 1 MPRRKQCEPKR

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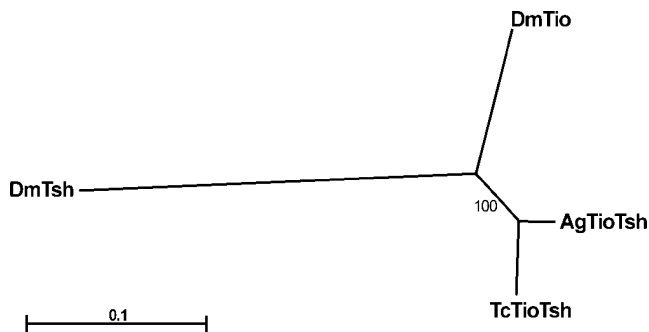
*spalt* does not appear to repress *Tc-tiotsh* in *Tribolium* embryos

In *Drosophila*, the limits of *tsh* expression are controlled either directly or indirectly by *spalt-major* (*salM*). In a *salM* mutant, the *tsh* expression domain expands both anteriorly and posteriorly (Roder et al. 1992). To assess whether the broader expression of *Tc-tiotsh* (compared to *tsh*) might reflect a difference in *Tribolium spalt* (*Tc-sal*) expression, we examined the embryonic expression pattern of the single *Tribolium sal* gene (Tomoyasu et al. 2005). We find that the *Tc-sal* expression domain (Fig. S3) significantly overlaps that of *Tc-tiotsh*, suggesting that *Tc-sal* does not repress *Tc-*

*tiotsh* expression. It is possible that *Tribolium* has lost the regulatory interaction between these genes. Alternatively, regulation of *tsh* by *sal* may have evolved more recently, after the divergence of these lineages.

*Tc-tiotsh* expression is reduced in embryos lacking Hox genes

In *Drosophila*, some aspects of *tsh* expression are regulated by the Hox genes (Roder et al. 1992). We have examined expression of *Tc-tiotsh* in embryos homozygous for *Df* (*HOMC*), a deletion that removes most of the *Tribolium* Hox cluster. *Tc-tiotsh* expression is severely reduced in the ventral region of these embryos (Fig. 4f). These results



**Fig. 3** Phylogenetic analysis of insect Teashirt family members. The two *Drosophila* paralogs cluster together in an unrooted tree produced by maximum likelihood analysis, suggesting that these genes resulted from a recent duplication in the *Drosophila* lineage. The bootstrap value of 100 indicates that the tree is very robust

suggest that reduction of *Tc-tiotsh* expression might contribute to the transformations seen in *Df(HOMC)* embryos. However, our functional studies of *Tc-tiotsh* (presented below) reveal no evidence for this.

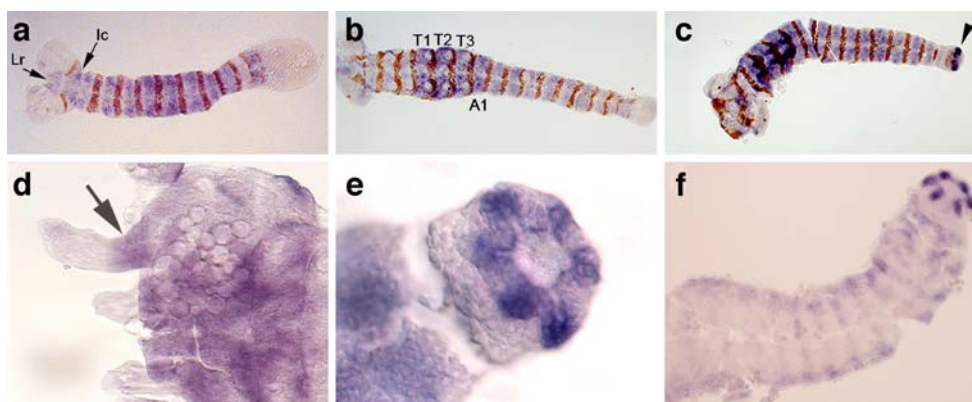
#### *Tc-tiotsh* functions in the embryonic labial and thoracic segments

