Restricted *teashirt* expression confers eye-specific responsiveness to Dpp and Wg signals during eye specification in *Drosophila*

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Summary

In *Drosophila*, the eye primordium is specified as a subdomain of the larval eye disc. Here, we show that the Zn-finger transcription factor *teashirt* (*tsh*) marks the region of the early eye disc where the eye primordium will form. Moreover, *tsh* misexpression directs eye primordium formation in disc regions normally destined to form head capsule, something the eye selector genes *eyeless* (*ey*) and *twin of eyeless* (*toy*) are unable to do on their own. We present evidence that *tsh* induces eye specification, at least in part, by allowing the activation of eye specification genes by the *wingless* (*wg*) and *decapentaplegic* (*dpp*) signaling pathways. Under these conditions, though, terminal eye differentiation proceeds only if *tsh* expression is transient.

Key words: *Drosophila*, Eye disc, Eye determination, Teashirt, Wingless, Decapentaplegic, Eyes absent, Homothorax, Atonal

Introduction

During development, groups of cells become singled out as organ primordia through the combined action of organ-specific selector genes and a limited number of signaling pathways (reviewed by Mann and Morata, 2000). Nevertheless, the expression domains of selectors genes are often broader than the territories that actually become the primordia. This is the case during the specification of the *Drosophila* compound eye. The fly eye derives from a bilayered epithelial sac called the eye imaginal disc. Throughout the three larval stages (L1 to L3), undifferentiated disc cells become progressively committed to different organ fates: eye, antenna, maxillary palp and head capsule. When the eye disc is formed, early during L1, all its cells express the so-called 'eye selector' genes: the *Pax6* paralogs *eyeless* (ey) and *twin of eyeless* (*toy*) (Czerny et al., 1999; Gehring, 2002). Thus, other factors, in addition to *Pax6* genes, must contribute to the selection of the eye primordium identity within the eye disc.

During L2, cell morphology in the two adjacent epithelial layers of the eye disc becomes distinct. The eye derives from the columnar layer called the main epithelium (ME), or disc proper. The overlaying squamous layer, the peripodial epithelium (PE), contributes to the head capsule that surrounds the eye (Haynie and Bryant, 1986; Jurgens and Hartenstein, 1993). These cell morphological changes are paralleled by changes in the expression of key genes. The expression of *eyes absent* (*eya*) in the posterior of the ME during L2 is considered the first hallmark of eye primordium specification. The initiation of *eya* expression is immediately followed by that of *sine oculis* (*so*) and *Dachshund* (*Dac*). *eya*, *so* and *Dac* are collectively known as 'early retinal genes', as their co-expression is necessary to lock-in the eye fate within the eye field, possibly by acting together as a transcriptional complex (Desplan, 1997; Kenyon et al., 2003; Kumar and Moses, 2001; Pichaud et al., 2001). The homologs of *ey/toy* and the early retinal genes also play crucial roles during vertebrate eye development (reviewed by Chow and Lang, 2001).

The expression of *hedgehog* (*hh*) and *hh*-dependent *decapentaplegic* (*dpp*) transcription at the posterior margin of the disc is key for the definition of the eye primordium, as they activate the expression of *eya* and *so*. The eye-inducing functions of *dpp* also include the posterior repression of *wingless* (*wg*), which would otherwise block eye development by promoting the alternative head-capsule fate. Therefore, *wg*, which is expressed in the anterior regions of the disc, and *dpp*, which is expressed first along the posterior margin and later at the MF, antagonize each other as eye-repressor and eye-activator, respectively (reviewed by Dominguez and Casares, 2005; Pappu and Mardon, 2004).

Early in L3, after the definition of the eye primordium within the eye disc, retinal differentiation begins in the posterior region of the eye primordium (Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Heberlein et al., 1993). Once initiated, retinal differentiation proceeds as a wave in a posterior-to-anterior direction. The front of this differentiation wave is marked by an indentation of the main epithelium called morphogenetic furrow (MF). Thus, during wave progression, undifferentiated cells are anterior to the MF, while differentiating cells are posterior to it (reviewed by Treisman and Heberlein, 1998). The progression of the MF is driven by the joint action of *dpp*, expressed within the furrow, and by *hh*, expressed in cells posterior to the furrow. The induction of the proneural gene *atonal* (*ato*) by *hh* is the first step towards the definition of the R8 photoreceptor, the founder neuron of

the mature eye units, or ommatidia. (Dominguez, 1999; Dominguez and Hafen, 1997). The expression of the selector genes *ey* and *toy*, which is initially widespread, is repressed in differentiating cells and thus their expressions become restricted to the undifferentiated region of the eye disc, anterior to the MF (Czerny et al., 1999).

