Three putative murine Teashirt orthologues specify trunk structures in *Drosophila* in the same way as the *Drosophila teashirt* gene

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Summary

***Drosophila teashirt* (*tsh*) functions as a region-specific homeotic gene that specifies trunk identity during embryogenesis. Based on sequence homology, three *tsh*-like (Tsh) genes have been identified in the mouse. Their expression patterns in specific regions of the trunk, limbs and gut raise the possibility that they may play similar roles to *tsh* in flies. By expressing the putative mouse Tsh genes in flies, we provide evidence that they behave in a very similar way to the fly *tsh* gene. First, ectopic expression of any of the three mouse Tsh genes, like that of *tsh*, induces head to trunk homeotic transformation. Second, mouse Tsh proteins can rescue both the homeotic and the segment polarity phenotypes of a *tsh* null mutant. Third, following ectopic expression, the three mouse Tsh genes affect the expression of the same target genes as *tsh* in the *Drosophila* embryo. Fourth, mouse Tsh genes, like *tsh*, are able to induce ectopic eyes in adult flies. Finally, all Tsh proteins contain a motif that recruits the C-terminal binding protein and contributes to their repression function. As no other vertebrate or fly protein has been shown to induce such effects upon ectopic expression, these results are consistent with the idea that the three mouse Tsh genes are functionally equivalent to the *Drosophila tsh* gene when expressed in developing *Drosophila* embryos.**

Key words: *teashirt*, Homeotic, Mouse, *Drosophila*, *wingless*, *Wnt*

Introduction

During evolution, several genetic programs for the control of regional specification of body parts have been conserved. Anteroposterior (AP) axis patterning by the Hox genes (reviewed by McGinnis and Krumlauf, 1992; Carroll, 1995) and eye morphogenesis by *Pax6* (reviewed in Pichaud and Desplan, 2002) are striking examples of conservation.

In addition to the Hox genes, region-specific homeotic genes have been identified. Among them, *Drosophila teashirt* (*tsh*) encodes a transcription factor with three atypical zinc finger motifs (Cx2Cx12HMx4H) and is crucial for patterning the trunk (three thoracic and eight abdominal segments) during embryogenesis (Fasano et al., 1991; Röder et al., 1992; de Zulueta et al., 1994). In addition, *tsh* is required for midgut morphogenesis (Mathies et al., 1994), the development of the proximal part of the adult appendages (Erkner et al., 1999; Wu and Cohen, 2000; Soanes et al., 2001) and for patterning of the adult eye (Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002).

During *Drosophila* embryogenesis, segmentation and patterning activities give rise to distinct head, gnathal, trunk and tail segments. In the embryo, *tsh* is expressed in the trunk where Tsh protein is crucial for several developmental processes. Tsh collaborates with certain Hox proteins to specify trunk identity (Röder et al., 1992; de Zulueta et al., 1994). In *tsh* loss-of-function mutants, the anterior-most trunk segment (T1) is transformed into a labial head segment (Fasano et al., 1991; Röder et al., 1992). In the absence of Tsh and Hox activities, all ventral trunk segment identities are replaced with those found in the head (Röder et al., 1992). Tsh is required to establish the identity of T1 and to repress head identity in cooperation with the Hox protein Sex combs reduced (Scr) (de Zulueta et al., 1994). Ectopic expression of *tsh* results in the transformation of the labial head segment into T1, while the antennal and maxillary head segments also acquire a trunk identity in that they develop anterior denticle belts, alternating with a posterior naked cuticle domain (de Zulueta et al., 1994), as seen in the normal larval trunk.

wingless (*wg*) is a segment polarity gene required for intrasegmental patterning. *tsh* acts as a modulator of Wg signalling in the trunk during embryogenesis (Gallet et al., 1998; Gallet et al., 1999). Around stage 10/11, Tsh protein accumulates to a high level in the nuclei of posterior cells receiving Wg signal that will form the naked cuticle. By contrast, in the anterior part of the segment, where Wg does not signal, lower levels of nuclear Tsh are detected, which in part contribute to the patterning of denticles (Gallet et al., 1998). Very high-level production of Tsh replaces denticles with naked cuticle. In addition, the maintenance of *wg* expression is controlled by *tsh* in the ventral part of the trunk (Gallet et al., 1998).

tsh is also crucial for eye development, which is controlled by *Drosophila Pax6 eyeless* (*ey*) and *twin of eyeless* (*toy*) genes. Both *ey* and *toy* have the ability to induce ectopic eye formation when ectopically expressed during larval development (Halder

et al., 1995; Czerny et al., 1999). Other transcription factors are involved downstream of these genes: *sine occulis* (*so*), *eye absent* (*eya*) and *dachshund* (*dac*). Homologues of the entire cascade of genes exist in vertebrates where *Six*, *Eya* and *Dach* appear to play important roles in eye development (Halder et al., 1995; Pignoni et al., 1997; Shen and Mardon, 1997). The fly eye arises from a larval structure called the eye-antennal imaginal disc where Tsh, Toy, Ey, Eya and Dac are detected in overlapping domains. It has been postulated that Tsh is required to prevent the premature expression of downstream transcription factors So, Eya and Dac (Bessa et al., 2002). Depending on the cellular context, ectopic *tsh* expression can either induce ectopic eyes or repress endogenous eye morphogenesis. In addition, *tsh* and *ey* mutually induce each other's expression (Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002).

The molecular mechanisms underlying *tsh* function are poorly understood. However, it has been shown that Tsh can repress transcription (Alexandre et al., 1996; Waltzer et al., 2001; Saller et al., 2002). Tsh binds to a specific enhancer of the *modulo* (*mod*) gene, a target of *Scr* and *Ultrabithorax* (*Ubx*) (Graba et al., 1994). Tsh inhibits *mod* expression in the epidermis of the T1 segment (Alexandre et al., 1996). In the midgut mesoderm, *tsh* is required for the transcriptional repression of *Ubx* that is mediated by high levels of Wg in collaboration with the co-repressors Brinker (Brk) and C-terminal Binding Protein (CtBP) (Waltzer et al., 2001; Saller et al., 2002). In this case, no sequence similar to the *mod* enhancer bound by Tsh could be identified and indeed direct binding of Tsh to the *Ubx* enhancer could not be detected in vitro. Instead, Tsh requires Brk to repress Wg signalling for *Ubx* expression in the midgut. Brinker binds the *Ubx* enhancer and then recruits Tsh and CtBP into a ternary repressor complex.

Three putative Tsh genes (*Tsh1*, *Tsh2* and *Tsh3*) have been identified in the mouse. The conservation between *Drosophila* and murine Tsh amino acid sequences is very low (35%) and essentially restricted to the region of the three atypical zinc-finger motifs (Cx2Cx12HMx4H) and an acidic domain towards the N terminus. The murine Tsh proteins possess in addition two, more classical (Cx2Cx12Hx3-4H), zinc-finger motifs at their C-terminal end, which are not found in Tsh. The expression patterns of *Tsh1* and *Tsh2* at different stages of mouse embryogenesis are reminiscent of *tsh* as they appear in restricted regions of the trunk, the gut and the limbs (Caubit et al., 2000). *Tsh3* is detected in a temporal and spatial pattern that is similar, though not identical, to *Tsh1* (L.F., N. Coré and X.C., unpublished).

