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Direct interaction between Teashirt and Sex combs reduced proteins, via Tsh's acidic domain, is essential for specifying the identity of the prothorax in *Drosophila*

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Abstract

teashirt (tsh) encodes a zinc-finger protein that is thought to be part of a network that contributes to regionalization of the *Drosophila* embryo and establishes the domains of Hox protein function. *tsh* and the Hox gene *Sex combs reduced* (*Scr*) are essential to establish the identity of the first thoracic segment. We used the development of the first thoracic segment as a paradigm for *Scr* dependent regional morphological distinctions. In this specific context, we asked whether Tsh protein could have a direct influence on Scr activity. Here we present evidence that Tsh interacts directly with Scr and this interaction depends in part on the presence of a short domain located in the N-terminal half of Teashirt called "acidic domain". In vivo, expression of full length Tsh can rescue the *tsh* null phenotype throughout the trunk whereas Tsh lacking the Scr interacting domain rescues all the trunk defects except in the prothorax. We suggest this provides insights into the mechanism by which Tsh, in concert with Scr, specifies the prothoracic identity.

Keywords: Teashirt; Sex combs reduced; Hox; Prothoracic identity; Trunk; Transcriptional repression; *Drosophila*

Introduction

The homeotic selector genes (Hox) are required to specify segmental identity along the anterior–posterior body axis (Lewis, 1978; Wakimoto and Kaufman, 1981a). Mutations in fly Hox genes lead to alterations in segmental identity, without affecting segment number. The Hox proteins are transcription factors that contain a DNA binding domain called the homeodomain. The discrepancy between the weak in vitro DNA binding specificity of the different Hox proteins and their highly specific action in vivo is thought to be due, at least in part, to their interaction with cofactor proteins. Cofactors form complexes with Hox proteins and are thought to improve the affinity and specificity of Hox proteins for specific DNA regulatory elements. Among the few co-factors known, Homothorax (Hth) and Extradenticle (Exd) and their respective vertebrate orthologues Meis and Pbx are best characterized (Mann and Affolter, 1998). Previously we reported that *teashirt (tsh)* encodes a zinc-finger protein and functions as a genetic co-factor during Hox specification of the trunk (thorax–abdomen) segment identity (Alexandre et al., 1996a; de Zulueta et al., 1994; Fasano et al., 1991; Roder et al., 1992). In vivo Tsh was shown to interact with Hth during embryonic development (Bessa et al., 2002). Recent data suggest that Tsh is part of a network that contributes to regionalization of the *Drosophila* embryos and establishes the domains of Hox protein function (Robertson et al., 2004). *tsh* is essential to establish the T1

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identity as does the Hox gene *Sex combs reduced (Scr)* that is expressed in the posterior maxillary (Mx) and labial (Lb) head segments and in the anterior part of the adjacent prothoracic (T1) segment (Kuroiwa et al., 1985; Martinez-Arias et al., 1987; Wakimoto and Kaufman, 1981b). *tsh* is regionally expressed only in the trunk (Alexandre et al., 1996a; Fasano et al., 1991) and is required to define a basic trunk identity (Roder et al., 1992). *tsh* loss of function mutations lead to the homeotic transformation of T1 into Lb identity (Fasano et al., 1991; Roder et al., 1992) and to ectopic expression of *Scr* in the T1 segment (Fasano et al., 1991). Conversely, ectopic expression of Tsh results in homeotic transformation of the Lb into T1 identity (de Zulueta et al., 1994) but does not affect *Scr* expression (Alexandre et al., 1996a). During embryonic development, the Scr protein is detected within the labial and the prothoracic segments (Mahaffey and Kaufman, 1987; Riley et al., 1987). Loss of function mutations in *Scr* are embryonic lethal and lead to homeotic transformations, with the T1 taking on mesothoracic (T2) identity, and the Lb resembling a Mx segment (Wakimoto and Kaufman, 1981a). When *Scr* is ubiquitously mis-expressed during embryonic development, both the T2 and metathoracic (T3) segments are transformed to T1 identity (Gibson and Gehring, 1988). Finally, when *tsh* and *Scr* are simultaneously expressed everywhere as many as six segments acquire T1 identity, three in the thorax and three in the head (de Zulueta et al., 1994). Together, these results strongly suggest that when co-expressed *tsh* and *Scr* are sufficient to induce T1 fate.

We thus decided to investigate if Tsh could be a co-factor for Scr. Here, we address this issue by performing an in vivo and in vitro structure–function analysis of the Tsh protein. We conclude the following: (1) Tsh interacts directly with Scr; (2) in vitro, 37 residue, acidic-rich, domain of Tsh is required for this interaction; (3) in vivo, we show that this domain is functionally important; (4) ubiquitously provided full length Tsh rescues a *teashirt* null phenotype whereas Tsh lacking the Scr interacting domain is able to rescue all the trunk defects except in the prothorax. These observations lead us to propose that Tsh in concert with the Hox protein Scr promotes the specification of the prothoracic identity.

Materials and methods

tsh deletion constructs

A full length *tsh* cDNA flanked by two *Not*I sites was PCR generated; this construct, *Not*I-tsh-cDNA, was subsequently used to generate a series of constructs, using the restriction enzymes indicated, with in frame deletions of the Tsh protein: TshΔ*Bgl*II–*Afl*II, TshΔ*Eco*RI, TshΔ*Bsp*MI, TshΔ*Kpn*I, TshΔXba–*Afl*II, TshΔ*Bam*HI TshΔ*Msc*I, TshΔ*Eco*NI, TshΔ*Sph*I, TshΔ*Afl*II–*Cla*I, TshΔ*Stu*I–*Nhe*I, TshΔ*Nsi*I–*Nhe*I (Fig. 1A). The TshΔacid construct was made with a single primer pairs (details are available upon request). TshΔPLDLS was published in Manfroid et al. (2004). These constructs were used in the yeast two-hybrid system and to establish UAS transgenic fly lines.

Yeast two-hybrid constructs

The yeast two-hybrid system uses transcriptional activity of reporter genes (*LacZ* or *Leu*^{as}) to measure protein–protein interactions (Fields and Song, 1989; Golemis and Gyuris, 1994). The materials used for the study, including yeast strain EGY48 (*MAT trp1 ura3 his3 lexAop-LEU2*), the plasmids pEG202, JG4–5 and pSH 18–34 (Golemis and Gyuris, 1994; Gyuris et al., 1993).

Drosophila stocks

Transgenic lines were established using standard procedures (Rubin and Spradling, 1982). *tsh* constructs were cloned into the pUAST vector and expression was driven by 69B-Gal4 (Brand and Perrimon, 1993).