We used RNAi to ascertain the probable LOF phenotype of *Tc-tiotsh*. Pupae injected with *Tc-tiotsh* dsRNA produced very few eggs upon reaching adulthood, and none of these progeny showed an abnormal phenotype. To circumvent the apparent effect of *Tc-tiotsh* on oogenesis, we injected *Tc-tiotsh* dsRNA either directly into eggs or into the abdomens of non-virgin adult females (egg production from these

females quickly tapers off, further indicating an effect on oogenesis). Injection of *Tc-tiotsh* dsRNA by either technique produced embryos with defects in the labial and thoracic segments (Fig. 5, Table 2, and data not shown). During normal *Tribolium* embryonic development, the two labial appendages fuse at the ventral midline and migrate anteriorly to a position between the maxillary appendages (Fig. 5a). In moderately affected *Tc-tiotsh* RNAi larvae (Fig. 5b), the labial appendages are smaller than normal, and in the most severely affected embryos (Fig. 5c) these appendages fail to fuse. In all of these cases, the abnormal labial appendages fail to migrate to their normal position between the maxillary palps. These abnormalities produce secondary effects on the orientation of the other head appendages.

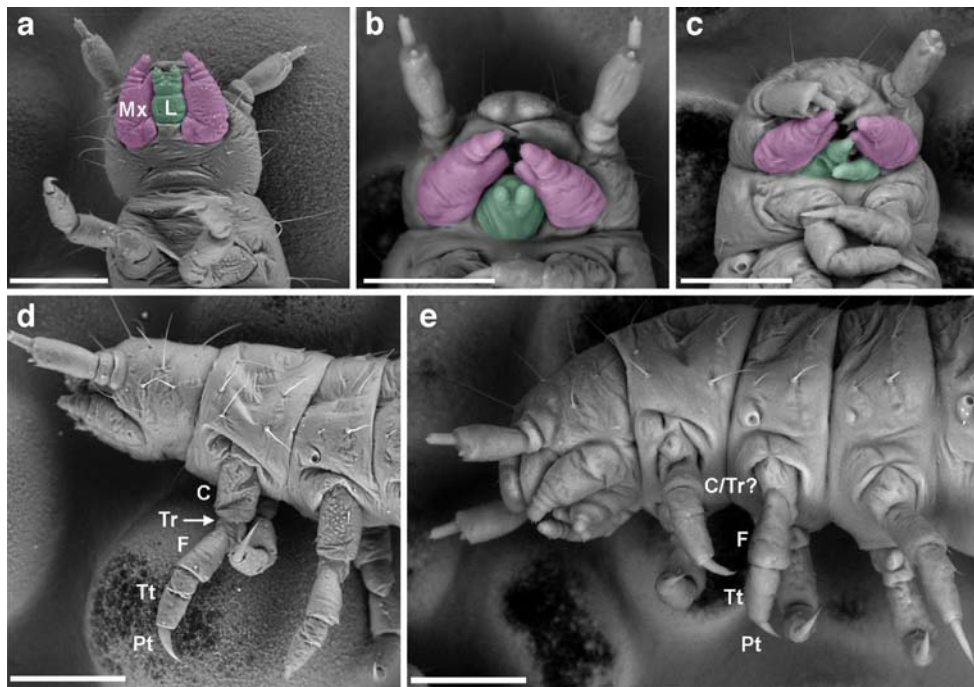
Short legs were the most common feature seen in *Tc-tiotsh* RNAi larvae, being present in all larvae with labial defects, as well as in some larvae with apparently normal labial appendages (Table 2). The reduction in leg length stems from effects on the proximal leg segments. The coxa and trochanter are reduced and apparently fused, and the femur is slightly shortened (Fig. 5e). This phenotype is similar to that observed in the adult legs of hypomorphic *Drosophila tsh* mutants (Erkner et al. 1999) and thus may indicate a conserved role for *tsh* family genes in proximal limb development.