Two other transcription factors are known to be expressed in late L2-early L3 eye discs: *teashirt* (*tsh*), which encodes a transcription factor harboring three widely spaced Zn-finger domains (Fasano et al., 1991); and *homothorax* (*hth*), a Meis-family homeobox gene (Pai et al., 1998; Rieckhof et al., 1997). The pattern of *tsh* expression in L3 eye discs is very similar to that of *ey/toy*, its expression being activated anterior to the MF and repressed posterior to it (Bessa et al., 2002; Fasano et al., 1991) (Fig. 1E). *hth* expression is repressed close to the MF via the action of *dpp* produced at the MF (Bessa et al., 2002). Therefore, the *tsh* territory can be further subdivided into two domains: a domain, far from the MF, in which *hth* expression maintains cells in an undifferentiated state and represses retinal selector gene expression (such as *eya*); and a domain abutting the MF, in which *hth* is repressed, leading to *eya* upregulation (Bessa et al., 2002). The latter is also known as the pre-proneural domain, as it precedes the onset of retinal differentiation (Greenwood and Struhl, 1999). In addition, *hth* expression is maintained in the peripodial epithelium and margin of the eye disc during its whole development (Pai et al., 1998; Pichaud and Casares, 2000). A detailed description of the dynamics of *ey* and *tsh* expressions is currently lacking.

Several lines of evidence suggest a role for *tsh* during eye development, although precisely what its role(s) are have not been fully clarified. *tsh* overexpression in the eye disc can induce ectopic eye development or block its normal formation, depending on the Gal4-promoter used (Manfroid et al., 2004; Pan and Rubin, 1998; Singh et al., 2004; Singh et al., 2002).

Interestingly, the steps leading to the specification of the eye primordium within the eye disc occur in only one of the two epithelial layers that compose the disc – the ME. This restriction is not explained by the model outlined above, as all L1 disc cells express the eye selector gene *ey*, and the signaling molecules *wg* and *dpp*, which are transcribed along the margins of the disc, should be able to reach both layers. Therefore, factors differentially expressed in the two disc layers must be responsible for making one of them either competent, or refractory, to eye-determining signals. Such factor(s) should be expressed specifically in one of the layers of the disc prior to the onset of eye-specific gene expression, and its expression might be able to alter the developmental potential of the other, if expressed ectopically.

Here, we show that *tsh* expression starts during L2 and is restricted to the ME. Ectopic expression of *tsh* in the peripodial cells transforms them into eye primordium-like cells, as judged by their cell morphology and gene expression; nevertheless, the final differentiation of these cells into retina occurs only if *tsh* expression is transient. Furthermore, our results indicate that *tsh* re-specification properties rely on its ability to make peripodial cells respond to *wg* and *dpp* by initiating the eye differentiation program. Thus, the asymmetric expression of *tsh* in one disc layer might allow eye primordium specification to occur in just that layer.

Materials and methods

Genotypes and genetic manipulations

Larvae were raised at 25°C, unless otherwise indicated. tsh^1 (called in this work *tshZ*) (Fasano et al., 1991) was used as reporter for *tsh* expression.

