Homeotic functions of the Teashirt transcription factor during adult *Drosophila* development

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Biology Open 2, 18–29
doi: 10.1242/bio.20122915
Received 22nd August 2012
Accepted 24th September 2012

Summary

During *Drosophila* development region-specific regulation of target genes by Hox proteins is modulated by genetic interactions with various cofactors and genetic collaborators. During embryogenesis one such modulator of Hox target specificity is the zinc-finger transcription factor Teashirt (Tsh) that is expressed in the developing trunk and cooperatively functions with trunk-specific Hox proteins to promote appropriate segment fate. This embryonic function of Tsh is characterized as homeotic since loss of embryonic Tsh activity leads to transformation of trunk segments toward head identity. In addition to this embryonic homeotic role, Tsh also performs vital Hox-independent functions through patterning numerous embryonic, larval and adult structures. Here we address whether the homeotic function of Tsh is maintained throughout development by investigating its contribution to patterning the adult abdomen. We show that Tsh is expressed throughout the developing abdomen and that this expression is dependent on the three Bithorax Hox proteins Ultrabithorax, Abdominal-A and Abdominal-B. Conditional reduction of Tsh activity during pupation reveals broad homeoic roles for this transcription factor throughout the adult abdomen. Additionally we show that, as during embryogenesis, the *tsh* paralog *tiptop* (*tio*) plays a partially redundant role in this homeotic activity.

Key words: *Drosophila*, Homeotic, Hox, Teashirt, Segmentation

Introduction

The zinc-finger transcription factor Teashirt (Tsh) has diverse, pleiotropic roles throughout *Drosophila* development. Initially identified for its homeotic function during embryogenesis (Fasano et al., 1991; Röder et al., 1992), *tsh* also has many Hox-independent patterning functions. In the embryo Tsh is expressed in the trunk segments (thorax and abdomen) and functions cooperatively with trunk-specific Hox proteins, encoded by a subset of the homeotic complex (HOM-C) genes, to direct fate away from head identity (de Zulueta et al., 1994; Fasano et al., 1991; Röder et al., 1992). In the absence of Tsh activity, thoracic and abdominal segments differentiate sclerotic cuticle indicative of gnathal structures. Furthermore, in common with trunk-specific Hox proteins, ectopic Tsh expression transforms head toward trunk identity (de Zulueta et al., 1994; González-Reyes and Morata, 1990). The collaborative homeotic function of Tsh and trunk-specific Hox proteins is underscored by their parallel contributions to repression of the homeodomain transcription factor Optix, restricting its expression to the labrum, maxillary and labial segments of the head (Coffier et al., 2008). These observations support the hypothesis that in *Drosophila* Tsh establishes a trunk ground state and that in combination with trunk Hox proteins promotes segment diversity through regulation of both independent and common transcriptional targets (de Zulueta et al., 1994). Tsh and the Hox protein Sex combs reduced (Scr) physically interact *in vitro* and both contribute to repression of the target gene *modulo* in the prothorax (Taghlí-Lamallem et al., 2007). Combined these observations support the hypothesis that during *Drosophila* embryogenesis Tsh functions collaboratively with Hox proteins to pattern segment identity, and at least in the case of Scr physical interactions may promote this homeotic activity. In this regard, Tsh displays similarity to two well-characterized Hox cofactors Extradenticle (Exd) and Homothorax (Hth); homeodomain proteins that bind to Hox proteins altering their DNA binding specificity and affinity (Chan and Mann, 1996; Chan et al., 1996; Mann and Chan, 1996; Ryoo and Mann, 1999; Ryoo et al., 1999).

*tsh* also has Hox-independent patterning roles throughout *Drosophila* development. For example, *tsh* is necessary to maintain late-embryonic *wingless* (*wg*) signaling (Gallet et al., 1998). In *tsh* null embryos the identity of posterior thorax and abdominal segments is not altered; however, *wg*-dependent regions of naked cuticle are reduced (Gallet et al., 1998). This *tsh*-dependent modulation of Wg signaling is mediated through physical interaction between Tsh and Armadillo (Arm); a key effector of the canonical Wg signaling pathway (Gallet et al., 1998; Gallet et al., 1999). *tsh* is also essential for development of a number of adult structures. In leg discs *tsh* patterns proximal segments (Bessa et al., 2009; Culi et al., 2006; Erkner et al., 1999; Wu and Cohen, 1999; Wu and Cohen, 2000) and dynamic transcriptional regulation of *tsh* in wing discs permits both blade specification (through its down regulation) and actively promotes proper morphogenesis of the proximal hinge and notum through continued expression in these domains (Azpiazu and Morata, 2000).
Results
Teashirt expression correlates with BX-C proteins in the pupal abdomen