To select *tsh* null embryos expressing FL Tsh or TshΔacid we performed the following crosses: *w; tsh*^{8}*/CyOActin5CGFP; 69BGal4/69BGal4 × w; tsh*^{8}*/CyOActin5CGFP; UAStsh/UAStsh* and *w; tsh*^{8}*/CyOActin5CGFP; 69BGal4/69BGal4 × w; tsh*^{8}*, UAStshΔacid/CyOActin5CGFP. tsh* null embryos (GFP negative) were selected under a fluorescence binocular microscope.

The mod-1050-*lacZ* transgene and expression patterns have been described (Alexandre et al., 1996b). Other mutations, aberrations and abbreviations are described in (Fasano et al., 1991; Lindsley and Zimm, 1992) or in FlyBase (http://flybase.bio.indiana.edu). UAS-Scr was a gift of M. Akam. Embryonic stages are as described by (Bate and Martinez-Arias, 1993).

Preparation of embryonic cuticles and in situ hybridization

Embryos were collected for approximately 12 h and aged for more than 24 h before preparing cuticles as described in Fasano et al. (1991). Antisense DIG RNA probes to *mod* and *LacZ* were generated as described in Alexandre et al. (1996b). *In situ* hybridization and antibody staining of whole-mount embryos were performed as in Kosman et al. (2004). *tsh* and *Scr* RNA probes were respectively labelled with digoxigenin (DIG) and dinitrophenyl (DNP). *tsh* and *Scr* RNA probes were respectively detected with primary antibodies Sheep anti DIG (1/300) and Rabbit anti DNP (1/500). For fluorescent detection, secondary antibodies conjugated with Alexa fluor dyes (Molecular probes), Alexa 555 donkey anti-sheep and Alexa 647 chicken anti-rabbit were used at 1/500. Images were obtained with a Leica SP2-AOBS confocal microscope.

Gst Fusion proteins and pull-down assays

GST-Tsh, GST-Scr, and Gst Antp fusion constructs were expressed in BL21 pLysS cells (Novagen) and were purified on glutathione-sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer's protocols. Reticulocyte lysate proteins were produced using the TNT reticulocyte lysate synthesis kit (Promega). ^{35}S-Methionine (Amersham Pharmacia Biotech) was included in reaction for the purpose of labelling the synthesized proteins. A 2 µg aliquot of GST-Tsh, GST-Scr or Gst Antp was incubated with 5 µl of ^{35}S-labeled SCR, HoxA5, Antp or tshΔacid in 500 µl of binding buffer (50 mM Tris pH7.6, 150 mM NaCl, 0.2% Tween, 200 ng/ml BSA, 20 µg/ml PMSF, 0.5 µg/ml pepstatin, 0.5 µg/ml benzamidine, 20 µg/ml antipaïne and 0.5 mM DTT) for 1 h at 4 °C under agitation. Beads were washed 4 times with 500 µl of binding buffer. After being washed the bound proteins were eluted with SDS gel loading buffer and analyzed by electrophoresis on 10% SDS-PAGE. The fraction of ^{35}S bound protein was detected by autoradiography.

Electrophoretic Mobility Shift Assays (EMSA)

Equal amounts of GST fusion protein were monitored by Coomassie Blue staining. Gel shift assays were performed with the mod-84 fragment. Double stranded synthetic oligonucleotide probe mod-84 was end-labelled with polynucleotide Kinase and ^{32}P ATP and gel purified. DNA-binding reactions were performed at 4 °C for 20 min with 1 to 10 ng of Tsh and 0.5 to 5 ng of Scr in a total volume of 10 ul containing 50 mM KCl, 15 mM Hepes pH 8, 5 mM $MgCl_2$, 1 mM DTT, 0.025% NP40, 5% glycerol, 1 ug BSA and 20,000 cpm of DNA probe. DNA–protein complexes were resolved at 4 °C on a native 5% polyacrylamide gel at 12V/cm using 0.25× TBE as running buffer. The bands were visualized by autoradiography. Tsh antibody (Alexandre et al., 1996b) was used at 1:100 final dilution. The mouse monoclonal antibody to Scr (Scr6H4) (Glicksman and Brower, 1988) was kindly provided by Dr. D. Andrew and used at 1:50 final dilution.