An important question is whether the proteins encoded by the vertebrate *tsh*-like genes are functionally conserved relative to *Drosophila* Tsh. To analyse this question, we expressed the mouse and *Drosophila* Tsh genes in *Drosophila* at different times and places during development. Abnormalities induced in the *Drosophila* larvae by ectopic expression of Tsh genes are very similar to those caused by ectopic expression of *tsh*. Furthermore, the three Tsh genes are able to rescue developmental defects of a *tsh* loss-of-function mutant. We also provide evidence that, just as *tsh*, all three Tsh genes participate in the network of eye determination genes. In addition, mouse Tsh and Tsh proteins all act as transcriptional repressors. Our results, in combination with expression data of Tsh genes in *Drosophila* and mouse embryos (Fasano et al., 1991; Caubit et al., 2000), indicate that, despite their evolutionary distance and sequence diversity, the three mouse Tsh genes and *Drosophila tsh* exert very similar activities in *Drosophila*.

Materials and methods

Mouse and *Drosophila teashirt* constructs

Murine *Tsh1*, *Tsh2* and *Tsh3* cDNAs (amino acids 15-stop) were subcloned in a modified pcDNA3 vector containing a Myc tag (Siomi and Dreyfuss, 1995). *Tsh1*, *Tsh2* and *Tsh3* sequences are available in GenBank database under the Accession Numbers AF191309, AF207880 and AY063491. Tsh cDNAs fused to the Myc tag were inserted downstream of Gal4 DNA-binding sites by subcloning into pUAST vector (Brand and Perrimon, 1993).

For transfection of mammalian cells, *Tsh1*, *Tsh2* and *Tsh3* cDNAs (amino acids 15-stop) were inserted in-frame downstream of the Gal4 DNA-binding domains (DBD; amino acids 1-147) within the pABgal expression plasmid (Baniahmad et al., 1992) (a gift from M. Muller, Sart-Tilman, Belgium). Deletion of the putative CtBP interaction motif (PIDLT) in mouse Tsh1 was performed by PCR. *Drosophila tshΔPLDLS* generated by PCR was inserted into pUAST vector. For GST-pull down assays, *Tsh1*, *Tsh2* and *Tsh3* cDNAs (amino acids 15-stop) were inserted downstream of the GST coding sequence in pGEX-4T2. For mouse Tsh1 antibody production, the N-terminal part of mouse Tsh1 (amino acids 31-218) was cloned in-frame downstream of GST in pGEX-4T1.

Mouse Tsh1 antibody production

Two rats (Lou strain) were initially immunized with 200 μg of protein suspended in complete Freund's adjuvant and boosted three times with 100 μg of protein suspended in incomplete Freund's adjuvant. Specificity was tested on purified full-length fusion mouse Tsh1 protein and on crude mouse embryonic extracts by western blotting.

Expression of Tsh and *tsh* genes in *Drosophila*

To generate the transgenic flies expressing mouse Tsh or TshΔPLDLS, embryos from a *Drosophila* strain carrying the y^1 w^{1118} mutations were injected with pUAST constructs as described elsewhere (Rubin and Spradling, 1982). w^+ germline transformants were isolated, and transgene insertions were mapped to individual chromosomes by standard genetic crosses. The results presented here are based on three representative homozygous lines for *Tsh1* and two for *Tsh2* and *Tsh3*. Mouse Tsh genes, *tsh* (from *P{UAS-tsh.G}*) and *tshΔPLDLS* were expressed ubiquitously in the epidermis under the control of the *P{GawB}69B* Gal4 driver (referred to as *69B-Gal4*) (Brand and Perrimon, 1993). For transgene expression in the eye, *P{GAL4-dpp.blk1}40C.6* (Staehling-Hampton and Hoffmann, 1994) and *P{GAL4-ey.H}* (Hazelett et al., 1998) drivers were used (referred to as *dpp-Gal4* and *ey-Gal4*, respectively).

All the ectopic expressions in flies were performed at 29°C except when otherwise stated.

Rescue of the *tsh* null mutant phenotype

For the rescue of tsh^8 null mutant (Fasano et al., 1991) by Tsh genes, tsh^8*/CyO; P{UAS-*Tsh*}* were obtained by standard genetic crosses. The expression of the transgenes was performed by crossing tsh^8*/CyO; P{UAS-*Tsh*}* males with tsh^8*/CyO; P{GawB}69B* females. Embryos were collected overnight and aged for 24 hours at 22, 25 or 29°C before preparing cuticles, as described by Fasano et al. (Fasano et al., 1991). tsh^8/tsh^8 cuticles from embryos expressing the transgenes were compared, on the basis of the respective phenotypes, with wild-type phenotype (tsh^8*/CyO*) from embryos expressing the transgenes as a control. Controls without the *69B-Gal4* driver displayed the expected cuticular phenotype of homozygous tsh^8 individuals (data not shown).

Western blot

69B>Tsh embryos were collected for 18 hours, dechorionated and lysed in 40 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% Triton, protease inhibitors (Roche Molecular Biochemicals). Protein concentration was determined using the Bradford assay (Bradford, 1976), and the proteins analysed by 8% SDS-PAGE. Expression of mouse Tsh proteins in *69B*>Tsh embryos was verified by western blotting with an anti-Myc antibody (A14, Santa Cruz Biotechnology) with an anti-Modulo antibody (MAb LA9) used to control the amount of loaded proteins.

Immunostaining and in situ hybridization on whole-mount embryos

Embryos were collected overnight and then fixed (Röder et al., 1992). In situ detection of *mod* transcripts was performed using a digoxigenin DNA-labelled probe (Krejci et al., 1989). Detection of *wg* mRNA was performed as described previously (Röder et al., 1992).

Immunodetection of mouse Tsh1 was realized using anti-mouse Tsh1 antibody used at 1/100. Tsh immunodetection was performed as described (Gallet et al., 1998). The secondary antibody was a FITC-conjugated goat anti-rat antibody (Jackson Laboratories). Nuclei were stained by propidium iodide. Embryos were mounted in Fluoromount-G (Southern Biotechnology Associates) and analyzed by laser confocal microscopy (Zeiss).