To investigate tsh adult homeotic activity we first analyzed Tsh protein expression in the developing pupal abdomen for which we have developed a reliable immunohistochemistry protocol (Wang and Yoder, 2011) and in which trunk-specific Hox proteins pattern individual segment identity. The adult abdominal epidermis is derived from imaginal histoblast cells that remain quiescent until proliferation is triggered at pupariation (Madhavan and Madhavan, 1980). Expanding histoblast cells then replace the large polyploid larval epidermal cells (LECs) to form the adult epidermis. Four bilateral pairs of histoblast nests contribute distinct cell populations to each abdominal segment and the three Hox genes of the Bithorax complex (BX-C) Ultrabithorax (Ubx), abdominal-A (abd-A) and Abdominal-B (abd-B) confer distinct fates to each segment (Celniker et al., 1990; Duncan, 1987; Karch et al., 1985; Sánchez-Herrero et al., 1985). For example, in the posterior abdomen Abd-B functions cooperatively with the sex determination transcription factor Doublesex (Dsx) repressing development of the terminal male abdominal segment (A7) and promoting male-specific pigmentation on A5 and A6 (Kopp et al., 2000; Wang and Yoder, 2012; Wang et al., 2011). Tergites and sternites (the dorsal and ventral cuticle plates of each segment) in the posterior abdomen have additional Abd-B dependent sex- and segment-specific morphology and A6 tergites of both sexes as well as female A7 are mostly devoid of trichomes (fine hair-like epithelial extensions) that cover more anterior tergites (Duncan, 1987; Celniker et al., 1990; Kopp et al., 2000; Sánchez-Herrero et al., 1985). Likewise, mitotic clonal analyses of the Hox cofactors evx and hth show strong homeotic transformations throughout the adult body (González-Crespo and Morata, 1995; Rauskolb et al., 1995; Ryoo et al., 1999).

The abdominal defects seen in tio rescue experiments and cursory analyses of adult tsh-lacZ enhancer trap expression suggest tsh should be expressed in the pupal/adult abdomen (Bessa et al., 2009; Bhojwani et al., 1995). We confirmed this prediction by immunohistochemistry. By 26 hr APF (after pupariation formation) histoblast nests in each abdominal segment have proliferated and fused into a continuous epithelium. At this stage of development individual abdominal segments are clearly distinguishable as they remain separated by narrow bands of LECs that will soon be removed by apoptosis as the adult epithelium continues development (Kopp et al., 1997). At 26 hr APF, Tsh protein is broadly expressed in both sexes throughout the pupal abdomen in all LECs and histoblast cells (Fig. 1A,B). Tsh expression is not uniform throughout the abdominal epithelium, but instead is slightly elevated in the anterior- and posterior-most segments A1 and A7 compared to mid-abdominal segments (Fig. 1A,B).

The spatially dynamic expression of Tsh suggests regionally distinct transcriptional regulation. BX-C proteins are expressed in discrete domains throughout the developing abdominal epithelium (Kopp and Duncan, 2002; Wang et al., 2011) and tsh is a known target of Hox proteins in the embryonic trunk (Röder et al., 1992). We therefore compared Tsh expression with Ubx, Abd-A and Abd-B. Abdominal Ubx expression is strongest in the anterior compartment of A1 with weaker expression extending into A2 (Fig. 1C) (Kopp and Duncan, 2002). Tsh A1 enrichment is coincident with the strongest Ubx abdominal expression in the anterior compartment of this segment (Fig. 1C). In the posterior abdomen Abd-B is expressed in a gradient from the posterior compartment of A4 through A7 with strongest expression in posterior A6 and A7 (Fig. 1D) (Kopp and Duncan, 2002; Wang et al., 2011). Here Tsh expression is likewise elevated coincident with highest Abd-B levels (Fig. 1D). Abd-A
is expressed at moderately lower levels than either Ubx or Abd-B and is restricted from, or its levels significantly reduced, where these neighboring Hox proteins are elevated (Fig. 1E) (Kopp and Duncan, 2002). In mid-abdominal segments, in the absence of high Ubx or Abd-B, Tsh levels are similarly attenuated, but nonetheless robust (Fig. 1A,B). The strict correlation between Tsh and BX-C protein levels and distribution in the pupal abdomen suggest that here, as in the embryo, tsh may be a target of these Hox proteins.

In both embryos and imaginal discs Tsh and Tio are expressed in overlapping patterns (Bessa et al., 2009; Datta et al., 2009; Laugier et al., 2005). We were unable to detect Tio in either the abdominal epithelium or imaginal discs with an anti-Tio antibody used in previous studies (data not shown) (Bessa et al., 2009; Datta et al., 2009; Laugier et al., 2005). We therefore used tio-GAL4 (Datta et al., 2009) to drive GFP and observed broad, but patchy and stochastic, abdominal expression coincident with Tsh protein (Fig. 1F,G). Like Tsh, uas-GFP,tio-GAL4 shows enhanced expression in A1 and A7 compared to mid-abdominal segments. We conclude that as in other tissues examined tsh and tio are expressed in overlapping patterns, likely reflecting similar regulatory control and a compensatory function of tio for tsh activity (Bessa et al., 2009; Laugier et al., 2005).