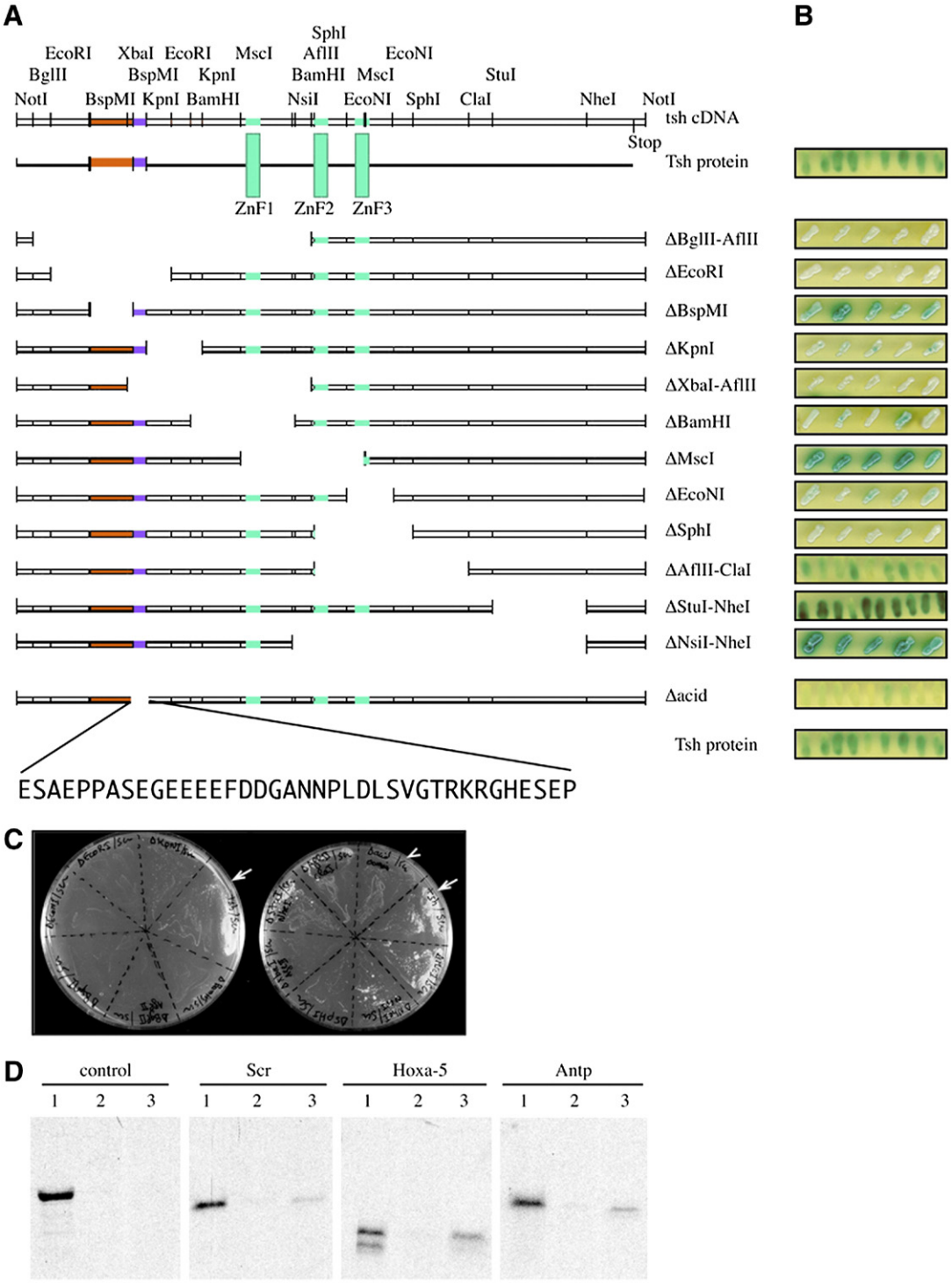

Fig. 1. Identification of an interaction between Tsh and Scr in the yeast two-hybrid system. (A) Schematic representation of the *teashirt* constructs. The top line shows the restriction sites of the *tsh* cDNA flanked by two *Not*I sites. The deletion constructs are shown below where gap corresponds to the deletion. The red boxes correspond to Alanine rich domains, the purple box is an acidic-rich domain and the three green boxes correspond to the three zinc finger motifs. (B) The interaction of Scr with Tsh constructs is monitored by β-galactosidase activity. Full length Tsh interacts with Scr. The constructs that delete the NH2 terminal part of Tsh loose their capacity to interact with Scr (i.e. TshΔ*Bgl*II–*Afl*II, TshΔ*Eco*RI, TshΔ*Xba*I–*Afl*II and TshΔacid). Note that the protein TshΔ*Sph*I construct is unable to interact with Scr, but the overlapping TshΔ*Eco*NI, TshΔ*Afl*II–*Cla*I and TshΔ*Nsi*I–*Nhe*I proteins do interact. The protein sequence of Tsh corresponding to the purple box in A is shown bellow the TshΔacid construct. (C) Reporter activities of the same constructs but on a selective medium (Leu^-) confirm the results obtain in the β-galactosidase test. Compare Tsh/Scr (arrow) with TshΔacid/Scr (arrow head). (D) GST interactions assays were performed with GST or GST-Tsh and ^{35}S-labelled Scr, HoxA5, Antp or luciferase as control. GST-Tsh binds to Hoxa-5 (the mouse ortholog of Scr) (middle panel) and to Antp (right panel). In all four panels, input (lane 1), 40% of the ^{35}S protein involved in the assay (lane 3); lane 2, control with the GST; lane 3, GST pull down).

Results

Tsh interacts directly with the Hox protein Scr

To test for direct interaction between Tsh and Scr we employed the yeast two-hybrid system (Fields and Song, 1989; Golemis and Gyuris, 1994). The DNA binding domain of LexA was fused in frame to full length Tsh and to various deletion mutants of Tsh (Fig. 1A), and then tested for interaction with Scr fused to the B42 transcriptional activation domain (Fig. 1B). On is own, none of the Tsh proteins activated reporter gene expression (data not shown). In contrast, Tsh and Scr together lead to activation of the reporter genes (*LexAop-LacZ or LexAop-LEU2*), supporting that they interact directly (Figs. 1B, C). The direct interaction between Tsh and Scr full length was confirmed by glutathione *S*-transferase GST-fusion protein–protein interaction (GST pull-down) studies. GST-Tsh was tested for its ability to retain in vitro ^{35}S-labeled Scr protein. Under these conditions, we also observed direct interaction between Tsh and Scr (Fig. 1D).

Next, we determined which part of Tsh was important for the interaction with Scr. To this aim, we examined the capacity of various Tsh deletion constructs to interact with Scr. Unknowingly, our analysis reveals that one deletion in the C-terminal half of Tsh (TshΔ*Sph*I) failed to interact with Scr (Fig. 1B). However, larger deletions (TshΔ*Afl*II–*Cla*I and TshΔNsi–*Nhe*I) uncovering this deletion and removing mainly the C-terminal half of Tsh strongly interacts with Scr (Fig. 1B).

This analysis also identified a series of constructs that delete larger intervals within the amino half of Tsh and fail to interact with Scr (TshΔ*Bgl*II–*Afl*II, TshΔ*Eco*RI and TshΔ*Xba*I–*Afl*II) and, two constructs (TshΔ*Bsp*MI and TshΔ*Kpn*I) that delete smaller domains interact with Scr (Fig. 1B). These results suggest that the interaction requires a sequence uncovered in common by the larger deletions (purple box, Fig. 1A). This deletion corresponds to a stretch of 37 amino acids rich in acidic residues (13/37 D or E residues, net charge of −9). To assess whether this motif contributed to Tsh/Scr interaction, we deleted it. Our data confirm that the absence of this region abrogates the Tsh/Scr interaction (Figs. 1B,C). The production of both Tsh and TshΔacid was verified by Western blot using an anti-Tsh antibody. Both Tsh and TshΔacid were detected (data not shown), ruling out the possibility that the lack of interaction between TshΔacid and Scr was due to a failure of production of TshΔacid. Finally, GST-Scr was tested for its ability to retain in vitro ^{35}S-labeled TshΔacid protein and no interaction was detected (data not shown).

Previous studies have shown that transgenic flies displayed Scr-like homeotic transformations after ectopic expression of HoxA5 and that HoxA5 participated in regulatory networks by activating down-stream target of Scr (Zhao et al., 1993b). To test the possibility that the Tsh–Hox interactions were conserved through evolution, we performed an in vitro protein-binding assay with GST-Tsh and ^{35}S-labeled HoxA5, the functional murine orthologue of Scr (Zhao et al., 1993a). Full length HoxA5 bound specifically, and as efficiently as Scr, to GST-Tsh but not to GST alone (Fig. 1D).

Finally, our developmental studies suggested that not only in the T1 but within the trunk Tsh is a potential Hox cofactor (de Zulueta et al., 1994; Fasano et al., 1991; Roder et al., 1992). Here we showed interaction between Tsh and Antp (Fig. 1D).