GST pull-down assays

GST fusion proteins were isolated from BL21 *E. coli* lysates using standard protocols. The production of GST-mouse Tsh fusion proteins was favoured by a 1-hour induction with 0.1 M IPTG. Resin-bound GST fusions were pelleted, washed in PBS containing protease inhibitors and resuspended in the same buffer containing 0.01% Triton x-100. Mouse Ctbp1 (pcDNA3.1.myc-Ctbp1, gift from E. Olson, Dallas), mouse Tsh1, Tsh2 and Tsh3 in pcDNA3.myc were synthesized using a coupled in vitro transcription/translation kit (Promega). For the binding assays, 10 μg GST fusion in 30 μl Gluthatione Sepharose-4B beads were incubated for 1 hour at 4°C with 5-15 μl [^{35}S]methionine labelled proteins in 500 μl 100 mM NaCl, 0.1% Triton x-100, 10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$ and protease inhibitors (Roche Molecular Biochemicals) and were eluted for 3 minutes in loading buffer. Proteins were analyzed by SDS-PAGE and autoradiographed.

Mammalian cell culture and transfections

MDCK (Madin-Darby Canine Kidney) cells were grown in DMEM (Stratagene) supplemented with 10% fetal bovine serum (BioWhittaker) and 1% penicillin/streptomycin (Stratagene) under a humidified atmosphere containing 9% CO_2. One day before transfection, 10^4 cells per well were seeded in 24-well plates. 18 hours later, cells were co-transfected with 0.25 μg of the reporter vector pGL2-5xUAS-Luc (gift from M. Muller, Sart-Tilman, Belgium), 0.025-0.1 μg pABgal-mouse Tsh1, 2 or 3, and 0.1 μg pcDNA3.1.myc-Ctbp1 and 3 μl FuGene 6 per μg of DNA according to the manufacturer's instructions (Roche Molecular Biochemicals). pABgal and pcDNA3.myc were added to keep the amount of expression plasmids constant. pSV-β-Galactosidase (Promega) was used to normalize for variations in transfection efficiencies. Cells lysis and measure of luciferase activity were performed (Caccavelli et al., 1998). For each experiment, at least three independent transfections in doublets were performed. Results are expressed as the mean±s.d. of three independent pools of transfected cells.

Results

Ectopic expression of murine and *Drosophila* Tsh genes induce identical homeotic transformations in *Drosophila*

In order to ask whether the mouse Tsh genes behave in a similar way to *tsh*, we expressed each of the Tsh genes during *Drosophila* embryogenesis using the UAS/Gal4 system (Brand and Perrimon, 1993). In the wild-type larval epidermis, each trunk segment comprises an anterior belt of denticles and a posterior region of naked cuticle (Fig. 1C). Each trunk segment has characteristics that allow them to be distinguished morphologically. For example, the T1 segment is characterized by an additional group of denticles forming the so-called 'beard' in the central part of the segment (arrow in Fig. 1C). Expression of the Tsh transgenes under the control of *69B-Gal4* (*69B*>Tsh genes) in the epidermis (Fig. 1A) results in transformation of the labial (the last head) segment into T1 segment (de Zulueta et al., 1994) (Fig. 1D-G). Additionally, the more anteriorly located antennal and maxillary head segments are also transformed and adopt a trunk identity in that they develop denticles and naked cuticle (arrowheads in Fig. 1D,E; out of focus in Fig. 1F,G) (de Zulueta et al., 1994). Therefore, ubiquitous expression of *69B>Tsh1*, *Tsh2* or *Tsh3* leads the head segments to develop epidermal features similar to those obtained following *69B>tsh* expression (compare Fig. 1E-G with Fig. 1D). Transformation of the labial segment into T1 segment is observed in all the larvae expressing *Tsh1*, *Tsh2* or *Tsh3* genes. However, the efficiency of transformation (detection of the beard) between the Tsh genes varies: *Tsh1* and *Tsh3*, like *tsh*, induce more ectopic T1 denticles typical of the beard, than does *Tsh2*, probably owing to the weaker expression levels of *Tsh2* (Fig. 1B). The presence of denticles and beard in the labial segment is consistent with its transformation towards a T1-like segment. In addition, detection of ectopic denticles and naked cuticle anterior to the labial segment shows that at least one other head segment is transformed and adopts a trunk-like identity. Moreover, the head fails to involute and cuticle derived from the embryonic head is largely lost (compare Fig. 1C with Fig. 1D-G). These results suggest that the three Tsh genes behave like *tsh* when expressed in wild-type *Drosophila*, producing head to trunk homeosis. Interestingly, despite a lower protein expression level, *Tsh2* is able to induce transformations similar to those observed for *tsh* and *Tsh1* and *Tsh3*.

Ectopic expression of mouse Tsh compensates for the loss of *Drosophila tsh*

As *tsh* is known to positively regulate its own expression (de Zulueta et al., 1994), Tsh genes might induce trunk identity through ectopic induction of *tsh*. To rule out this possibility of 'autoregulation', we expressed mouse Tsh genes in tsh^8 null mutant and tested whether they could compensate for the loss of *tsh* function. In tsh^8 null mutant larvae, as well as being shorter than wild-type larvae, T1 identity is replaced by labial identity (characterized by loss of T1 cuticle) (Fasano et al., 1991) and in the trunk, a weak segment polarity phenotype is observed (Fig. 2C) (Gallet et al., 1998). Ectopic expression of *tsh* using a heat shock promoter can rescue all of these defects (de Zulueta et al., 1994). At 22°C, ubiquitous expression of *69B>Tsh1*, *Tsh2* or *Tsh3* results in partial rescue of the T1 segment as well as of the trunk cuticle phenotypes, as illustrated for *Tsh1* (Fig. 2D). All the tsh^8/tsh^8*; 69B>Tsh1* or *Tsh3* larvae show significant but incomplete rescue of the T1 denticle belt phenotype but none of them displays beard-specific denticles. The size of the naked cuticle bands in the trunk appears normal. At 22°C, the phenotype of all mutant larvae expressing *Tsh2* is more reminiscent of the tsh^8