Spatially dynamic Teashirt expression is positively regulated by BX-C proteins
To test whether adult abdominal tsh expression is regulated downstream of the BX-C proteins, we analyzed Tsh expression in gain or loss of function genotypes as well as null mitotic clones for individual BX-C genes. In the abd-B regulatory gain of function allele Fab-7 Abd-B levels are elevated in A6 to levels comparable to wild type A7 (Fig. 2A) (Gyurkovics et al., 1990). As a result, A6 is transformed toward A7 fate adopting characteristic female A7 morphology and undergoing male-specific reduction. In abd-BFab7 pupae Tsh expression is also elevated in A6 (Fig. 2A) suggesting that it is positively regulated by Abd-B. The regulatory allele Uab5 can be considered both an abd-A gain of function and a Ubx loss of function allele as Abd-B expression expands anteriorly into A1 repressing Ubx (Fig. 2B) (Karch et al., 1990; Lewis, 1978). In Uab5 pupae Tsh A1 expression is reduced compared to wild type and expressed at levels equivalent to A2–A5 suggesting that high levels of Ubx promote elevated Tsh expression (Fig. 2B).

These abd-B and abd-A/Ubx alleles cause embryonic segmental transformations, therefore the altered Tsh expression we observed may reflect these earlier events rather than Hox-mediated regulation during development of the adult abdominal epithelium. We therefore also generated null mitotic clones of abd-A and abd-B in the developing pupal abdomen to test whether Hox proteins are required to maintain tsh expression during pupation. Consistent with a positive regulatory role, null abd-B clones in A7 lead to strong reduction of Tsh (Fig. 2C). Conversely, Tsh is strongly up regulated in null abd-A clones (Fig. 2D). These results are explained by the genetic interaction of posterior prevalence in which HOM-C proteins negatively
regulate transcription of more anteriorly expressed Hox genes (McGinnis and Krumlauf, 1992) As a result Ubx is up-regulated in abd-A mitotic clones (Fig. 2E). This increased Ubx expression likely promotes higher transcription of tsh. Combined, these data show that during pupal abdominal development the BX-C proteins are essential for proper maintenance of Tsh expression consistent with observations during embryogenesis (Fasano et al., 1991).

**Tsh has a homeotic role in patterning the adult abdomen**

tsh is required for patterning several adult tissues; however, none of these described functions can be characterized as homeotic (Bessa and Casares, 2005; Bessa et al., 2009; Datta et al., 2009; Erkner et al., 1999; Laugier et al., 2005; Pan and Rubin, 1998; Singh et al., 2002; Singh et al., 2004; Wu and Cohen, 1999; Wu and Cohen, 2002). Rather, in each case (eye, leg and wing imaginal disc) tsh functions in a Hox-independent fashion to regulate proliferation and patterning through genetic interactions with other key developmental genes (Bessa et al., 2002; Erkner et al., 1999).

Although expression of tio in the tsh domain can rescue tsh embryonic lethality several adult phenotypes, including severe abdominal developmental defects, were observed suggesting only partial compensation by tio for loss of tsh (Bessa et al., 2009). We repeated this experiment to more closely analyze adult abdominal phenotypes. Pupae of this genotype do not eclose and die at various stages of late pupal development. In the most advanced pupae, tergites and sternites are developed and have differentiated bristles and trichomes; however, they fail to fuse at the dorsal and ventral midlines (Fig. 3D). Similar phenotypes are observed upon perturbing the cell cycle of abdominal histoblasts (Ninov et al., 2007; Ninov et al., 2009) suggesting that tsh contributes to regulating histoblast proliferation, consistent with observations in other developing adult tissue (Bessa et al., 2002).

Although these proliferation defects prevent thorough analysis of segment identity we found two phenotypes that suggest tsh contributes to homeotic patterning of the adult thorax and abdomen. In wild type flies Ubx represses most thoracic tissue derived from the dorsal T3 disc with the exception of halteres. As a result, only a thin band of laterotergite separates the wing-bearing T2 mesothorax and abdominal A1 (Fig. 3E) (Hittinger et al., 2005). In strong Ubx loss of function adults the T3 laterotergite is transformed toward T2 identity characterized by expanded size and the presence of bristles. We observed similar T3 transformations in these tsh-GAL4/tsh++;uas-tio/+ pupae (Fig. 3F). Additionally, in wild type D. melanogaster males Abd-B and Dsx cooperatively repress male A7 development (Kopp et al., 2000; Wang and Yoder, 2012; Wang et al., 2011). As a consequence neither tergite nor bristles develop between male A6 and the genitalia (Fig. 3G). However, some tsh-GAL4/tsh++;uas-tio/+ males develop small A7 tergites identifiable by the presence of small cuticle plates and associated bristles.
widespread recombination experiments. Additionally, the induction of FRT linked chromosomes for mitotic stages of development preventing analyses of adult segment located close to the centromere of chromosome 2 preventing morphology (Bessa et al., 2009). We therefore used the GAL4:UAS system to conditionally knock down tsh activity by driving expression of a double-stranded-tsh construct (UAS-ds-tsh) throughout the developing pupal abdomen. Because tio functions redundantly with tsh in other tissues, we also performed tsh-RNAi experiments in a tio loss of function background. Our results show that tsh functions broadly to help establish proper segment identity throughout the abdomen and that as during embryonic and imaginal disc development tio plays a redundant role in this homeotic activity.