We also noted that *Tc-tiotsh* RNAi larvae show a pronounced curvature of the head and thoracic segments, such that the head bends forward (Fig. 5e). This phenotype apparently results from reduction in length (anterior to posterior) of the ventral portions of the thoracic segments and posterior head (Fig. 5b,c,f).



**Fig. 4** *Tc-tiotsh* expression in *Tribolium* embryos. Engrailed expression is shown in brown, while *Tc-tiotsh* expression is in purple. Embryos are oriented with anterior to the left (except in panel **d** in which anterior is to the top). **a** During germband elongation, *Tc-tiotsh* is expressed in the gnathal and trunk segments as well as two procephalic regions (the intercalary segment [*Ic*] and the labrum [*Lr*]). **b** When germband elongation is completed, *Tc-tiotsh* expression is strongest in the thoracic (T1–T3) and first abdominal (A1) segments. **c** Slightly later, strong

*Tc-tiotsh* expression appears in the posterior terminal region of the embryo (arrowhead). **d** *Tc-tiotsh* is expressed in only the proximal portion (arrow) of the developing legs. **e** In embryos undergoing germband retraction, *Tc-tiotsh* expression is present in the six Malpighian tubule primordia that evert from the hindgut. **f** In a *Df(HOMC)* homozygote lacking most Hox genes, *Tc-tiotsh* expression in the segmented portion of the germband is greatly reduced. In contrast, expression in the Malpighian tubule primordia remains strong



**Fig. 5** Scanning electron micrographs of *Tc-tiotsh* RNAi larvae. For all panels, the scale bar represents 50 $\mu$ m. **a–c** Ventral views of larval heads. Maxillary (*pink*) and labial (*green*) appendages have been shaded. **d–e** Lateral views with leg segments labeled as follows: coxa (*C*), trochanter (*Tr*), femur (*F*), tibia (*Tt*), and pretarsus (*Pt*). **a** Wild-type larval head showing the proper position of the maxillary (*Mx*) and labial (*L*) appendages. **b** Moderately affected *Tc-tiotsh* RNAi

larva in which the labial appendages (especially the proximal region) are highly reduced. **c** Severely affected *Tc-tiotsh* RNAi larva in which the labial appendages have failed to fuse. Note that in both **b** and **c**, the ventral, posterior region of the head (presumed to derive from the labial segment) is almost completely absent. **d** Wild-type larva. **e** *Tc-tiotsh* RNAi larva with short legs. On each leg, the trochanter is presumably fused with the reduced coxa

*Tc-tiotsh* is important for proper development of adult trunk segments

We examined the role of *Tc-tiotsh* in adult development using larval RNAi (Tomoyasu and Denell 2004). The resulting adults display a number of defects in the thoracic and abdominal segments (Fig. 6). Most noticeably, these individuals have abnormalities of the T2 notum that prevent the elytra from closing properly (data not shown). We also

