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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.
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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.

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