To circumvent embryonic tsh requirements and potentially lethal pupal pleiotropy we used a dsx-GAL4 driver which, as we have previously shown, drives broad expression in all abdominal histoblast cells (Rideout et al., 2010; Wang and Yoder, 2012). Importantly, this driver has very limited embryonic expression and is absent from the LECs, therefore we can be confident that observed phenotypes are due to tsh requirements during pupal development and do not result from altered homeotic activity during embryogenesis. We confirmed by immunohistochemistry that Tsh expression is significantly reduced, but not completely eliminated, within all developing abdominal segments in dsx-GAL4/UAS-ds-tsh pupae (data not shown). Additionally, all pupae fully mature and eclose allowing detailed cuticle characterization.

We predicted that if Tsh has adult homeotic activity then reducing its expression throughout the pupal abdomen should produce transformations similar to exd and hth mutant clones; anterior and posterior segments should be transformed toward mid-abdominal fates (González-Crespo and Morata, 1995; Rauskolb et al., 1995; Ryoo et al., 1999). Analyses of adult dsx-GAL4/UAS-ds-tsh cuticles confirmed these predictions. Reduction of Tsh activity partially restores male A7 development (100%; n=19) (Fig. 4B, arrowheads) and leads to fusion of female A7 laterotergites (100%; n=22) (Fig. 4D, arrowhead), both characteristics of A7 transformation toward anterior segment identity. Additionally, in wild-type adults, A6 tergites of both sexes, and A7 of females, are mostly devoid of trichomes that cover tergites on A1–A5 (Fig. 4C). However, in dsx-GAL4/UAS-ds-tsh A6 trichomes are restored in both sexes (100%; n=25) (Fig. 4D). The ventral sternite cuticle of male A6 also has characteristic wild-type morphology that is transformed anteriorly in dsx-GAL4/UAS-ds-tsh flies. Wild-type male A6 sternites are U-shaped and lack sensory bristles found on more anterior sternites (Fig. 4A). However, in dsx-GAL4/UAS-ds-tsh males, A6 sternites are rectangular (100%; n=19), like more anterior segments, and occasionally develop 1–2 sensory bristles (58%; n=19: 9 individuals with one bristle; 2 individuals with 2 bristles) (Fig. 4B).

In addition to posterior abdominal transformations, segment A1 is also transformed upon reduction of Tsh activity. Consistent with exd clonal analyses this transformation is toward more posterior segment identity. In wild-type flies segment A1 does not generate a ventral sternite (Fig. 4E); however, dsx-GAL4/UAS-ds-tsh adults show sclerotized ventral cuticle plates (100%; n=19) that occasionally develop a single sensory bristle (26%; 5/19 individuals) (Fig. 4F).

Together these observations suggest that during pupation, as during embryogenesis, tsh functions cooperatively with trunk-specific Hox genes to pattern segments of the adult abdomen. The homeotic phenotypes described here are weaker than those reported for exd mitotic clones and gynandromorphs (González-Crespo and Morata, 1995; Rauskolb et al., 1995). Furthermore, one abdominal character, male-specific pigmentation, which is under direct regulation of Abd-B (Jeong et al., 2006; Williams et al., 2008), showed no phenotypic alteration in dsx-GAL4/UAS-ds-tsh.

![Fig. 3. Developmental defects of tio rescue flies suggest adult homeotic function for tsh. (A,B) Adult abdominal cuticle preparations of wild type male and female flies. (C) Dorsal cuticle preparation of wild type adult male A5–A6. (D) Dorsal cuticle preparation of tsh-GAL4/tsh−;UAS-tio adult male A4–A6 shows proliferation defects resulting in failed fusion of lateral hemistergites. (E) The T3 laterotergite of wild type flies (marked by a yellow line) is a thin band of tissue separating T2 and A1. (F) In tsh-GAL4/tsh−;UAS-tio flies the T3 laterotergite is expanded (outlined by a yellow border) and often generates bristles (arrowhead). Dashed line outlines the haltere. (G) Wild type males lack an A7 tergite. As a result no sclerotized cuticle or bristles are located between the A6 tergite and the genitalia. (H) In tsh-GAL4/tsh−;UAS-tio males a small A7 tergite develops (outlined) with supernumerary bristles (arrowheads). (H1) shows the same image with outline and arrowheads removed for clarity. ga, genital arch derived from the genital disc. In A–F anterior is up. Scale bars: 200 μm (A,B), 100 μm (C–F), 50 μm (G,H).](image-url)
adults. Because Tsh protein in dsx-GAL4/UAS-ds-tsh pupae is reduced but not eliminated, it is likely the degree of the observed homeotic phenotypes reflects activity of residual Tsh. Additionally, we have previously shown that target gene expression under control of this driver is spatiotemporally dynamic (Wang and Yoder, 2012). Prior to 26 hr APF, dsx-GAL4 promotes robust expression in all A7 histoblast cells with weaker and ventro-laterally restricted expression in more anterior segments. After 28 hr APF, this driver promotes strong and ubiquitous expression in all abdominal histoblasts. Therefore, moderate or weak tsh-RNAi phenotypes in mid-abdominal segments, especially in dorsal tissue, may reflect these spatiotemporal dsx-GAL4 dynamics. To circumvent this issue we used two additional drivers (abd-A-GAL4 and pannier-GAL4) to express ds-tsh. abd-A-GAL4 drives strong expression in segments A2–A6 (with weaker expression in A7) and pnr-GAL4 drives