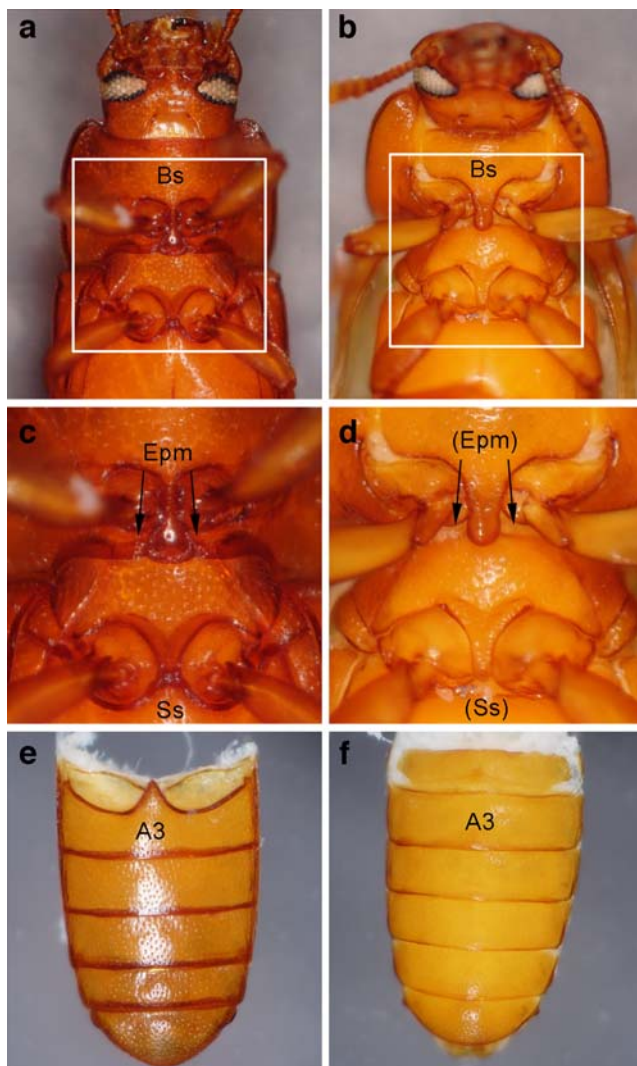
observed mild defects in the proximal legs. In addition, adults resulting from *Tc-tiotsh* larval RNAi show abnormalities in many of the ventral sclerites (sternites) (see El-Kifl 1953 for a description of wild-type structures). In the ventral prothorax (T1) of RNAi-treated individuals, the epimera, which are normally located posterior to the T1 coxae, are missing and the basisternum is reduced in size resulting in the exposure of membranous areas (Fig. 6b,d). In addition, the shape of the sternellum (the process

**Table 2** *Tc-tiotsh* RNA interference cuticular phenotypes

dsRNA fragment	Severity of Effect			
	None	Mild	Moderate	Severe
1358–1786	12/74 (16.2%)	4/74 (5.4%)	14/74 (18.9%)	44/74 (59.5%)
1358–1786	2/58 (3.4%)	14/58 (24.1%)	39/58 (67.2%)	3/58 (5.2%)
1848–2581	0/24 (0%)	4/24 (16.6%)	20/24 (83.3%)	0/24 (0%)

All results were obtained by injection of dsRNA into eggs. dsRNA fragment names refer to the nucleotides of GenBank sequence AF356647 included in the fragment. Severity of effect was classified as follows. Larvae with mild effects had short legs resulting from reduction and fusion of proximal segments. Moderately affected larvae had reductions in the ventral head and labial appendages in addition to short legs. Severely affected larvae were distinguished from those with moderate effects by the failure of the labial appendages to fuse at the ventral midline.





**Fig. 6** Adult phenotypes resulting from *Tc-tiotsh* larval RNAi. All images are ventral views with anterior to the top. **a** Wild-type adult head and thorax. **b** Head and thorax of *Tc-tiotsh* RNAi individual. The T1 basisternum (Bs) is smaller than normal. **c** Enlarged view of region outlined in **a**. **d** Enlarged view of region outlined in **b**. The T1 epimera (Epm) and the T3 meso-spinasternum (Ss) are reduced or missing. **e** Wild-type adult abdomen. **f** Abdomen of *Tc-tiotsh* RNAi adult. The third abdominal segment (A3) lacks its anterior process, and an additional heavily sclerotized segment is present anterior to A3

extending between the T1 legs) is abnormal. Ventral T2 is not noticeably affected, but the T3 sternite is missing its anterior process (meso-spinasternum) (Fig. 6d). The anterior abdominal segments are also affected by *Tc-tiotsh* larval RNAi. In wild-type adults, the first and second abdominal segments (A1–A2) form an internal portion of the T3 leg sockets and are only lightly sclerotized, while A3 is distinguished by a sharply pointed process extending between the T3 legs (Fig. 6e). In *Tc-tiotsh* RNAi individuals, an additional anterior region (A1 and/or A2) is heavily sclerotized and A3 lacks its anterior process, thus resem-

bling the more posterior abdominal segments (A4–A6) (Fig. 6f).

## Discussion

We have identified and cloned the lone member of the *tsh* gene family from *Tribolium castaneum*. Our analysis of this gene provides important insights into the evolution of the *tsh* gene family at the levels of both sequence and function.