Labial to prothoracic homeosis depends on the acidic domain of Tsh

In the wild-type larval epidermis, each trunk segment has characteristics that allow them to be distinguished morphologically. For example, the T1 segment is characterized by the presence of a "beard" of denticles (arrowhead in Fig. 2A). We previously showed that expression of the *tsh* transgene under the control of *69B-Gal4* (*69B>tsh*) in the epidermis results in transformation of the labial (the last head) segment into T1 segment (Manfroid et al., 2004; Fig 2B). We decided to use this phenotype as an in vivo functional assay to score the biological effects of the Tsh derivative proteins. To this aim, we generated transgenic flies with all Tsh constructs shown in Fig. 1A. Analysis of the cuticles showed that homeosis of the labial segment depends on the presence of the acidic domain and the zinc finger motifs (see Table 1). For example, TshΔ*Msc*I interacts with Scr (Fig. 1A) but its misexpression does not lead head to trunk homeosis (not shown). At 18 °C and 25 °C, 69B>*tsh* larval cuticles show a clear transformation of Lb into T1, as visualized by the appearance of a prothoracic denticle belt and a beard (Fig. 2B). Interestingly, in 69B>tshΔacid larvae morphogenesis of the head skeleton is affected but we never observed homeosis of the labial segment (Fig. 2C). The finding that TshΔacid protein is completely devoid of transformation activity strongly suggests that the acidic domain of Tsh is essential for Tsh homeotic function.

The acidic-rich domain of Tsh acts specifically in the prothorax

To determine if the acidic domain was required for all aspects of *tsh* function within the trunk, we expressed TshΔacid in a *tsh*^{8} null background. In *tsh*^{8} mutant, the T1 is transformed into Lb identity and in the rest of the trunk, a weak segment polarity phenotype is observed (Figs. 2D, D′) (Fasano et al., 1991; Gallet et al., 1998). All *tsh*^{8}/*tsh*^{8}; *69B>tsh* larvae show a complete rescue of the trunk phenotype and homeotic transformation of head segments (Figs. 2E, E′). In *tsh*^{8}/*tsh*^{8}; *69B>tshΔ acid* larvae, TshΔacid was unable to rescue the T1 phenotype but rescues all the defects in the other trunk segments (Figs. 2F, F′). Moreover, TshΔacid was unable to induce homeosis of the head segments (compare Figs. 2E and F). The ubiquitous production of TshΔacid was monitored and confirmed with Tsh polyclonal antibody (data not shown). These results show that the TshΔacid protein is functional and more importantly that the acidic domain has a specific role for T1 identity that is not required in the rest of the trunk.

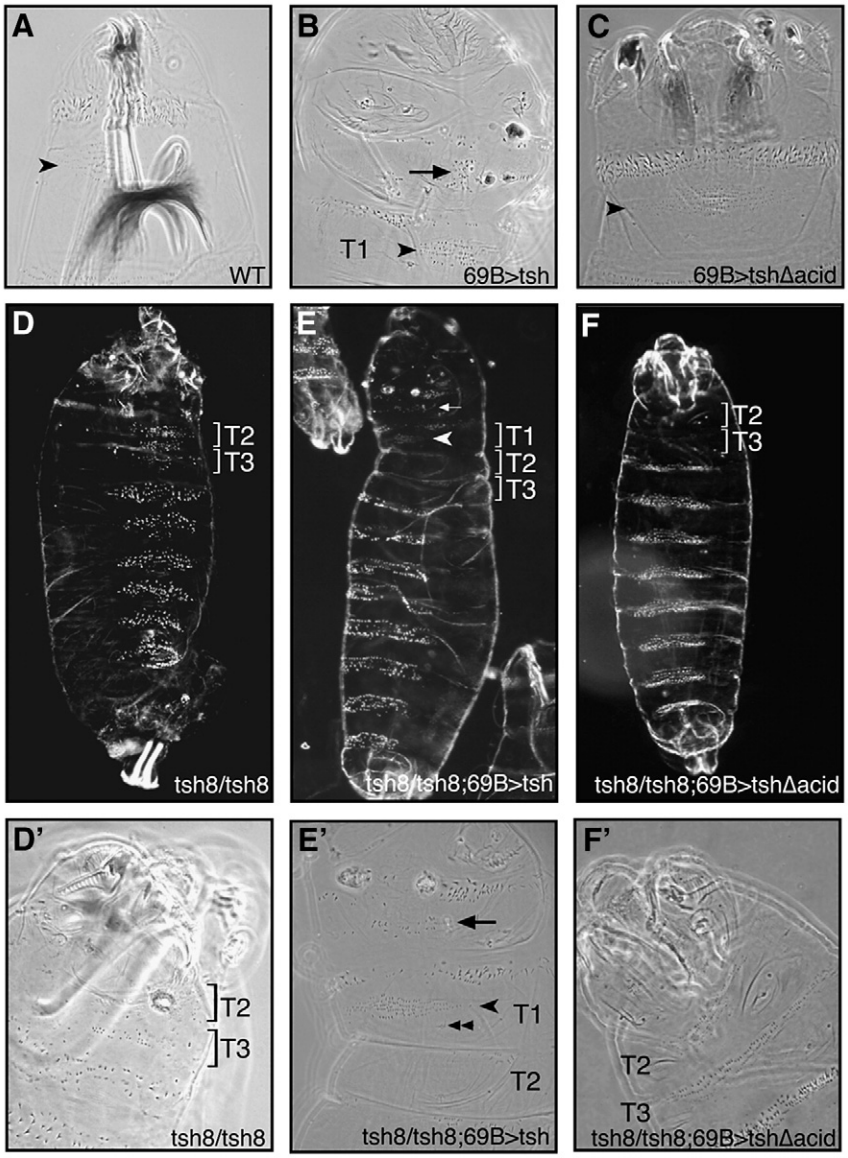

Fig. 2. The acidic-rich domain of Tsh has a specific role for T1 identity but not for the rest of the trunk. (A) Wild type larval cuticle (phase contrast) of the trunk-head region showing the T1 segment and its beard (arrow head). The head segments are invaginated inside the larva. (B) Ectopic expression of FL Tsh with the epidermal 69B-Gal4 driver (69B>*tsh*) showing the homeotic transformation of the labial segment toward T1 at 18 °C (At higher temperatures, FL Tsh induces naked cuticle (Gallet et al., 1998) masking the production of a clear T1 pattern), and the disruption of the head skeleton. Arrow shows an ectopic beard in the transformed labium. (C) 69B>*tshΔacid* at 29 °C during embryogenesis. No homeosis of the labial segment is detectable, but the head skeleton is affected. (D, D′) tsh^{8} ventral larval cuticle (dark field, D) and close-up (phase contrast, D′). The T1 is transformed into labial identity and the cuticle of the other trunk segments is disorganized. (E, E′) In tsh^{8}/tsh^{8}; 69B>*tsh* cuticle at 25 °C, the *tsh* null phenotype is rescued and the labial segment is transformed into T1. At this temperature, overexpression of Tsh mimics ectopic Wingless signalling, explaining why denticle belts seem badly rescued. Arrowhead: beard in the endogenous T1; arrow: ectopic beard in the labial; double arrow head: restored Keilin's organ in the T1. (F, F′) In all ($n=60$) tsh^{8}/tsh^{8}: 69B>*tshΔacid* cuticles at 29 °C there was no rescue of the T1 phenotype but rescue was observed in the rest of the trunk. In all views, anterior is to the top.