Fig. 4. Adult homeotic transformations associated with reducing tsh expression during pupal abdominal development. (A) Ventral cuticle of adult wild type male. A6 sternites (center) have a characteristic horseshoe shape and are devoid of bristles. Neither A7 tergites nor sternites develop. As a result of A7 reduction during pupation A7 spiracles are associated with A6, which therefore has two spiracles adjacent to each tergite (arrowheads). (B) In dsx-GAL4/UAS-ds-tsh males A6 sternites are rectangular, similar to more anterior sternites (compare A with Fig. 3A) and sternites are reduced in size or absent. (C) Control male dorsal cuticle showing tergites A4–A6. The genotype is pnr-GAL4, abd-BMcp/TM6B. abd-BMcp is a regulatory gain-of-function allele resulting in expanded expression of abd-B into A4. As a result A4 is darkly pigmented similar to wild type A5–A6; however, note absence of pigmentation in patches of A4. (D) In pnr-GAL4, abd-BMcp/UAS-ds-tsh pigmentation is reduced along the ventral midline in A4–A6. Also, note reduction in bristle number within the domain of tsh reduction. Scale bars: 200 μm (A), 100 μm (B,C).

A7 tergites are fused and pigmented at the dorsal midline indicating transformation toward anterior identity. Additionally, in both sexes, trichomes are present on A6 as seen in magnification of the boxed area (C’). (D) In dsx-GAL4/UAS-ds-tsh females

Fig. 5. Additional adult homeotic phenotypes revealed through earlier reduction of tsh expression. (A) In abd-A-GAL4/UAS-ds-tsh, tsh expression is knocked down throughout the abdomen, likely from early embryogenesis throughout pupal development. The result is a strong homeotic transformation of segments A2–A7 toward A1 identity. Bristles adopt a short and stout morphology characteristic of A1. Additionally, the band of pigmentation at the posterior margin of each tergite is restricted to the most dorsal domains as in wild type A1 (compare A with Fig. 3B) and sternites are reduced in size or absent. (B) Control male dorsal cuticle showing tergites A4–A6. The genotype is pnr-GAL4, abd-BMcp/TM6B. abd-BMcp is a regulatory gain-of-function allele resulting in expanded expression of abd-B into A4. As a result A4 is darkly pigmented similar to wild type A5–A6; however, note absence of pigmentation in patches of A4. (C) In pnr-GAL4, abd-BMcp/UAS-ds-tsh pigmentation is reduced along the ventral midline in A4–A6. Also, note reduction in bristle number within the domain of tsh reduction. Scale bars: 100 μm (A, 100 μm (B,C)).
expression in a stripe of cells along the dorsal midline, including the dorsal-most histoblast cells. Both drivers are expressed from embryogenesis through adult development, and therefore promote expression of *ds-tsh* throughout all stages of histoblast proliferation and development. These experiments show that *tsh* plays an important role in patterning all adult abdominal segments. Strikingly, *abd-A-GAL4/UAS-ds-tsh* transforms adult A2–A7 toward A1 identity. Tergite bristles are fewer in number than in wild type A2–A7 and adopt a short, stout bristle morphology characteristic of A1 (Fig. 5A). Additionally A2–A7 tergite pigmentation is modified toward A1 identity (Fig. 5A). *ds-tsh* expressed along the dorsal midline under control of *pnr-GAL4* not only restores trichomes in tergite A6 but is also partially effective at repressing male-specific pigmentation (Fig. 5C). Also, as observed in *exd* thoracic mitotic clones, reduction of *tsh* expression in *pnr-GAL4* flies suppresses bristle development (Fig. 5C) (González-Crespo and Morata, 1995).

Partial redundancy between *tio* and *tsh*

Reduction of *tsh* expression by RNAi in a *tio* null background dramatically enhances larval segment and adult leg phenotypes compared to *tsh*-RNAi alone reflecting partially redundant functions between these paralogous genes (Bessa et al., 2009; Laugier et al., 2005). To investigate similar redundancy between *tsh* and *tio* during adult abdominal development we reduced *tsh* expression in a *tio* null background (*tio473;dsx-GAL4/UAS-ds-tsh*). In this genotype adult survivorship was reduced compared to wild type and *dsx-GAL4/UAS-ds-tsh* (percent eclosed 73%; 24/33 pupae scored). However, all unclosed flies reached pupal development and could be removed from the pupal case for cuticle analysis.

Neither male nor female A7 transformations were clearly enhanced in *tio473;dsx-GAL4/UAS-ds-tsh* abdomen. As in *dsx-GAL4/UAS-ds-tsh* A7 tergite development was partially restored in all males and all female A7 laterotergites fused at the dorsal midline (Fig. 6A,B). Likewise, we observed no dramatic increase in the number or density of trichomes restored on A6 tergites of either sex (Fig. 6B). We therefore used bristle number on A1 and male A6 sternites to compare the degree of transformation in these two genotypes. In *dsx-GAL4/UAS-ds-tsh* 47% of males (9/19) develop a single bristle on sternite A6 while 10% (2/19) possess 2 bristles. In *tio473;dsx-GAL4/UAS-ds-tsh* 80% of males (n=21) generate one or more A6 sternite bristles and the number of bristles is increased compared to *dsx-GAL4/UAS-ds-tsh* (single bristle=3, two bristles=12 and three bristles=4) (Fig. 6A). As in *dsx-GAL4/UAS-ds-tsh* 100% (n=21) of *tio473;dsx-GAL4/UAS-ds-tsh* flies develop an A1 sternite; however, the number of flies with A1 sternite bristles (74%; n=19) and the number of bristles is increased (single bristle=4, two bristles=8, 2 three bristles=2 and 4 bristles=1) (Fig. 6C). The increased penetrance of homeotic transformation in *tio473;dsx-GAL4/UAS-ds-tsh* indicates that as during embryogenesis and imaginal disc development, *tio* function can partially compensate for loss of *tsh*.