### Evolution of *tsh* family genes

Searches of several non-drosophilid insect genome sequences identified only a single *tsh* family member in each genome. These results corroborate previous suggestions (based on more limited data) that most insects have only a single *tsh*-related gene and that this is the ancestral state (Herke et al. 2005; Laugier et al. 2005). A key implication of this model is that the *Drosophila* genes *tio* and *tsh* are the result of a recent duplication event that occurred sometime after the divergence of mosquitoes and flies. Since that duplication, *tsh* has apparently diverged from the ancestral sequence faster than has *tio* (although both have diverged more than the *Tribolium* and *Anopheles* homologs). More rapid divergence by one member of a recently duplicated gene pair is not unprecedented. For example, the *Hox3* paralogs *zerknüllt* (*zen*) and *bicoid* (*bcd*) are believed to have arisen by duplication late in the dipteran lineage (Brown et al. 2001; Stauber et al. 1999, 2002), but the sequence of *Zen* is more similar to that of the *Hox 3* proteins of other insects than it is to *Bcd*.

Much of the sequence conservation between Tsh family members is found in the zinc finger regions. However, a few additional conserved motifs are present, particularly at the protein termini. The C-terminal region is well conserved between Tc-TioTsh, Ag-TioTsh, and Tio, but this conserved region is not found in Tsh. The conservation of the N-terminal region is particularly interesting. The Tsh family members for which N-terminal sequence is available fall into two distinct classes (Fig. 2c). Ag-TioTsh and both *Drosophila* proteins share an extended region of identity that is completely different than the short motif (MPRRKQ) shared by the *Tribolium* and vertebrate proteins. This pattern of conservation suggests that an ancestral Tsh family member had the MPRRKQ motif, which was replaced with a different motif sometime before the separation of *Anopheles* and *Drosophila* within the dipteran lineage. It is not clear what the function of these disparate motifs might be, as the vertebrate Tsh proteins function very similarly to Dm-Tsh when expressed in *Drosophila* (Manfroid et al. 2004). Perhaps these motifs are important in a particular tissue or at a different stage in development



than those assayed. As additional insect Tsh family protein sequences become available, it will be possible to more accurately determine the point at which these N-terminal sequences began to diverge.

#### Evolution of *tsh* family gene expression

Although the sequences of other insect *tsh* family members are most similar to *tio*, their expression patterns (where known) seem to be more similar to *tsh*. The embryonic expression patterns of *tsh* and *tio* in *Drosophila* are strikingly different (Laugier et al. 2005). *tsh* is expressed throughout the ectoderm of trunk segments, while *tio* is expressed in only a few cells of the trunk ectoderm (as well as in the Malpighian tubules and parts of the CNS and gut). In *tsh* mutants, however, *tio* is more broadly expressed in the trunk, indicating that *tsh* represses *tio* expression in this region. Likewise, *tsh* is ectopically expressed in the stomodeal region when *tio* expression is knocked down by RNAi.

The *Tc-tiotsh* expression pattern includes similarities to both the *tsh* and *tio* expression patterns. The ectodermal expression in the segmented region of the germband is reminiscent of, although more extensive than, that of *tsh*. On the other hand, *Tc-tiotsh* is expressed in the Malpighian tubule primordia, similar to *tio*. The expression of *tio* near the stomodeum might also correspond to the expression of *Tc-tiotsh* in the labrum and intercalary segment, although it is difficult to compare expression patterns in the cephalic regions of these insects due to the derived structure of the *Drosophila* larval head.

It is interesting that the *Tc-tiotsh* expression pattern approximates the sum of the *tsh* and *tio* patterns. This may indicate that the expression pattern of the ancestral gene that gave rise to *tsh* and *tio* had an expression pattern similar to *Tc-tiotsh* and that parts of its expression pattern (and perhaps its function) have been split between its daughters. Such division of labor resulting from complementary loss of particular expression domains or functions is termed subfunctionalization and has been suggested to play a key role in the maintenance of duplicated genes (Force et al. 1999).