For targeted mis-expression, we used the UAS/GAL4 system (Brand and Perrimon, 1993). Lines used were UAS-*tsh* (Gallet et al., 1998), UAS-*ey* (Halder et al., 1995), UAS-*toy* (Czerny et al., 1999), UAS-tkv^{QD} (Nellen et al., 1996), UAS-*Axin A2* (Willert et al., 1999), *dpp*-GAL4 (Staehling-Hampton et al., 1994), *tsh*-GAL4 (Wu and Cohen, 2000), *ey*-GAL4 (Hazelett et al., 1998), *Arm*-GAL4 (Tolwinski and Wieschaus, 2001) and *hs-GAL4* {P[w(+mC)=GAL4-Hsp70.PB]89-2-1; Flybase}. *MS1096* (Milan et al., 1998) and *MD705* (gift from G. Morata) express Gal4 specifically in the PE/margin of the eye disc (see Results section), beginning in late L2.

tsh-ectopic expression clones were generated randomly in eye discs by heat shocking L1 or L2 larvae [24-48 hours and 48-72 hours after egg laying (AEL), respectively] for 30 minutes at 35.5°C from a *yw hsFlp122; tub>GFP, y+>GAL4* (Zecca and Struhl, 2002); UAS-*tsh*/*SM6^TM6B* stock, or from the following crosses: *yw hsFlp 122*; *tub>GFP*, *y+>GAL4*; UAS-*tsh*/*SM6^TM6B* females to UAS-*Axin* A2 or UAS-tkv^{QD}/*Y*; UAS-*Axin A2* males; and *yw hs-Flp122*; *act>y+>GAL4*, *UAS-lacZ/CyO* (Ito et al., 1997) females to UAS-*ey*; UAS-*toy*; UAS-tkv^{QD}/*Y*, +; or UAS-*Axin A2* males. Clones were marked negatively by the absence of GFP, or positively by detection of β-galactosidase (*lacZ*-marked) or Tsh antigens.

Mad^- loss-of-function clones were induced in the eye disc in larvae of the genotype *ey-Flp; FRT 40A* Mad^{B1}*/FRT 40A arm-Z* (Hazelett et al., 1998) and marked by the absence of β-galactosidase.

To induce a pulse of *tsh* expression, larvae of the genotype *hs-Gal4; UAS-tsh* were heat shocked for 45 minutes at 35°C during L2, after which they were returned to 25°C for the rest of their development. Discs were dissected from late L3 larvae.

tsh-knock-down was achieved by expressing a UAS-tshRNAi transgene [flies kindly provided by Georg Dietzl and Barry Dickson (IMBA, Vienna)], which carries an inverted repeat targeting the sequence GGCGGTGCTGCTGGTAGTGGCGCAGTGACCAAAG-CGAGGCATAACATTTGGCAATCGCACTGGCAAAACAAGGGT-GTGGCCAGTTCGGTGTTCAGATGTGTGTGGTGCAAGCAGAG-TTTCCCTACCCTGGAAGCCCTGACCACCCACATGAAGGACA-GCAAGCATTGCGGCGTGAATGTACCACCTTTTGGTAATCTG-CCAAGCAACAATCCTCAGCCGCAGCACCACCATCCAACTCC-ACCTCCACCGC in the *tsh* cDNA. UAS-tshRNAi was induced in two ways: (1) in clones, induced at 28-72 hours AEL in larvae of the genotype *yw hs-Flp122; act>y+>GAL4, UAS-lacZ;* UAS-tshRNAi; or (2) uniformly in the developing eye disc of larvae of the genotype ey-GAL4; arm-GAL4/UAS-tshRNAi. Larvae containing only one of these two GAL4 drivers plus the UAS-tshRNAi transgene gave rise to normal flies.

Immunostaining

Antibodies used were rabbit anti-β-galactosidase (Cappel), mouse anti-β-galactosidase (Sigma), guinea pig anti-Hth (Casares and Mann, 1998), rabbit anti-Tsh (Wu and Cohen, 2000), rabbit and rat anti-Ey (gifts from P. Callaerts), and rabbit anti-Ato (Jarman et al., 1993). The monoclonal antibodies against Armadillo (Riggleman et al., 1990), Dac (Mardon et al., 1994), Eya (Bonini et al., 1993), Elav (7E8A10; (O'Neill et al., 1994) and mouse 22C10 (Fujita et al., 1982) were obtained from the DSHB, University of Iowa. Anti-mouse, anti-rabbit and anti-guinea pig secondary antibodies, conjugated with Alexa 488, 568 or 647 are from Molecular Probes, and anti-rat secondary antibodies conjugated with FITC, Cy3 or Cy5 are from Jackson Laboratories. GFP signal was directly detected. Images were obtained with a SP2-AOBS Leica confocal system and processed with Adobe-Photoshop.