Homeotic activity through regulation of Hox target genes

Finally we addressed the mechanism of *tsh* homeotic activity during adult abdominal development. During embryogenesis *tsh* is positively regulated downstream of trunk-specific HOM-C genes but is not required for maintenance of Hox expression. Therefore *tsh* homeotic activity in the embryo is not a result of its regulating Hox expression. We asked whether BX-C protein expression in the pupal abdomen is likewise independent of *tsh* activity by investigating expression of each protein in *pnr-GAL4/UAS-ds-tsh* pupae. This genotype provides an internal control for assessing protein levels as only the dorsal-most cells of each histoblast nest express *ds-tsh*. We found no evidence that *tsh* is necessary for proper BX-C expression as neither Ubx, Abd-A nor Abd-B levels are altered upon *tsh* knockdown (Fig. 7A–C).

Next we investigated expression of known targets of one abdominal Hox protein to confirm that altered target gene expression correlates with specific homeotic phenotypes. Three Abd-B targets have been characterized that contribute to male-specific characters in the adult abdomen. The *bric-a-brac* (*bab*) locus encodes a pair of paralogous transcription factors involved in many developmental processes and Abd-B and Dsx directly repress *bab* in male A5–A6 promoting characteristic male-specific pigmentation of *D. melanogaster* (Williams et al., 2008). Additionally, we have shown that Abd-B and Dsx repress *wingless* in male A7 leading to loss of this segment in adults (Wang et al., 2011) and that Abd-B positively regulates *dsx* during early pupation (Wang and Yoder, 2012). We therefore predicted that Wg and Bab repression would be partially relieved and that Dsx expression would be reduced following knockdown of *tsh* expression in *pnr-GAL4/UAS-ds-tsh* pupae. Indeed, ectopic Wg expression was observed in male A7 (Fig. 8B,
arrowhead) and Bab was elevated along the A5–A6 dorsal midline in pnr-GAL4/UAS-ds-tsh males (Fig. 8D, arrowheads and magnified insets). However, we observed no down-regulation of Dsx in this genotype (Fig. 8C). The differences in response to tsh knockdown between these three Abd-B targets may reflect differential sensitivity of each gene to partial depletion of tsh. Alternatively, the difference in response by positively versus negatively regulated Abd-B targets may be evidence of the collaborative mode of Tsh homeotic activity (see Discussion).

Finally, we investigated whether phenotypic similarities between reduction of Tsh activity in the developing abdomen and Exd/Hth abdominal mitotic clones reflects functional interdependency between these homeotic collaborators. Exd functions as a homeotic cofactor through direct association with Hox proteins, leading to altered DNA binding specific and affinity. Exd nuclear localization is dynamic throughout development and its nuclear translocation requires physical interaction with Hth (Rieckhof et al., 1997). In the developing eye Tsh was found to directly interact with Hth, forming a putative trimeric complex along with Eyeless (Bessa et al., 2002). Such physical associations between Tsh and Hth may modulate the latter’s ability to promote Exd nuclear translocation. Furthermore, in wing and antennal discs ectopic Tsh can maintain Hth protein expression outside of its normal domain indicating a possible regulatory role for Tsh in Hth expression (Bessa et al., 2002; Bessa et al., 2009). However, in these tissues tsh-RNAi clones do not alter normal hth expression or Exd nuclear accumulation (Bessa et al., 2009). These data suggest that in this developmental context Tsh does not participate in hth transcriptional regulation. To investigate whether the Tsh adult homeotic activity we have characterized may be an ultimate consequence of regulating Exd subcellular distribution we investigated expression of both Hth and Exd in Tsh-RNAi pupae. In wild type pupae Hth is ubiquitously expressed in all abdominal histoblasts and LECs (Fig. 9A; data not shown). Upon reduction of Tsh activity in pnr-GAL4/UAS-ds-tsh pupae Hth expression is unaltered and remains at expression levels similar to wild type within histoblast cells (Fig. 9B). We therefore conclude that here, as in other imaginal tissue, Tsh does not regulate hth transcription. In wild type pupae Exd is also expressed in all abdominal histoblasts and LECs (Fig. 10A). Exd expression is much weaker compared to Hth and is enriched in ventral compared to dorsal histoblast cells (not shown). Because Exd expression is extremely weak in dorsal cells we compared its expression in ventral histoblasts between wild type and dsx-GAL4/UAS-ds-tsh pupae. As with Hth, neither Exd expression levels nor intracellular distribution were altered upon reducing Tsh activity. These observations are consistent with similar Exd expression analyses performed in wing and antennal imaginal discs (Bessa et al., 2009) and suggest that the homeotic function of Tsh is not mediated through promoting either expression or nuclear accumulation of Hth or Exd.