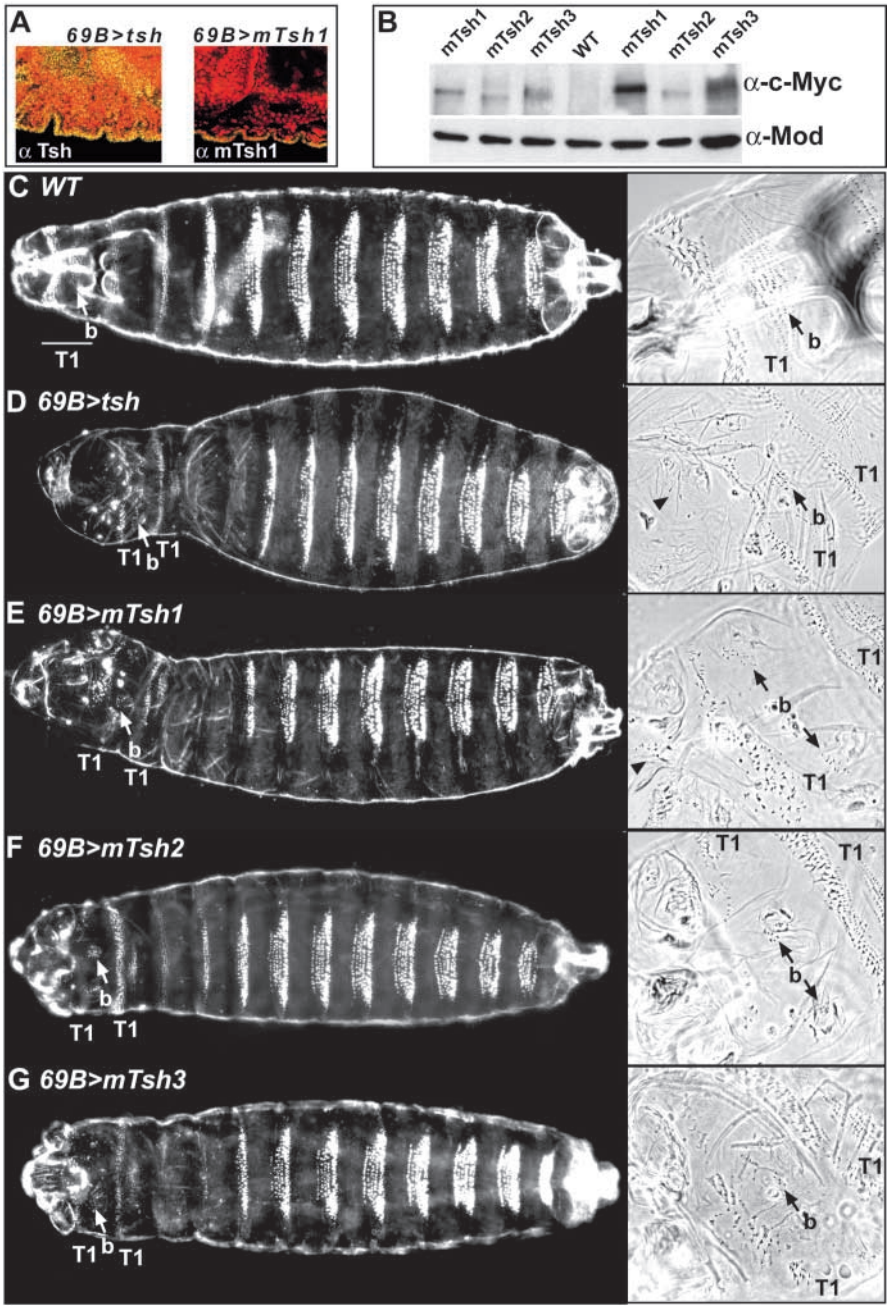

Fig. 1. Ubiquitous expression of mouse and *Drosophila* Tsh genes provokes identical homeotic transformations in the *Drosophila* embryo. (A) Ubiquitous mouse Tsh1 and ectopic Tsh proteins expressed by the *69B-Gal4* driver in wild-type embryos accumulate in the nucleus of epidermal cells. Confocal section through the nucleus of the epidermis of the head/thorax region of stage 12-13 *69B>tsh* and *69B>Tsh1* embryos. The localization of Tsh proteins is shown in green and the nuclei are stained by propidium iodide (red). (B) Comparison of the mouse Tsh proteins level upon ectopic expression in *69B*>Tsh *Drosophila* embryos by western blot analysis with an anti-c-Myc antibody. Two independent transgenic lines are shown for mouse Tsh1, 2 and 3. Mod expression levels serve as a loading control. (C) Wild-type larval cuticle (dark field, left) and close-up (phase contrast, right) of the trunk-head region showing the T1 segment and its beard (b). (D) Ectopic *tsh* expression with the epidermal *69B-Gal4* driver (*69B>tsh*) at 18°C showing the homeotic transformation of the labial head segment into the first thoracic segment. Arrows indicate the beard in the ectopic T1 segment, most visible on phase contrast pictures. Head involution is affected and most of the head skeleton is deleted; compare with C. (E-G) Labial to T1 homeosis induced by the three Tsh genes. (E) *69B>Tsh1*, (F) *69B>Tsh2* and (G) *69B>Tsh3* cuticles of larvae raised at 29°C. All the Tsh-expressing larvae are affected. Arrowheads indicate ectopic denticles resulting from the transformation of more anterior head segments into trunk (shown for *tsh* and *Tsh1*, similar result for *Tsh2* and *Tsh3* but out of focus, data not shown). Anterior is towards the left and dorsal on the top. T1, T1 thoracic segment.

phenotype (no T1 denticles and reduced naked cuticle domains), probably owing to its lower expression level, though the length of the larvae is significantly increased (not shown). At 29°C, where the *Gal4/UAS* system is more efficient, the three Tsh genes cause a more complete and fully penetrant rescue (Fig. 2E-G). At this temperature, the alternation in the trunk of normal naked cuticle domains with normal denticle belts demonstrates that the three Tsh genes are able to rescue completely the segment polarity phenotype of tsh^{8}. The T1 segment phenotype is almost completely rescued by the three Tsh genes, as attested by the presence of scattered denticles and a recognisable beard. In addition, clear homeosis occurs as attested by the presence of a second T1 segment, replacing the endogenous labial segment (additional T1). These results provide strong evidence that any of the Tsh genes can efficiently substitute for *tsh* during *Drosophila* embryogenesis.

Ectopic expression of the Tsh genes induces naked cuticle less efficiently than *tsh*

tsh overexpression in the trunk at 29°C leads to loss of ventral denticles, whereas, at 25°C, the denticles are only partially lost (Fig. 2H). In contrast to ectopic *tsh*, the pattern of denticle does not seem to be affected in *69B>Tsh1*, *Tsh2* or *Tsh3*, even at 29°C (Fig. 1E-G), suggesting that the mouse proteins may not be able to induce naked cuticle. However, the characteristic alternating pattern of denticles and naked cuticle is perfectly restored in tsh^{8} homozygotes expressing Tsh genes (Fig. 2E-G). Increasing the level of mouse Tsh by combining two insertions of each *UAS*-Tsh partially replaces denticles with