X-Gal histochemical staining

Late MS1096-Gal4/UAS-*lacZ* pupae were dissected and processed as described previously (Casares and Mann, 2000).

Results

tsh expression is restricted to the main epithelium of L2 eye discs

tsh transcription and protein distribution throughout eye disc development were assayed using the reporters *tsh*-Z and *tsh-gal4*, and an anti-Tsh antiserum (Fig. 1), respectively. *tsh* is not expressed in embryonic eye-antennal disc primordia (Fasano et al., 1991) (Fig. 1A). *tsh-gal4* expression is very similar, both spatially and temporally, to that of *tsh-Z*, although a bit patchy (not shown). Therefore, we describe only *tsh-Z*. In L1 discs, which show no signs of morphological differentiation, *ey* expression is widespread in both prospective PE and ME layers. At this stage, no or very weak *tsh-Z* expression is detected (Fig. 1B). Expression of *tsh* first begins in early L2 discs at the time of Eya induction (Kenyon et al., 2003), specifically in the ME layer (Fig. 1C). It is around this time that the morphologies of the PE and ME epithelia become distinct (compare Fig. 1B,C). In early third instar discs the eye primordium expresses both *ey* and *tsh* (Bessa et al., 2002; Halder et al., 1998). The *tsh* expression domain is confined to the ME, while *ey* is also expressed along the disc margin and PE (Fig. 1E) (Bessa et al., 2002). Before retinogenesis, *tsh* expression domain is subdivided into two subdomains: in the