Repression of modulo, a target gene of Scr, depends on the presence of the acidic domain of Tsh

In vivo, Hox proteins establish region-specific differences in gene expression along the antero-posterior axis that will contribute to establish segment identity diversity. During development, *tsh* and *Scr* are co-expressed in ventral and lateral epidermis of the anterior T1 segment (Figs. 3A–C) and they are needed to establish the prothoracic identity. The interaction between Tsh and Scr proteins raised the possibility that Tsh may help the Hox protein Scr in establishing differences in gene expression along the antero-posterior axis. In the labial segment, *Scr* positively controls the epidermal expression of *modulo* (*mod*), whereas Tsh represses *mod* expression in the T1, restricting epidermal expression of *mod* to the Lb (Fig. 3D) (Alexandre et al., 1996b; Garzino et al., 1992; Graba et al., 1994; Laurenti et al., 1995). In a tsh^{8} null mutant, the expression of *Scr* and *mod* in the labial segment is not affected but the absence of Tsh allows ectopic expression of *Scr* and *mod* in the ventral part of the T1 segment (Alexandre et

Table 1
Results of ectopic expression of the constructs and yeast two-hybrid interaction tests

Teashirt constructs	Acidic motif	Deleted ZnF	Lab->T1	Interaction
Tsh	p	None	+	+
Δ*Bgl*II–*Afl*II	a	1	−	−
Δ*Eco*RI	a	None	−	−
Δ*Bsp*MI	p	None	Weak	+
Δ*Kpn*I	p	None	+	Weak
Δ*Xba*I–*Afl*II	a	1	−	−
Δ*Bam*HI	p	1	Weak	Weak
Δ*Msc*I	p	1, 2, 3	−	+
Δ*Eco*NI	p	3	+	Weak
Δ*Sph*I	p	2, 3	−	−
Δ*Afl*II–*Cla*I	p	2, 3	−	+
Δ*Stu*I–*Nhe*I	p	None	+	+
Δ*Nsi*I–*Nhe*I	p	2, 3	−	+
Δacid	a	None	−	−

Interaction of Tsh constructs with Scr in yeast and their capacity to induce homeosis. In the "acidic motif" column, presence (p) or absence (a) of the acidic motif within the construct is stated. In the "deleted Znf "column, the Zinc finger motif(s) deleted in the construct is (are) indicated (e.g. 1 stand for Znf1). In the "Lab->T1" column, the capacity of the construct to induce homeosis of the labial segment into T1 when ectopically expressed under the control of the 69B-Gal4 driver at 29 °C is indicated (homeosis: +; no homeosis: −; trunk transformation: weak). In the "interaction" column, the capacity of the construct to interact with Scr in the yeast two-hybrid system is indicated (interaction: +; no interaction: −; light blue staining and/or slow growth on Leu− medium: weak).

al., 1996b). In *69B>tsh* embryos, expression of Tsh in the Lb represses *mod* without affecting *Scr* expression (Fig. 3E) (Alexandre et al., 1996b). In *69B>tshΔacid* embryos, the expression of *mod* and *Scr* was not affected, demonstrating that Tsh mediated repression of *modulo* depends on the presence of the acidic domain (Figs. 3F, J).

modulo is a direct target of Scr

We previously reported that a regulatory sequence of l050 bp (mod-1050) was able to drive expression of the *lacZ* reporter gene in a similar pattern to *mod* (Fig. 3G) (Alexandre et al., 1996b). Moreover, electrophoretic mobility-shift (EMSA) and DNase I footprinting assays allowed us to show that the mod-1050 regulatory sequence contains Tsh binding sites (BS) that are located in a 84 base pair sub-fragment (mod-84) (Alexandre et al., 1996b). These results allowed us to conclude that *mod* was a direct target of Tsh (Alexandre et al., 1996b). In an *Scr* mutant background, *mod* is not expressed in the labial segment, but as of yet Scr has not been shown to bind directly to *mod* regulatory sequences (Alexandre et al., 1996b). All together, these data prompt us to test whether or not *mod* could be a direct target of Scr and thus potentially a common candidate gene for Tsh and Scr.

A computer-assisted search allowed us to identified two putative binding sites for Hox proteins within mod-84 and one is well conserved in five different *Drosophila* species (Fig. 4A). Next we analysed Scr DNA binding activity and showed that mod-84 produced one complex in EMSA assay (lane 7, Fig. 4B). Supershift EMSA identified Scr in the complex observed in the EMSA (lane 6, Fig. 4B). This result shows that Scr binds to *mod* regulatory sequence and supports that as a transcription factor, Scr controls directly in vivo the expression of *mod*.

Consistent with this result, we showed that, in the labial epidermis, not only mod-1050 regulatory element drives expression of the *lacZ* reporter gene in a similar pattern to *mod* but it also responds to Tsh as the endogenous *mod* gene; expression of mod-1050-*lacZ* reporter gene was repressed in 69B>*tsh* and unchanged in 69B>tshΔacid embryos (Figs. 3G–I).

Thus, our in vivo analysis showed that TshΔacid ectopically produced in the labial segment was unable to repress the expression of the *mod* gene and mod-1050-*LacZ* reporter gene. A simple explanation to these observations could be that TshΔacid does not bind to DNA. To rule out this possibility, TshΔacid DNA binding activity was analysed by EMSA. We found that mod-84 produced one complex in EMSA with TshΔacid and supershift EMSA identified TshΔacid in the complex (Fig. 4B). These results suggest that the absence of repression of *mod* and mod-1050-*LacZ* by TshΔacid is not due to the lost of DNA binding properties.