Among other insects, expression patterns of *tsh* family members have been reported only for the firebrat, *Thermobia domestica*, and the milkweed bug, *Oncopeltus fasciatus* (Herke et al. 2005; Peterson et al. 1999). In both cases, these genes are expressed in the gnathal, thoracic, and first abdominal segments, with very weak (if any) expression in the remainder of the abdomen. Both insects also show expression in the posterior tip of the embryo (see below). *Oncopeltus* has additional expression in the procephalon. The leg expression pattern in firebrats has not been described, but Herke et al. (2005) reported that milkweed

bugs show expression throughout the legs, which fades from the distal tips later in development.

Interestingly, all of the insect *tsh* family genes whose expression has been described are expressed in the post-abdominal region. However, these domains have been interpreted differently. In firebrats, expression in this region is reported to represent the anlagen of the cerci and the median caudal filament (appendages of the 11th abdominal segment) (Peterson et al. 1999). The posterior expression of *tsh* has been interpreted as the future anal opening (Fasano et al. 1991), and that of *Oncopeltus tiptop* is described only as the caudal region (Herke et al. 2005). Our results show that *Tc-tiotsh* is expressed in the Malpighian tubule primordia. Likewise, *tio* is expressed in the developing Malpighian tubules (Laugier et al. 2005). Thus, Malpighian tubule expression may have been part of the ancestral expression pattern of this gene family. It is possible that the posterior expression domains in other insects will, upon closer examination, be found to represent the developing Malpighian tubules as well.

#### Evolution of *tsh* family function

*Tc-tiotsh* is required for the proper development of the proximal portion of developing larval legs. *Drosophila* larvae lack external appendages, but *tsh* is expressed in the proximal regions of leg imaginal discs and hypomorphic *tsh* alleles cause proximal leg defects (Erkner et al. 1999). These results suggest that proximal leg expression and function were probably a feature of a *tsh*-like gene in the ancestor of beetles and flies. In contrast, Herke et al. (2005) reported that knockdown of *Oncopeltus tiptop* caused transformations of the distal legs to antennae. Based on this observation, they concluded that *Oncopeltus tiptop* acts as a distal leg selector gene, and suggested that *tio* or *tsh* might play a similar role in *Drosophila*. *tsh* expression is known to be restricted to the portion of the imaginal disc that will form the body wall and proximal leg segments (Gonzalez-Crespo and Morata 1996). Similarly, *Tc-tiotsh* is expressed in the proximal region of embryonic legs and leg imaginal discs (Fig. 4d and data not shown). We have also examined the expression of *tio* in *Drosophila* leg imaginal discs and found that expression is restricted to the body wall and proximal portion of the developing leg. Thus, it is highly unlikely that the “*tiptop*-like” distal leg-specifying function suggested by Herke et al. (2005) is performed by *Tio*, either alone or in combination with *Tsh*.

We cannot rule out additional, internal functions for *Tc-TioTsh*. In particular, the expression of *Tc-tiotsh* in the Malpighian tubules is intriguing, as *Drosophila tio* (and possibly other homologs) show similar expression.

The striking effects of larval *Tc-tiotsh* RNAi would not have been predicted based on *Drosophila* experiments, as

relatively few adult effects of LOF *tsh* mutations have been reported. Other than the proximal leg defects that have been discussed above, the only other adult phenotypes reported are defects in wing posture (Soanes et al. 2001) and in structures that develop from the eye-antennal disc, namely, the eyes (Singh et al. 2002) and the maxillary palps (Bhojwani et al. 1997). This dearth of adult phenotypes could be a consequence of the pre-adult lethality of many *tsh* allele combinations. However, it might also be due to functional redundancy with *tio*. We examined *tio* expression in *Drosophila* imaginal discs and found that it is indeed expressed in a pattern very similar to that of *tsh* (data not shown). Thus, loss of both *tio* and *tsh* during *Drosophila* imaginal development might produce a more severe phenotype.