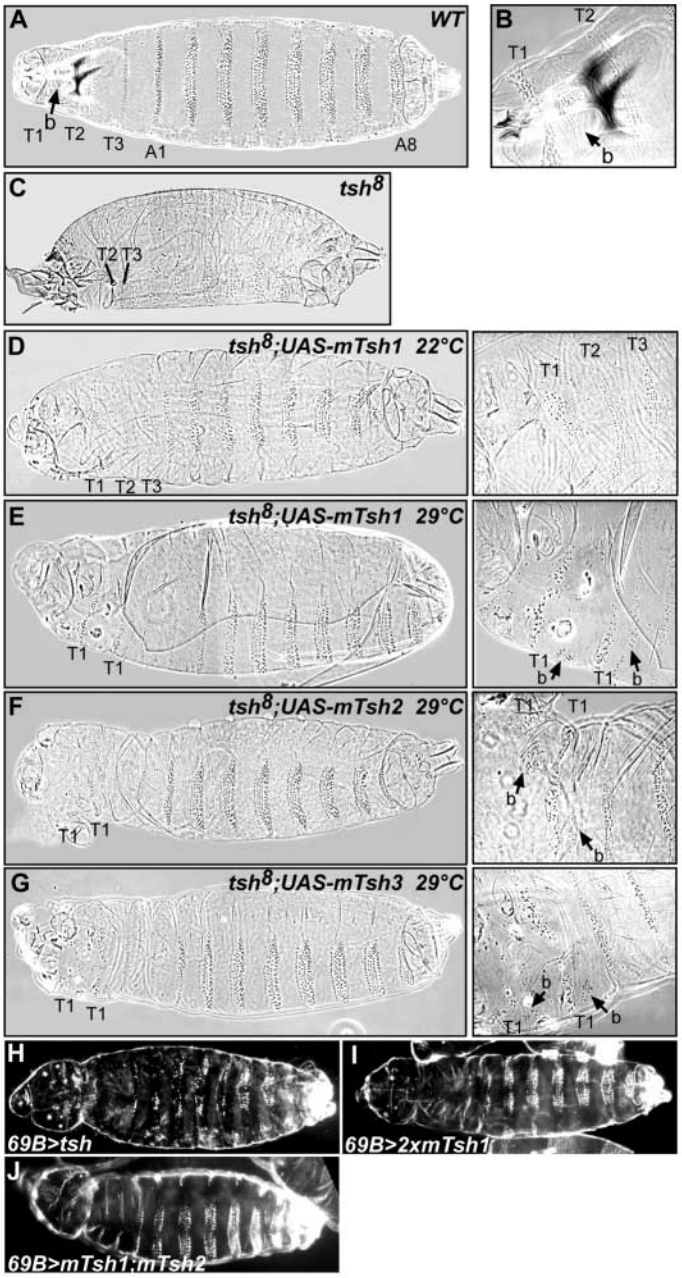

Fig. 2. Murine Tsh genes rescue the cuticular phenotype of the *tsh*^{8} null mutant. Both the homeotic transformation and the segment polarity phenotype are restored. (A) Wild-type larval cuticle showing the three thoracic (T1, T2, T3) and the eight abdominal segments. (B) Close-up of the T1-T2 region. Arrows indicate the beard (b) in the T1 segment. (C) Homozygous *tsh*^{8} loss-of-function cuticular phenotype; the T1-specific denticles are missing (homeotic transformation of the T1 segment into the labial segment) (Röder et al., 1992) and naked and denticled cuticular regions are reduced in the trunk. (D-G) Rescue of *tsh*^{8} by Tsh genes. Close-up of T1-T3 region is on the right. (D) At 22°C in *tsh*^{8} homozygotes, *69B>Tsh1* expression results in partial rescue of *tsh*^{8} phenotype. The T1 segment is weakly restored (denticles form only on one side in this photograph) and the size of naked cuticle is comparable with wild type (compare with A-C). At 22°C, *Tsh3* display similar levels of rescue (not shown), whereas *Tsh2* has very little effect (see text). (E-G) At 29°C, the three Tsh genes perfectly restore the naked cuticle regions in the trunk although the T1, on the basis of the morphology of the beard and T1 denticle belt, is not completely rescued (compare with B). This defective differentiation was used to distinguish *tsh*^{8} homozygotes. Homeotic transformation of the labial segment into T1 segment is highlighted by ectopic denticles in the head (additional T1 and arrows). (H,I) Increased dose of mouse Tsh reveals a segment polarity phenotype similar to *tsh*. (H) *69B>tsh* larvae raised at 25°C exhibit ectopic naked cuticle in the trunk (in contrast to *69B>tsh* at 18°C in Fig. 1D), particularly along the ventral midline. (I) Combination of two independent insertions of *UAS-Tsh1* (*69B>2xTsh1*) also causes ectopic naked cuticle (similar results were obtained with *Tsh2* and *Tsh3*, data not shown). Most of the denticles of the thoracic and ectopic T1 segments are absent. (J) Combination of *UAS-Tsh1* with *UAS-Tsh2* (*69B>Tsh1;Tsh2*) gives similar results (as for *UAS-Tsh1* with *UAS-Tsh3* and for *UAS-Tsh2* with *UAS-Tsh3*, not shown).

naked cuticle as illustrated for *Tsh1* (*69B>2xTsh1*, Fig. 2I). Similar to *69B>tsh* larvae cultured at 25°C, in all the *69B>2xUAS-Tsh1* larvae some thoracic denticles are lost and in the abdomen, denticles are replaced by naked cuticle along the ventral midline. However, the loss of denticles observed is less dramatic than that seen with *tsh*, indicating that, even at high expression levels, mouse Tsh genes are less efficient than *tsh* in specifying naked cuticle. Two insertions of *UAS-Tsh2* or *UAS-Tsh3* give the same results (not shown). Expression of two different Tsh genes together, as shown for *UAS-Tsh1* with *UAS-Tsh2*, is similarly inefficient at suppressing denticles (compare *69B>Tsh1;Tsh2* in Fig. 2J with Fig. 2I).

Tsh genes expression affects the expression of *tsh* target genes

The ability of Tsh genes to induce the same transformations as ectopic *tsh* strongly suggests that mouse and *Drosophila* Tsh genes act on the same target genes. One gene controlled by *tsh* is *wg*. *wg* is expressed in stripes in each segment until stage 10 of development (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Yoffe et al., 1995), whereupon expression is lost from ventral regions of the labial and maxillary head segments but maintained in homologous positions of the trunk (Fig. 3A). *tsh* is required for the maintenance of *wg* at this stage in the ventral part of each trunk segment. Consequently, following ectopic expression of *tsh*, *wg* is maintained in the ventral part of the labial and maxillary head segments (Fig. 3B) (Gallet et al., 1998). Similarly, *Tsh1*, *Tsh2* and *Tsh3* are able to maintain *wg* expression in these head segments. Like *tsh*, the maintenance is particularly marked in the labial segment (Fig. 3C, see figure legend for quantitative data). The detection of *wg* in the labial segment correlates with the ability of Tsh genes to induce labial to T1 transformation.