These data showed that in the labial segment the ectopic co-expression of Tsh and Scr leads to the repression of *mod* and this repression depends on the presence of the acidic domain of Tsh. We wonder if this artificial situation could reflect a real mechanism at work in the prothoracic segment. At stage 11, double *in situ* hybridization experiments showed that *tsh* and *Scr* were expressed in ventral and lateral cells of the T1 segment, suggesting that during embryonic development co-regulation of *mod* could take place (Fig. 3C).

To address the co-regulation of *mod* by Tsh and Scr within the T1, we mutagenised the Tsh BS, the Scr BS and both Tsh and Scr BS sites within the mod-84 regulatory element. These mod-84 variants were substituted for the endogenous mod-84 within the mod-1050-*lacZ* fusion gene and the effects were analyzed in transgenic embryos. In all cases, no expression of the LacZ reporter gene was detected in transgenic embryos, suggesting that Tsh and Scr regulators are indeed important for *mod* expression (data not shown).

Discussion

On the basis of morphologic differences and/or similarities, the *Drosophila* embryo can be divided into three regions; the head, the tail and the trunk (or thorax and abdomen) in between. Very importantly, the Hox genes *Antp*, *Ubx*, *abd-A* and the *tsh* gene function to establish trunk patterns and to prevent head ones to form (Roder et al., 1992). Here we demonstrated for the first time that the Tsh protein interacts directly with the Hox proteins Scr and Antp. Finally, investigating the mechanism by which the Tsh protein could contribute with Scr to establish the prothoracic identity, we showed that the *mod* gene is likely to be a direct target of Scr.

The acidic-rich domain of Tsh is only required in the prothorax

The analysis of different genetic contexts has lead to the model that co-expression of *tsh* and *Scr* is a prerequisite to

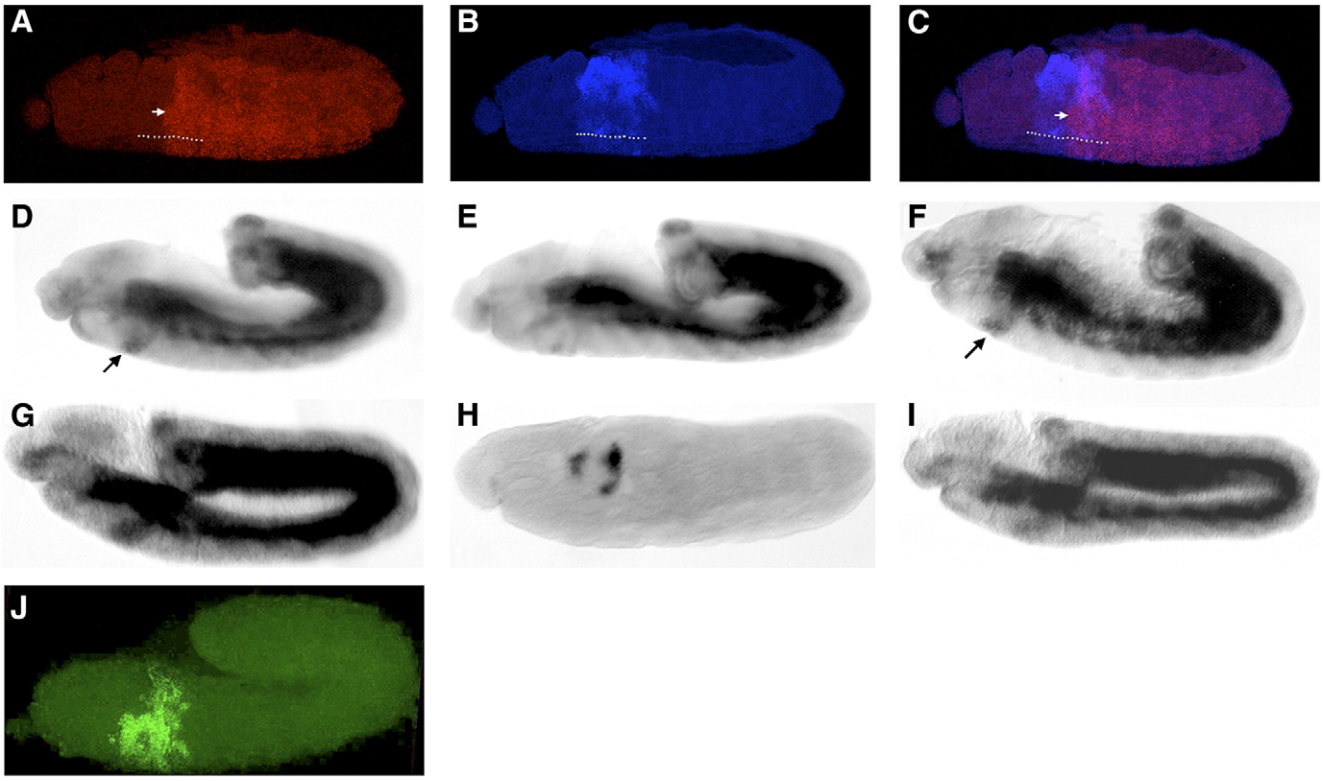

Fig. 3. In vivo, *mod* and mod-1050-*LacZ* are repressed by Tsh but not by TshΔacid. Double fluorescence *in situ* hybridization performed for *tsh* (red) and *Scr* (blue) at stage 11. (A) *tsh* is detected in the trunk. The anterior limit of expression of tsh corresponds to the border between the T1 and the labial segment (arrow in panel A). (B) *Scr* is detected in the labial segment and in the T1. In both segments, Scr expression is stronger laterally than ventrally. (C) Merged images. *tsh* and *Scr* are detected in the T1 segment and arrow in panel C indicates the border between the labial and the T1 segment. In all pictures, the dotted line marks the ventral midline. (D) In wild type embryo, *modulo* is expressed in the visceral mesoderm and in the labial segment (arrow). (E) In 69B>*tsh*, *mod* is repressed in the labium. (F) In 69B>*tshΔacid*, there is no repression of *mod* in the labial (arrow). (G) mod-1050-*LacZ* expression reproduces *mod* expression in the visceral mesoderm and the labial segment. (H) In 69B>*tsh*, mod-1050-*lacZ* is repressed by Tsh in the labium. Note that mod-1050-*LacZ* is strongly repressed in the mesoderm. (I) In 69B>tshΔacid, mod-1050-*LacZ* is unchanged. (J) In 69B>*tshΔacid*, expression of Scr is not affected (compared with panel B).

induce T1 fate. Here we demonstrated that Tsh interacts directly with Scr and this interaction depends on a acidic domain of Tsh. To analyse the *in vivo* relevance of this interaction, we performed two different tests. First, we ectopically provided the Tsh and TshΔacid proteins in the labial segment. We showed that, whereas in both cases *Scr* expression was not affected, only the Tsh protein was able to induce T1 fate in the presence of Scr. Next we used a *tsh* null genetic background in order to analyse the capacity of both Tsh and TshΔacid to rescue the prothoracic identity when provided; we showed that only Tsh rescue the T1 phenotype.