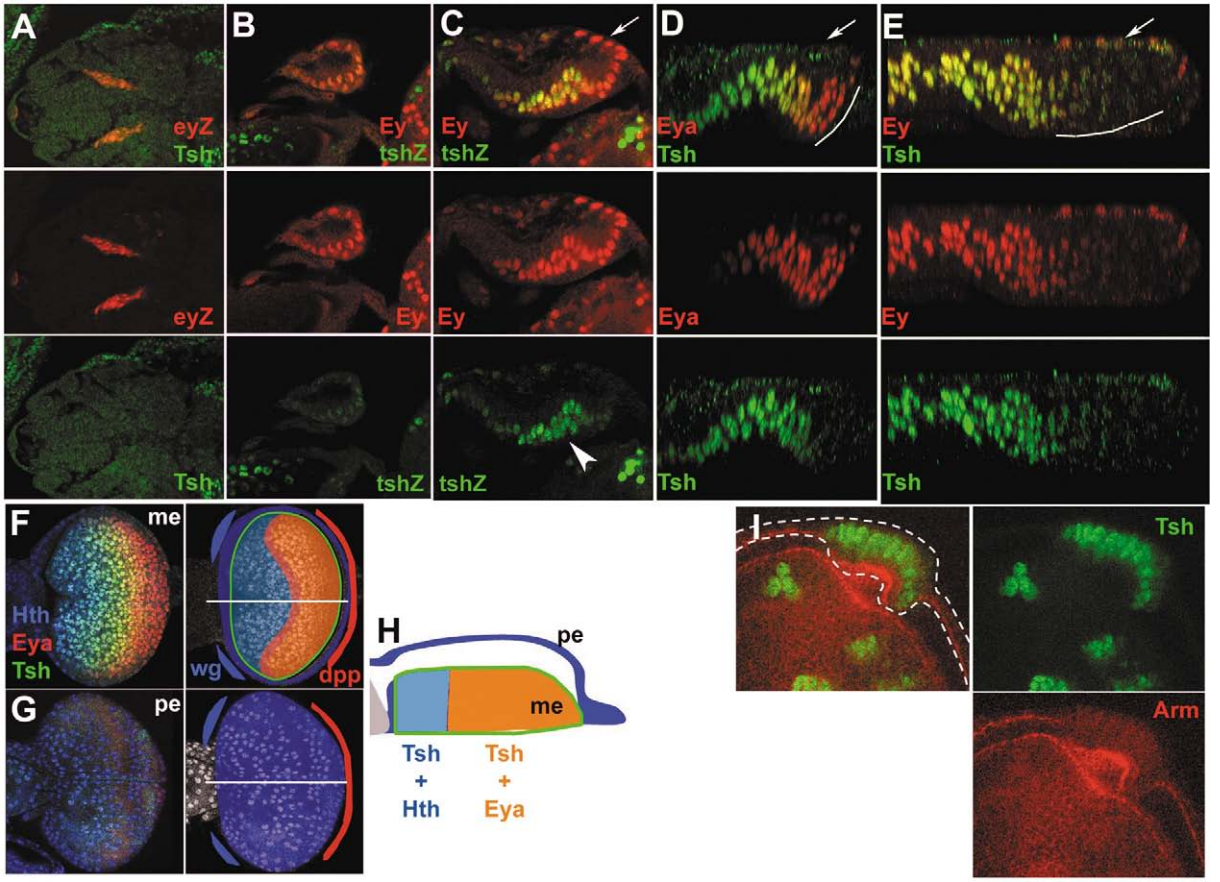
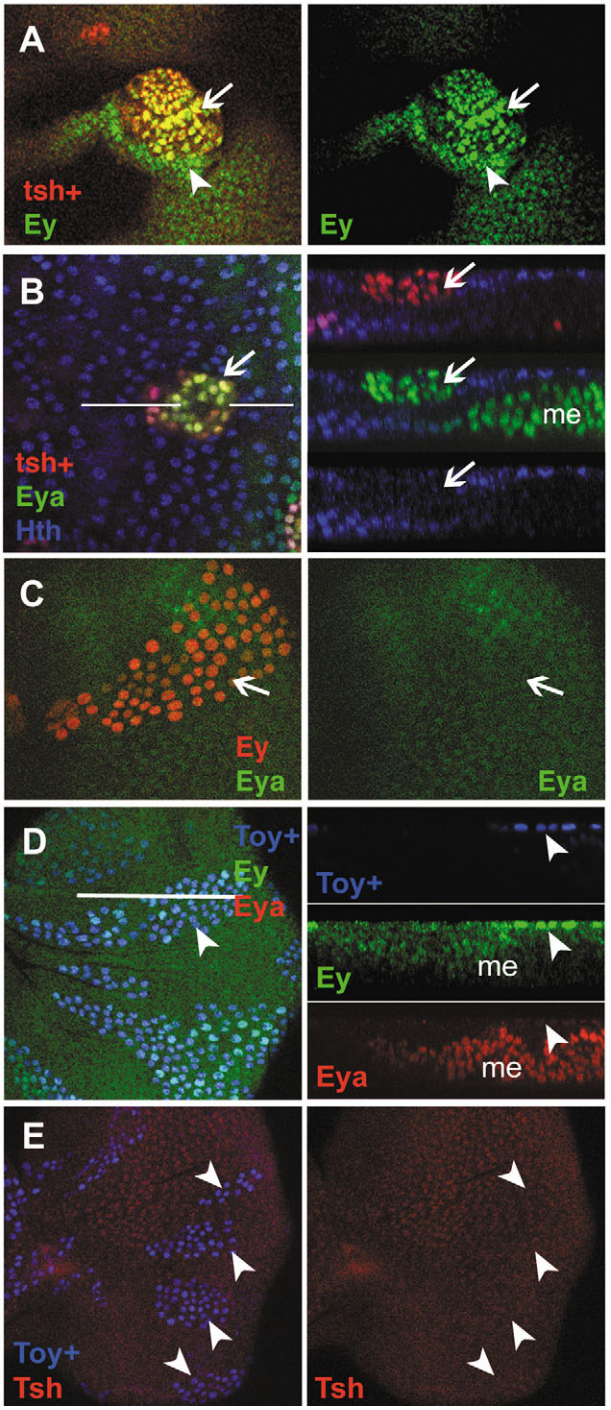