**Discussion**

Products of the *Drosophila* HOM-C genes positionally specify segment identity along the anteroposterior axis through independent and combinatorial actions reflecting their spatially restricted expression domains. *In vitro*, individual Hox proteins exhibit weak target specificity due to high affinity for a shared and abundant target DNA binding sequence (Ekker et al., 1994; Gehring et al., 1994; Hoey and Levine, 1988). However, *in vivo* Hox proteins possess a high degree of target specificity and regulate unique suites of genes within their respective domains. Several non-exclusive mechanisms are proposed to aid in
We have described the first detailed characterization of Tsh expression in the *Drosophila* pupal abdomen and provided the first evidence that Tsh functions as a homeotic patterning protein during adult *Drosophila* development. Additionally, our analyses show that as during embryogenesis and imaginal disc development the *tsh* paralog *tio* functions redundantly with *tsh* for these patterning functions. Our data show that the homeotic functions of *tsh* are not limited to embryogenesis and the choice between trunk versus head identity. Rather, conditional reduction of *tsh* expression during pupation reveals a crucial collaborative role with the HOM-C genes throughout development as exemplified by its necessity to establish proper fate in all adult abdominal segments.

Here we note an interesting correlation with observations following RNAi against the single *tsh/tio* homolog in the red flour beetle *T. castaneum* (*Tc-tiotsh*). Reduction of *Tc-tiotsh* during embryogenesis does not promote a trunk toward head transformation, suggesting the embryonic trunk-specifying activity of *Drosophila* *tsh* may not be conserved throughout insects (Shippy et al., 2008). Likewise, neither thoracic nor abdominal segmental transformations were observed upon RNAi against the *tsh/tio* homolog in the Hemipteran insect *O. fasciatus* (Herke et al., 2005). Rather, in basal insects the principle patterning function of the *tsh* homolog appears to be restricted to leg segmentation. However, when *Tc-tiotsh* is reduced during larval development adult segments display strikingly similar phenotypes to those described here, such as the apparent transformation of ventral A2 and A3 toward more posterior identity (Shippy et al., 2008). Therefore functional interactions between *tsh* homologs and trunk specifying Hox proteins appear to be evolutionarily conserved at least within the holometabolous insects.

Combined with earlier observations of the homeotic activity of Tsh during embryogenesis (Fasano et al., 1991; Röder et al., 1992), these data deepen similarities between the interactions of Hox proteins with Tsh and the Hox cofactors Exd/Hth. Like Exd/Hth, we have shown that Tsh is required from embryogenesis throughout adult development to aid in Hox-mediated patterning. Though not as severe, the abdominal segmental transformations described here are similar to those produced by *exd* and *hth* mitotic clones (González-Crespo and Morata, 1995; Rauskolb et al., 1995; Ryoo et al., 1999). Additionally, our analyses of *Hth* and *Exd* expression in *tsh-RNAi* pupae suggest that the phenotypic similarities are not the result of *Tsh*-mediated regulation of *hth* or *exd* transcription or nuclear localization of their protein products. The difference in degree of transformation between our analyses of *tsh* and those of *exd/hth* may reflect incomplete penetration due to residual *tsh* expression. However, this may also be indicative of differences in the modes of cooperative gene regulation between Hox proteins and these transcription factors. For example, *exd* contributes to both positive and negative regulation of Hox target genes (Mann et al., 2009); however, the only characterized Hox/Tsh targets are repressed through these collaborative interactions (Alexandre et al., 1996; Coiffler et al., 2008; Robertson et al., 2004). We found that only products of repressed Abd-B targets (Wg and Bab1) showed altered expression following *tsh* knock down. Expression of Dsx, which is activated downstream of Abd-B, was not affected. While again, this failure to alter regulation of Dsx may be a consequence of incomplete *tsh* knock down another possibility is that Tsh serves as a dedicated Hox corepressor similar to characterized Hox/Engrailed interactions (Gebelein et al., 2004).

Fig. 8. Knock down of *tsh* expression leads to dysregulation of a subset of *Abd-B* targets. (A) Wild type 26 hr APF male pupa co-labeled for Tsh (green) and Wg (red). Wg expression is repressed by Abd-B and Dsx in male A7. (B) Male *pnr-GAL4/UAS-ds-tsh* pupa at 26 hr APF also co-labeled for Tsh and Wg. Tsh expression is strongly reduced, but not eliminated, in the dorsal-most half of each dorsal nest (left panel). Wg expression is de-repressed in A7 (middle panel, arrowhead). Inset in each panel are the dorsal-most cells of A7 showing reduction of Tsh expression and concomitant de-repression of Wg. (C) High levels of Abd-B in A7 positively regulate Dsx expression; however, reduction of Tsh (left panel and green in merge) does not alter Dsx levels (middle panel and red in merge). (D) Dsx expression in wild type male at similar developmental stage for comparison. (E) Bab1 expression in *pnr-GAL4, abd-B^{cer+/+}* male pupa. Bab1 is expressed in A1–A3 and repressed by Abd-B in A4–A6. (F) In *pnr-GAL4, abd-B^{cer+/UAS-ds-tsh}* males expression of ds-Tsh along the dorsal midline de-represses Bab1 expression in A4–A6 (arrowheads). In all panels dorsal is up and anterior is left. Scale bars: 100 μm.