In contrast to *wg*, *mod* is known to be a direct target of Tsh through direct binding to specific cis-elements in the *mod* 5′ regulatory region (Alexandre et al., 1996). *mod* is expressed in the labial segment and repressed by Tsh in the T1. In *tsh*^{8} mutants, *mod* is de-repressed in the T1 segment. Ectopic Tsh inhibits *mod* expression in the labial segment (Fig. 3D,E), further suggesting that Tsh acts as a transcriptional repressor. Similarly, *mod* expression in the labial segment was reduced in *69B>Tsh1*, *Tsh2* and *Tsh3* embryos, with a lower penetrance for *Tsh2* (Fig. 3F), indicating that, to some extent, mouse Tsh proteins can also mediate transcriptional repression. Again, despite the lower expression level of mouse Tsh2 compared

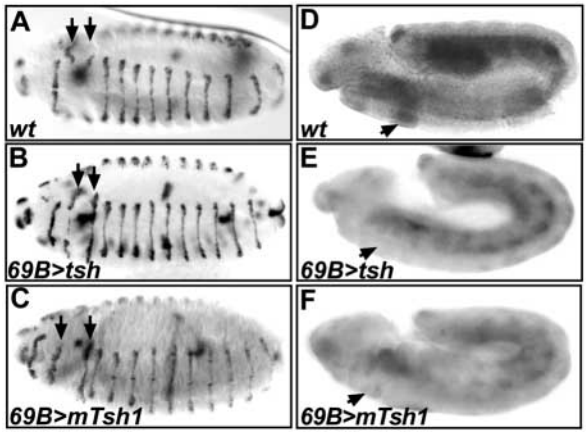

Fig. 3. Ectopic expression of Tsh genes in *Drosophila* alters the expression patterns of two *tsh* target genes. (A-C) Regulation of *wg* expression by Tsh genes. (A) *wg* mRNA in a 12-13 stage wild-type embryo showing absence of *wg* expression in the ventral parts of the labial and maxillary segments (arrows). (B) Maintenance of *wg* expression in the head segments by ectopic *tsh* and (C) in *69B>Tsh1* embryos (arrows, identical results with *Tsh2* and *Tsh3*). The maintenance of *wg* expression by *tsh* and the three Tsh in the labial is completely penetrant. By contrast, the maxillary segment is maintained in almost 75% of *69B>tsh* (*n*=212), *69B>Tsh1* (*n*=305) and *69B>Tsh3* (*n*=159), and in ~25% of *69B>Tsh2* (*n*=170). (D-G) Regulation of *mod* expression by Tsh genes. (D) *mod* expression revealed by in situ staining in a stage 11 wild-type embryo. *mod* mRNA are detected in the labium (arrow). (E) Reduced *mod* expression in the epidermis of the labial segment upon ectopic expression of *tsh* (*69B>tsh*) or Tsh genes as illustrated in F by *Tsh1*. Reduction of *mod* expression occurs in roughly 75% of *69B>Tsh1* (*n*=291) and *69B>Tsh3* (*n*=170) and 50% of *69B>Tsh2* (*n*=230) embryos.

with mouse Tsh1 and mouse Tsh3, our results suggest that the amount of mouse Tsh2 is sufficient to affect the expression of the same target genes as the two other mouse Tsh proteins.

Ectopic expression of Tsh genes in the eye

tsh is also necessary for eye specification. Targeted expression of either *ey*, *eya*, *dac* or *tsh* can cause ectopic eye development in non-eye tissues. However, *tsh* also acts as a repressor of eye development (Bessa et al., 2002; Singh et al., 2002). In *dpp>tsh*, ectopic eyes are induced ventrally in the antennal part of the eye-antennal disc (Fig. 4A) (Pan and Rubin, 1998; Singh et al., 2002), whereas expression of *tsh* using the *ey-Gal4* driver compromises eye development (Fig. 4B) (Bessa et al., 2002). Using the same drivers, we expressed Tsh genes in the eye-antennal discs. This causes the same kind of phenotypes as ectopic *tsh* (Fig. 4C,D). The poor penetrance of the phenotypes observed with *Tsh2* correlates with its lower expression level.

These results indicate that mouse Tsh genes encode proteins, which can apparently interact productively with the target proteins and/or genes of *Drosophila* Tsh that are required for eye development.

Mouse Tsh proteins are transcriptional repressors

Repression of *mod* by Tsh indicates that, like Tsh, mouse Tsh proteins can act as transcriptional repressors. In addition, the proteins are all nuclear (Fig. 1A) consistent with the idea that mouse Tsh proteins act as transcriptional regulators. A transcriptional repressing activity has been previously attributed to the N-terminal half of Tsh (Waltzer et al., 2001). Moreover, Tsh can bind CtBP and in a complex with Brk and CtBP, represses *Ubx* transcription in the visceral mesoderm (Saller et al., 2002). In the N-terminal part of Tsh, lies a consensus motif for the interaction with CtBP (PLDLS). Deletion of this motif prevents the binding of CtBP to Tsh (Fig. 5A). We made a *UAS-tshΔPLDLS* transgene that specifically lacks the sequence encoding the PLDLS motif in order to test whether this motif is crucial for repressor activity of Tsh in vivo. Expression of TshΔPLDLS protein is unable to repress *mod* expression in the labium (Fig. 5A compare with Fig. 3E). This indicates that repression of *mod* transcription by Tsh depends on the PLDLS motif presumably via its interaction with CtBP.

As mouse Tsh genes also inhibit *mod* expression (Fig. 3E,F), we asked whether mouse Tsh proteins display transcriptional repression activities when targeted to a promoter in a heterologous in vitro test. To this end, the DNA-binding domain of Gal4 was fused to each of the mouse Tsh genes and each fusion protein was expressed in mammalian cells. Waltzer et al. (Waltzer et al., 2001) have shown that in human Saos-2 cells, Gal4-Tsh fusion protein represses two- to 10-fold the luciferase reporter gene in a dose-dependent manner. In an analogous assay, we show that a similar fold repression of the reporter gene activity is observed upon expression of Gal4-mouse Tsh1, Tsh2 and Tsh3 fusion proteins (Fig. 5B) showing

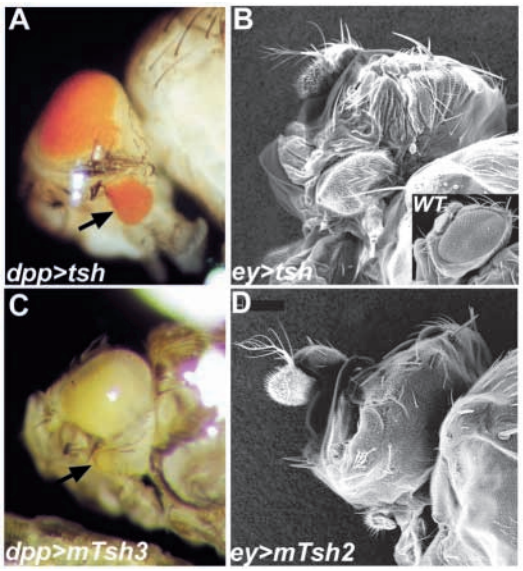

Fig. 4. Targeted expression of Tsh genes in the fly eye cause the same defects as misexpressed *tsh*. (A,C) Ectopic eye development in *dpp>tsh* (A) and *dpp>Tsh3* (C) ventral to the normal eye in the pharate adult raised at 25°C (arrows). About half *dpp>Tsh1* (not shown), *dpp>Tsh3* flies (*n*=36 and *n*=41 respectively) and one third of *dpp>Tsh2* flies (*n*=57, not shown) show ectopic eye development. (B,D) Scanning electron micrographs of the heads of *ey>tsh* (B) and *ey>Tsh2* (D) flies raised at 25°C showing impaired eye development. A wild-type eye is shown in the inset in B. About one third of *ey>Tsh1* (*n*=45, not shown) and *ey>Tsh3* (*n*=29, not shown), and 15% of *ey>Tsh2* (*n*=63) flies do not develop eyes. *tsh* expression leads to eye defects in more than 80% of the flies (*dpp>tsh*, *n*=28 and *ey>tsh*, *n*=33).