In the course of these experiments, we also noticed that even if TshΔacid did not rescue the T1 identity, it was able to rescue the identity of the other trunk segments of a *tsh* null mutant. For example, the T2 phenotype suggested a possible interaction with Antp; we showed that Tsh indeed interacts directly with Antp.

All together these results support that the acidic-rich domain of Tsh is specifically required *in vivo* for the identity of the prothorax and suggest that other parts of the Tsh protein could be involved in the interaction with other Hox proteins expressed in the trunk. Other experiments will be needed to further investigate this last observation.

Tsh has a transcriptional repressor activity

Genetic data strongly supported that Tsh and Scr play a critical role to establish the prothoracic identity. At the molecular level, it has been suggested that Tsh and Scr could share common target genes but so far this remains hypothetical (Alexandre et al., 1996b; Andrew et al., 1994; de Zulueta et al., 1994; Roder et al., 1992). In order to test this hypothesis we needed to solve two major problems. First, very few direct in vivo-regulated target genes have been identified for any given Hox proteins and this stands for Scr. Second, the identification of a common target gene for Tsh and Scr was not obvious as they are thought to have opposite regulatory function; in the labial segment, *Scr* is known to activate salivary gland gene expression whereas in the prothorax *tsh* contributes to their repression (Andrew et al., 1994). As it has been suggested that Scr "yields a different identity depending upon which co-factor is present" we reasoned that a good target gene should be differentially regulated in the labial and prothoracic segments (Robertson et al., 2004).

Previous analysis of the control of the expression of *modulo* suggested that *mod* could be such a target gene. *In vivo*, expression of *mod* is under the positive control of *Scr* in the

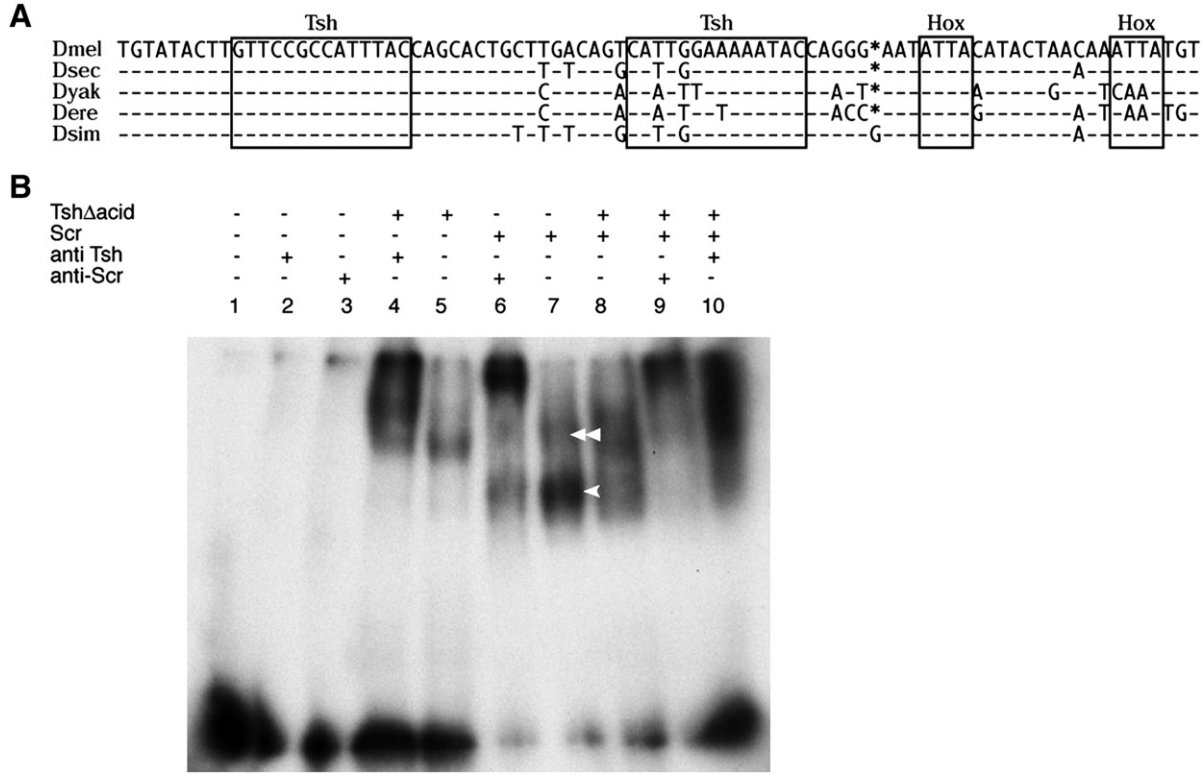

Fig. 4. The Hox Scr protein binds to mod-84. (A) mod-84 sequences from five *Drosophila* species were aligned. Confirmed Tsh and putative Hox binding sites are boxed. Conserved nucleotides are represented with dash. * indicates a gap. (Dmel: *Drosophila melanogaster*; Dsec: *Drosophila sechellia*; Dyak: *Drosophila yakuba*; Dere: *Drosophila erecta*; Dsim: *Drosophila simulans*). (B) In vitro-synthesized Scr protein was subjected to EMSA with labeled mod-84 probe. Scr forms one major complex (white arrowhead lane 7) and a minor complex (double white arrowhead lane 7). EMSA supershift demonstrates that minor and major complexes both contain Scr (lane 6). This same antibody does not shift the probe in absence of Scr (lane 3). TshΔacid forms one complex (lane 5). The TshΔacid protein alone (lane 5) and the Scr protein alone (lane 7) exert a higher mobility shift than the mixed TshΔacid and Scr proteins (lane 8). The nature of the retarded complexes was accessed by co-incubation with antibodies against Scr (lane 9) and Tsh (lane 10). EMSA supershift confirms that TshΔacid and Scr can bind together to mod-84 (lanes 9, 10).