The principal question prompting our studies of the *Tribolium Tc-tiotsh* gene was whether the trunk-specifying function reported for *tsh* in *Drosophila* was conserved in other insects. However, insights from our work and other recent reports compel us to first reexamine whether *tsh* itself is really a trunk-specifying gene. The key observations, which originally led to this conclusion, were the presence of head-like sclerites in embryos lacking *tsh*, and the positive correlation between expression of *tsh* and the presence of denticle belts (a trunk-specific feature) (de Zulueta et al. 1994; Fasano et al. 1991; Roder et al. 1992). The later discovery that *tsh* and *disco* form mutually exclusive domains in the trunk and head, respectively, seemed to support this view (Robertson et al. 2004). Below we synthesize recent studies that may lead to new interpretations of these observations.

A number of mutant phenotypes in *Drosophila*, including those of *tsh* and some Hox genes, feature small patches of sclerotic tissue. The previous interpretation of such patches as ectopic head tissue has been called into question. Mace et al. (2005) showed that the sclerites that form in a segment lacking Hox gene expression are correlated with breaches in epidermal integrity and the resulting activation of a wound response pathway. If in fact, the sclerotized cuticle seen in *tsh* mutants is scar tissue rather than head cuticle, the interpretation of *tsh* as a trunk-specifying gene would be less convincing, but still possible.

The case for *tsh* acting as a trunk specifier was apparently strengthened when Robertson et al. (2004) proposed the attractive hypothesis that a system of zinc finger proteins acted in conjunction with the Hox genes to specify body regions. Within this system, *disco* specified gnathal segments and *tsh* specified trunk segments. However, the same laboratory recently reported that the conserved role of *disco* is to specify the medial portion of appendages, and suggested that the boundary between *disco* and *tsh* expression is actually an artifact of maggot morphology (Patel et al. 2007). That is, the reduced

*Drosophila* larval head might lack proximal, *tsh*-expressing tissue (body wall) while retaining medial appendage tissue, whereas the trunk retains *tsh*-expressing tissue but has lost most appendage tissue. Taking this scenario a step further, ectopic expression of *tsh* might “restore” the body wall tissue of the head, creating larger, more complete segments. As the genes controlling segment formation also control denticle belt patterning, these enlarged head segments would have denticle belts as well. This interpretation is consistent with the observation that the additional denticle belts produced by ectopic *tsh* expression are interspersed with head-specific structures (de Zulueta et al. 1994). When considered in this light, perhaps *tsh* is no more a trunk identity gene than are other genes involved in segment formation and denticle patterning such as *wingless*, *rhomboid*, or *shavenbaby*. On the other hand, *tsh* does seem to provide the spatial information that limits full segment formation to the trunk, and thus in *Drosophila* could be considered a trunk-specifying gene.

In contrast, *Tc-tiotsh* does not appear to act in any way as a trunk identity gene. This is not to say that *Tc-tiotsh* has no role in the development of trunk segments, but rather that we find no evidence that it specifies trunk vs head fates. In embryos depleted of *Tc-tiotsh* the ventral portions of the labial and thoracic segments are reduced, but not visibly transformed. Interestingly, some of the labial abnormalities observed in *Tc-tiotsh* RNAi larvae are reminiscent of those produced by loss of function alleles of *prothoraxless* (*Tc-Antp*) (Shippy, unpublished data). Knockdown of *Tc-tiotsh* during imaginal development produces adults with patterning defects in the thoracic and abdominal sclerites, including possible transformations of anterior abdominal sclerites to a more posterior identity. These phenotypes, together with the reduction in *Tc-tiotsh* expression seen in Df(HOMC) embryos, suggest that *Tc-tiotsh* is regulated to some extent by Hox genes and may carry out a subset of the functions of these genes in both the head and the trunk.

Our studies of *Tc-tiotsh* indicate that trunk specification is not a universal function of *tsh* family members. It is possible that this function has been lost in *Tribolium*. However, expression pattern data from other insects and functional analysis in *Oncopeltus* argues against this conclusion. A more likely scenario is that *tsh* family members acquired a role in defining trunk at some point after the divergence of the coleopteran and dipteran lineages. Analysis of *tsh* function in additional holometabolous insects should help pinpoint when this event occurred.

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