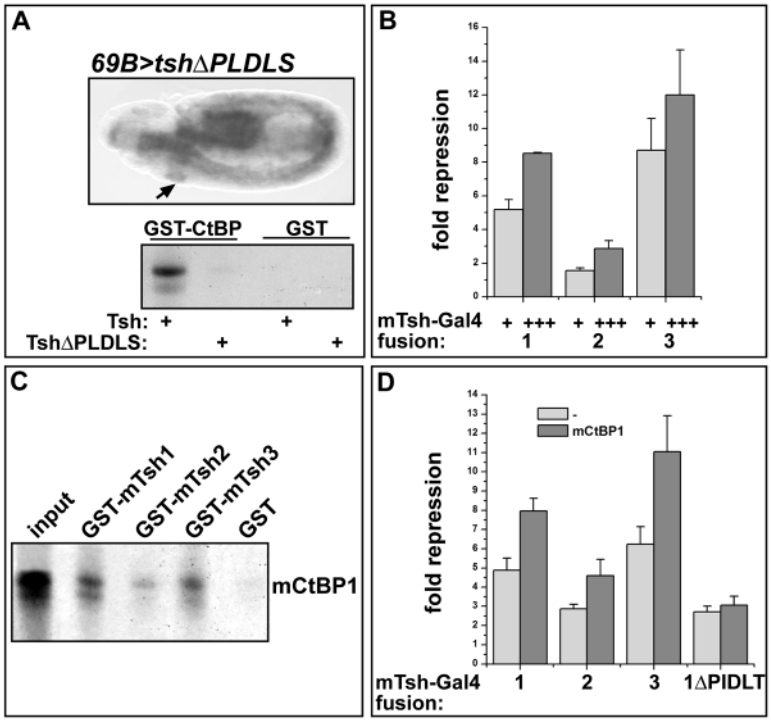

Fig. 5. Tsh proteins are CtBP-dependent repressors. (A) Deletion of the CtBP-interaction motif in Tsh abolishes the interaction with CtBP in vitro (bottom) and fails to suppress *mod* expression in the labial segment of *69B>tshΔPLDLS* embryos (top, compare with Fig. 3E). (B) pABgal-mouse Tsh1, 2, and 3 (0.025 and 0.1 μg) alone repress pGL2-5xUAS-Luc reporter activity (0.25 μg). (C) GST-pull down assay showing the interaction between in vitro translated mouse Ctbp1 and GST-mouse Tsh fusion proteins. Non-specific interaction is shown with GST alone. (D) In an independent experiment from B, co-expression of 0.1 μg mouse Ctbp1 with 0.1 μg Gal4-mouse Tsh fusions leads to a synergistic inhibition. Deletion of the putative CtBP interaction motif in pABgal-mouse Tsh1 reduces the activity of the reporter from 5 to 2.5-fold and abolishes the synergistic repression in the presence of mouse Ctbp1. Mouse Ctbp1 alone does not affect the reporter activity (not shown). Data are presented as fold repression relative to luciferase activity, normalized to the β-galactosidase activity, of the reporter in presence of empty pABgal and pcDNA3.myc.

that mouse Tsh proteins, like Tsh, mediate repression of basal transcription when targeted to DNA.

Interestingly, the three mouse Tsh proteins present a putative motif for the interaction with CtBP, PIDLT. We tested whether the mouse Tsh proteins could interact with mouse CtBP1 by performing GST pull-down assays (Fig. 5C). All three mouse Tsh proteins interact with mouse Ctbp1. In mammalian cells, although the in vitro affinity for mouse Ctbp1 and the repressor potential vary between the mouse Tsh proteins, co-expression of mouse Ctbp1 with the three Gal4-mouse Tsh fusions potentiates the repression of the reporter (Fig. 5D). Deletion of the PIDLT motif in Gal4-mouse Tsh1 affects its basal repression activity, but a significant level of inhibition of the reporter persists. Importantly, this deletion leads to loss of the synergistic inhibition by mouse Ctbp1 (Fig. 5D). As mouse Ctbp1 is ubiquitously expressed, it is probable that it is similarly ubiquitously expressed in other mammals and it is thus likely that endogenous CtBP in canine MDCK cells partially contributes to the basal repression by mouse Tsh1 in the absence of transfected mouse Ctbp1. Our results indicate that mouse Tsh proteins present intrinsic, Ctbp-dependent, transcriptional repressing activity. Because, however, residual inhibiting activity is seen upon deletion of the PIDLT motif, mouse Tsh1, and presumably mouse Tsh2 and mouse Tsh3, must also repress transcription in a CtBP-independent manner.

Discussion

Tsh genes and AP patterning

We were concerned with the functional conservation of the Tsh genes (Caubit et al., 2000). The present study provides evidence that the murine *tsh*-related genes are functionally equivalent to *tsh* at least in the tests we describe here in *Drosophila*. First, ectopic expression of the three mouse Tsh proteins, like *tsh*, results in homeotic transformation of the labial head segment into the T1 trunk segment. One or two other head segments take on trunk identities as they differentiate denticles and naked cuticle in place of certain head structures following ectopic Tsh (or *tsh*) expression. Second, and more importantly, Tsh genes efficiently (although not completely in the T1 segment) rescue the phenotype of tsh^{8} null mutants in a very similar way to that described for *tsh* (de Zulueta et al., 1994) (this work): both the segment polarity and the T1 to labial homeotic phenotypes are rescued. Tsh and *tsh* also affect the expression of *wg* in the ventral labium at stage 11 and repress the expression of the direct *tsh* target gene *mod*. We also provide evidence that mouse and *Drosophila* Tsh proteins behave similarly as transcriptional repressors: they can reduce *mod* expression and they inhibit transcription in a heterologous test in mammalian cells. Throughout these tests, the weaker phenotypes or penetrance exerted by *Tsh2* compared with *Tsh1* and *Tsh3* appears to correlate with its lower expression level compared with the other Tsh transgenes.

The ability of *tsh* and Tsh genes to induce T1 identity indicates that both collaborate with the same genes and/or proteins that determine segment identity. The identity of the T1 segment is specified by the combined action of *tsh* and the Hox gene *Scr* (Röder et al., 1992; de Zulueta et al., 1994), whereas the labial segment forms where *Scr* is expressed in the absence of Tsh. This observation, together with the capacity of Tsh protein to directly interact with Scr (L.F. and O. Taghli, unpublished), suggest that Tsh may modify the transcriptional regulatory properties of the Scr protein to allow the expression of target genes essential for the T1 segment identity. The fact that ectopic expression of *Tsh1*, *Tsh2* or *Tsh3* can transform the labial segment into T1 provides strong evidence that Tsh can specify T1 identity in cooperation with *Scr* and suggests

that mouse Tsh proteins can substitute for Tsh and regulate specific target genes responsible for morphological features of the trunk.