labial segment. Here we showed that Scr binds to mod-84, suggesting that in the labial segment Scr activates *mod* expression. In the prothorax, *tsh* represses *mod* and in a *tsh* null mutant, *Scr* is strongly expressed in the ventral epidermis of the T1 and ectopic expression of *mod* is detected in this domain (Alexandre et al., 1996b). From these data, it is possible to propose that within the prothorax Tsh represses *mod* independently of Scr. On this line, we have previously shown that the acidic region contains a binding site for the co-repressor CtBP and the association of CtBP with Tsh depends on the presence of this motif (Manfroid et al., 2004). In addition to the CtBP binding site, Tsh possesses a repressor motif which is reminiscent of the alanine–proline rich sequences found in the repressor domains of the transcriptional repressor proteins Kruppel, Even-skipped, Engrailed and Ultrabithorax (Galant and Carroll, 2002; Han and Manley, 1993; Hanna-Rose and Hansen, 1996; Hanna-Rose et al., 1997; Licht et al., 1994). This putative repressive domain is precisely deleted in the TshΔ*Bsp*MI construct and in 69B>TshΔ*Bsp*MI embryos *mod* is not repressed (data not shown). Nevertheless, in the yeast two-hybrid system TshΔ*Bsp*MI protein interacts with Scr and *in vivo* it induces T1 identity (at 29 °C) showing that this construct retains some function. These data indicate that acidic rich and alanine-proline rich domains are together necessary for full regulation of a subset of genes, whereas a subset of genes involved in homeogenesis require an intact acidic rich domain. Thus, the capacity of Tsh to induce T1 identity can be separated in part from its ability to repress target genes such as *mod* suggesting that the transcriptional activity of Tsh depends on the cellular context. This hypothesis is supported by the effect of ectopic expression of Tsh on the *disco* expression in the labial segment. In armGal4>*tsh* embryos, disco mRNA diminishes in the epidermis of the gnathal lobes except in the central part (Robertson et al., 2004). Maintenance of *disco* in the center of the labial segment corresponds with the transformation of the labial sens organ toward a Keilin's organ (de Zulueta et al., 1994; Robertson et al., 2004). This result supports that Tsh represses gnathal and promotes trunk identity.

As we showed that Tsh interacts with the Hox protein Scr, and that Tsh and Scr proteins bind to mod-1050 regulatory elements, we explored the possibility that Tsh and Scr promote trunk identity through the control of the expression of common target genes. Loss of Scr induces loss of *mod* whereas ectopic *Scr* causes ectopic spots of *mod* expression in the head but not in the trunk where Tsh acts as a repressor. As in absence of Scr *modulo* is not expressed, repression of *mod* by Tsh either in the labial or prothoracic segment has always been observed in the presence of Scr. Here we showed that when TshΔacid is expressed in the labial segment, *mod* is not repressed and this is not due to the lost of DNA binding properties of TshΔacid,

suggesting that 1—the interaction between Tsh and Scr might be important for the repression of *mod* or 2—is the CtBP binding motif involved in repression of *mod*? We also showed that Tsh lacking the acidic domain failed to interact with Scr and was unable to promote T1 identity with Scr in the prothorax and labial segment when ectopically provided. These data suggest that the combined activities of Scr and Tsh are crucial for repressing *mod* expression in the prothorax. As Scr interacts with Tsh, one could speculate that Scr is involved in the repressor activity of Tsh or Tsh modifies the activity of Scr on the target gene *mod.*

Unfortunately, the use of transgenic embryos expressing a reporter gene under the control of mod-1050 mutagenized regulatory element did not allow us to conclude that in the prothorax repression of *mod* expression depends on the binding of both Tsh and Scr. So, concerning the mechanism that leads to the repression of mod in the epidermis of the prothorax we are still left with two possibilities: Tsh is a repressor of *mod* or Tsh binds to Scr and modifies the regulatory properties of Scr on *mod*. In other words, even if we have demonstrated that Tsh interacts with Scr, it remains to be established if they act as a complex or in parallel pathways.

The role of the Tsh–Hox complexes

In the trunk, if Tsh is a co-factor for Hox proteins, how can different Hox proteins establish different segment identities with the same co-factor (for example, Scr and Antp with Tsh)? It has been shown that *Split ends* (*Spen*), *tsh* and *Antp* function in a combinatorial manner to repress the development of head-like sclerites and to promote the development of thoracic identity (Wiellette et al., 1999). In *tsh Antp* double mutant embryos, the first two thoracic denticle belts are completely absent and replaced with cuticle typical of the head skeleton (Roder et al., 1992). Conversely ectopic expression of *Antp* induces a strong transformation of T1 to T2 (Gibson and Gehring, 1988; Gibson et al., 1990). In vitro, we showed that Tsh interacted directly with the Hox protein Antp.

One role of the Tsh–Scr and Tsh–Antp complexes could be to control specific subset of target genes, in order to specify the identity of the T1 and T2 segments. The capacity of TshΔacid to rescue the T2 defects of a tsh^{8} null mutant suggests that the collaborative activity of Tsh/Scr and Tsh/Antp does not involve the same domain of Tsh.

Are Tsh–Hox complexes evolutionary conserved?

The remarkable evolutionary conservation of the Hox complexes and their constituent genes is well-known (Gellon and McGinnis, 1998). It seems likely that Teashirt is also conserved, as putative vertebrate orthologues have been cloned (Caubit et al., 2000; Manfroid et al., 2004). The anterior limit of expression of *Tshzl* and *Tshz2* seems to correspond to the somites that will give rise to the first cervical vertebra (Atlas) and to the first thoracic vertebra, respectively (Caubit et al., 2000). Interestingly, the analysis of the skeleton of *HoxA5* (an orthologue of *Scr)* mutant mice reveals that the most frequent morphological abnormality is the posterior transformation of the last cervical vertebra (C7) into the likeness of a thoracic vertebra (Jeannotte et al., 1993). Thus, the position of the anterior limit of expression of *Tshz2* could reflect its requirement to restrict the domain of activity of *HoxA5* in the somites and setting a morphological border. Interestingly, ectopic expression of *HoxA5* in *Drosophila* embryos activates ectopic expression of salivary gland target genes just like its functional homologue *Scr* (Zhao et al., 1993a). This ectopic expression leads also to the transformation of the larval thoracic segments T2 and T3 toward T1 (Zhao et al., 1993a), suggesting that the mammalian protein not only recognizes the correct *Scr* target sites on DNA but also interacts with the proper Scr cofactors. Given that Tsh interacts directly with HoxA5 in vitro, we would like to suggest that Tsh is one of these cofactors; the capacity of Hoxa5 to induce *Scr*-like transformations depends on the presence of Tsh.

Finally, we recently provided evidence that in flies the three putative mouse Tshz genes behave in a similar way to the fly *tsh* gene (Manfroid et al., 2004) suggesting that the results obtained during *Drosophila* embryogenesis could be applicable to vertebrates embryogenesis. Given the many similarities among the mammalian and *Drosophila Hox* genes, lessons we learn from the regulatory interactions involving *Hox* genes used to control *Drosophila* organogenesis are likely to be applicable to tissue specification in higher organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.04.028.

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