generating this target specificity including, but not limited to, dimerization of individual Hox proteins (Papadopoulos et al., 2012), collaborative regulation with other transcription factors (Gebelein et al., 2004; Williams et al., 2008) and physical interaction with cofactors that alter Hox DNA binding specificity (Chan and Mann, 1996; Mann and Chan, 1996). Tsh has been proposed to function as such a cofactor based upon its broad effect on embryonic trunk-specific Hox activity and its observed physical interaction with Scr (Robertson et al., 2004; Taghli-Lamallem et al., 2007). In this single example of target co-regulation by Tsh and Scr the binding sites of the respective transcription factors are not immediately adjacent and no cooperative binding was observed, and therefore it has been cautioned that without additional data Tsh should be considered a genetic collaborator rather than a cofactor (Mann et al., 2009).
Our data do not provide evidence to either support or refute the Hox cofactor status of Tsh. However, regardless of the mode of interaction between Tsh and Hox proteins, our observations of similar homeotic phenotypes provide evidence that Hox/Tsh and Hox/Exd/Hth interactions likely regulate overlapping suites of target genes and therefore function cooperatively in at least some of their actions. Characterization of target regulation through analysis of Hox, Tsh and Exd/Hth responsive cis-regulatory elements (CREs) will be necessary to determine whether Hox/Tsh and Hox/Exd/Hth act on the same or distinct CREs and, if on the same elements, whether their actions are independent or collaborative.

Materials and Methods

Fly stocks

The following stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University: ds-tsh (tsh-RNAi) is a pVALIUM20-derived transgene generated by the Transgenic RNAi Project at Harvard Medical School.
Biology Open

GAL4 functional endogenous dsx
Adi Salzberg (Rappaport Institute, Haifa) and the Bloomington Stock

References

The authors have no competing interests to declare.

Antibodies

Antibodies against Abd-B (clone 1A2E9), Exd (clone B11M), Ubx (clone F53.38) and Wg (clone 4D4) were obtained from the Developmental Studies Hybridoma Bank (Ginker and Nicolson, 1989; Neumann and Cohen, 1996; White and Mlodzik, 1984; Aspland and White, 1997). Antibodies against Tsh, Tio, Bbl and Abd-A were kindly provided by S. Cohen, L. Fasano, T. Williams, A. Salzberg and I. Bank (Celniker et al., 1989; Neumann and Cohen, 1996; White and Wilcox, 1984; Williams et al., 2008; Kurant et al., 1998). Fluorescent secondary antibodies were kindly provided by S. Cohen, L. Fasano, T. Williams, A. Salzberg and I. Bank (Celniker et al., 1989; Neumann and Cohen, 1996; White and Wilcox, 1984; Williams et al., 2008; Kurant et al., 1998). The authors have no competing interests to declare.

Immunohistochemistry and cuticle preparations

Pupae were dissected, fixed and stained as previously described (Wang and Yoder, 2011). Dilutions of primary antibodies were as follows: mouse-anti-Abd-B (1:250), mouse-anti-Ubx (1:100), mouse-anti-Wg (1:250), rabbit-anti-Tio (1:20–1:500), rabbit-anti-Bab1 (1:100), mouse-anti-Abd-A (1:250), mouse-anti-Ubx (1:100), mouse-anti-Abd-A (1:250), mouse-anti-Hh (1:1000) and mouse-anti-Exd (1:5). For Hh and Exd images samples were incubated with primary antibody for two days in PBS +0.3% Triton X-100. All secondary antibodies were diluted 1:250 in PBS. Samples were then washed and counterstained with 4'-6-diamidino-2-phenylindole (DAPI) and equilibrated in 75% glycerol. Samples were mounted in 0.8 mm depression well slides and imaged using a Nikon 90i fluorescence microscope equipped with an Optigrid structured illumination accessory (Qioptiq). Cuticles were prepared as previously described (Wang et al., 2011).

Mitotic clones

To generate aberrant clones and Abd-A mitotic clones the following stocks were constructed: hsFlp; FRT82B, Abd-Afl/+; FRT82B, Ubx-GFP and hsFlp; FRT82B, Abd-Afl/+; FRT82B, Ubx-GFP. White pre-pupae were then collected and subjected to a cold-shock at 37°C for 30 min and returned to 25°C until times indicated prior to preparation for dissection and immunohistochemistry.

Acknowledgements

The authors thank Justin Kumar (University of Indiana), Ernesto Sanchez-Herrero (Universidad Autónoma de Madrid), Laurent Fasano (CNRS, Marseille), Ian Duncan (Washington University), Adi Salzberg (Rappaport Institute, Haifa) and the Bloomingtom Stock Center (Indiana University) for reagents and fly stocks, and J. O’Donnell for comments on the manuscript. This work was supported by Grant IOS-0919891 from the National Science Foundation (to J.H.Y.).

Competing Interests

The authors have no competing interests to declare.

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