Several studies have shown that mouse and human Hox genes can carry out equivalent functions to their *Drosophila* counterparts when introduced in flies [*Hoxb6/Antp* (Malicki et al., 1990; McGinnis et al., 1990), *Hoxb4/Deformed* (Malicki et al., 1992), *Hoxa5/Scr* (Zhao et al., 1993), *Hoxb1/labial* (Lutz et al., 1996)]. Interestingly, ectopic expression of mouse *Hoxa5*, which is functionally homologous to *Scr*, can activate ectopic expression of a salivary gland target gene, and induce the homeotic transformation of the larval thoracic segments T2 and T3 towards T1 (Zhao et al., 1993). These data imply that, in addition to regulating specific Scr target genes, the mammalian protein Hoxa5 is also able to interact with putative Scr co-factor(s). Although functional analysis of Tsh genes are necessary in mouse, it is tempting to hypothesize that Tsh, together with the Hox genes, may be part of common developmental genetic mechanisms for patterning the invertebrate and vertebrate trunk. The expression patterns of the Tsh genes (Caubit et al., 2000) during mouse embryogenesis are consistent with a function in trunk versus head boundary specification in vertebrates.

Mouse Tsh proteins, like *Drosophila* Tsh, act as modulators of Wg signalling in *Drosophila*

We observed that denticles are replaced with naked cuticle when the dose of Tsh is increased by combining two insertions. In addition, Tsh genes rescue the segment polarity phenotype of tsh^{8} null mutants. These results indicate that Tsh genes can operate in the gene network involved in formation of naked cuticle. In tsh^{8} null mutants, *wg* expression is not maintained in the trunk and the reduced naked cuticle domains resemble late *wg* loss-of-function phenotype. The rescue of the tsh^{8} cuticular phenotype by Tsh genes suggests that these genes are sufficient to ensure *wg* transcription and/or signalling in the posterior part of each segment as seen in wild-type flies. Although the maintenance of *wg* in the tsh^{8} trunk was not assessed upon Tsh and *tsh* expression, we showed that Tsh genes, like *tsh*, are able to maintain its expression in the gnathal segments. This would suggest that, like *tsh*, Tsh genes could control *wg* expression in the trunk as well as in the head. An autoregulatory loop involving Tsh in the Wg signalling pathway has been postulated for the maintenance of *wg* expression (Gallet et al., 1998), suggesting that in addition to regulating *wg* expression, mouse Tsh genes, like *Drosophila* Tsh, might modulate Wg signalling. Given the striking conservation of the Wg and Wnt signalling components between species, one could hypothesize that at least some aspects of *tsh* activity in Wg/Wnt signalling may be conserved from *Drosophila* to vertebrates. However, further investigation is required to assess for a role of Tsh genes in Wnt signalling in vertebrates.

Mouse Tsh genes, like *Drosophila tsh*, affect the development of the fly eye

Our data also demonstrate the ability of Tsh genes to operate in the formation of the adult fly eye. Interestingly, ectopic expression of *ey* or its vertebrate homologue *Pax6* induce ectopic eyes in *Drosophila* (Halder et al., 1995), demonstrating their equivalence in the development of complex sensory structures. *Pax6* also plays a crucial role in vertebrate eye formation (Chow et al., 1999; Ashery Padan et al., 2000). The Pax/Dac/Eya/Six regulatory network first identified in the context of the *Drosophila* eye has been shown to be involved in vertebrate somitogenesis. Indeed, this regulatory relationship extends to other members of these families: Pax3, Six1, Eya2 and Dach2 (Heanue et al., 1999; Kardon et al., 2002). In the fly, *tsh* in cooperation with *homothorax* (*hth*), a negative regulator of eye development, prevents premature expression of the downstream genes *so*, *eya* and *dac* (Singh et al., 2002; Bessa et al., 2002). In addition to a genetic interaction in the developing *Drosophila* eye, a direct protein interaction has been described between Tsh and Hth and its partner Extradenticle (Exd) (Bessa et al., 2002). Our results suggest that, like *tsh*, mouse Tsh genes can regulate the activity and/or expression of some genes involved in formation of the fly eye. In vertebrates, numerous *hth* and *exd* homologues are found: *Meis1-3* and *Prep1* for *Hth*, and *Pbx1-3* for *Exd*. One of the aims of future work will be to investigate whether Tsh proteins are involved in similar protein/gene networks in vertebrates.

Mouse Tsh proteins have a transcriptional repressor activity

Comparison of the organization of Tsh with Tsh-related proteins in mouse and humans (Caubit et al., 2000) (L.F. and X.C., unpublished) suggests that common functional features are probably defined by the region encompassing the three zinc-finger motifs and by the presence of a motif known to interact with CtBP. Interestingly, mouse and *Drosophila* Tsh proteins display intrinsic transcriptional repression activity. Our results and those of others (Saller et al., 2002) suggest that the repression ability of Tsh proteins is partly due to their interaction with the co-repressor CtBP. In the visceral mesoderm, Tsh is recruited to the *Ubx* enhancer in a repressor complex containing Brk and CtBP (Saller et al., 2002), wherein Tsh does not seem to bind directly to DNA, but rather Brk is the DNA-binding partner. In the ectoderm, however, Tsh directly binds to the *mod* enhancer and represses transcription in vivo (Alexandre et al., 1996). We show that the association of CtBP with Tsh is dependent on the CtBP-interacting motif (PLDLS) located in the N-terminal part of Tsh, and this CtBP/Tsh complex contributes to the observed repression. An analogous motif (PIDLT) is found in the C-terminal part of the three mouse Tsh proteins. Despite the different context encompassing the PIDLT motif in the mouse proteins (C-terminal), we show that this motif is functional and essential for the repressor function of mouse Tsh1. Although we only directly addressed the role of this motif in mouse Tsh1, Tsh2 and Tsh3 repression activity is equally potentiated by mouse Ctbp1, suggesting that mouse Ctbp1 is a co-repressor acting with all mouse Tsh proteins. Interestingly, the PIDLT motif lies within a region of the three mouse Tsh proteins where the sequence similarity is low and thus appears to be a highly conserved functional domain in a variable region. In addition, it is worth noting that, in mammalian cells, some repression activity persists in mouse Tsh1 after deletion of the CtBP-interacting motif, implying that other mechanisms of transcriptional repression are used by mouse Tsh. In contrast to Tsh, which contains a repressor domain rich in Ala (L.F. and O. Taghli, unpublished), analysis of the mouse Tsh protein sequences fail to reveal a comparable feature or any known motif that could account for the mouse Tsh1ΔPIDLT repressor activity.

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