Fig. 1. *tsh* expression in the eye disc starts during L2 and is restricted to the columnar ME. In all images, anterior is towards the left. (A) Dorsal view of a late ey-Z embryo. The eye disc primordium, visualized with an *ey*-Z reporter (anti-β-galactosidase, red) does not express *tsh* (anti-Tsh, green). (B-E) vertical optical sections of L1 (B), L2 (C) and early L3 (D,E) eye discs; the PE is marked by an arrow. (D,E) Region where the retina is already differentiating is marked by the curved line. Merged images and separate channels are shown. Ey (anti-Ey, red) is expressed in both eye disc layers during the whole development of the disc (B,C,E). *tsh*, which is monitored by a *tsh*-Z reporter (anti-β-galactosidase, green), is not expressed in L1 discs (B), but becomes upregulated in L2 discs in the columnar layer (C; arrowhead) and maintained there in early L3 discs (E). (D) Tsh expression overlaps Eya in the ME (anti-Eya, red). (F,G) Confocal images through the ME (F) or PE (G) layers of early L3 discs, stained for Hth (blue), Tsh (green) and Eya (red). (F) *tsh* is expressed only in the main epithelium (F), where it is co-expressed with Hth in an anterior subdomain (cyan) and with Eya in a posterior one (yellow-orange). In the scheme on the right, the domain of *tsh* expression is outlined in green, and the anterior and posterior subdomains are colored in cyan and orange, respectively. The approximate domains of *wg* and *dpp* expression at this stage are depicted as the blue and red bars, respectively. (G) In the PE of a similar stage disc, neither *tsh* nor *eya* is expressed. This is represented in the scheme on the right. (H) Idealized vertical cross-section through the line in F and G. The color codes as in F and G. pe, peripodial epithelium; me, main epithelium. (I) *tsh*-expressing clones in the disc margin or PE induce the columnarization of the cells. Tangential confocal section through a disc, showing a *tsh*+ clone (marked with anti-Tsh, green) in the dorsal PE. The membranes are labeled with Arm (red). *tsh*+ cells overproliferate and exhibit a columnar morphology, contrasting with the more squamous morphology of neighboring, Tsh non-expressing, cells. The broken line in the merged image delineates the tissue.

anterior part of the eye primordium, *tsh* expression domain overlaps that of *hth,* and in its posterior part it overlaps *eya* (Fig. 1D,F,H). At this stage, *tsh* is still absent from the PE (Fig. 1G,H). In late L3 discs, *tsh* expression remains co-expressed with *ey* in cells anterior to the MF (Fig. 1E).

The specification of the eye primordium within the ME of L2 discs correlates with *tsh* expression, suggesting that *tsh* might be involved in this specification. If this is the case, we expect that ectopic *tsh* expression will transform PE cells into an eye primordium, characterized by: (1) columnar morphology of the epithelial cells; (2) eye-specific gene expression; and (3) eye-specific response to key signaling pathways. We have analyzed each of these points in turn by inducing the expression of *tsh* in marked clones of cells in the PE.



tsh induces a columnar cell shape in peripodial cells

Cells expressing *tsh* in the margin of the disc or in the PE overproliferate (Bessa et al., 2002; Singh et al., 2002) (Fig. 1I), adopt a columnar shape, with elongated nuclei, and are more densely packed than non-expressing cells (Fig. 1I). Some of these clones further show a sorting behavior, by which the *tsh*-expressing cells arrange themselves as hollow sacs with their apical sides pointing inwards, as monitored by expression of armadillo/β-catenin, which localizes to adherens junctions (not shown). Such a sorting behavior is usually considered to be the consequence of the cells adopting a new identity (McNeill, 2000).

tsh, but neither *ey* nor *toy*, induces *eya* expression in peripodial cells

In order to test if *tsh* is sufficient to induce eye primordium identity in PE cells, we analyzed the expression of the eye selector gene *ey*, as well as that of the early retinal genes *eya* and *Dac* in *tsh*-expressing clones. *tsh*-positive cells show increased Ey expression (Fig. 2A). In addition, PE *tsh*-expressing clones that lie close to the posterior margin activate *eya* (see Fig. 2B) and the *eya* target *Dac* (data not shown), indicating that these cells adopt an eye primordium-like fate. PE clones overexpressing *ey* are not able to induce *eya* (Fig. 2C), neither are similar *toy*-expressing clones, in which *ey* expression is upregulated (Fig. 2D). In these PE clones, *tsh* expression is not induced (Fig. 2E). Therefore, we conclude that neither *ey* upregulation nor the joint overexpression of *toy* and *ey* are able to re-specify the peripodial epithelium. In addition, overexpression of *eya* in PE clones do not turn *Dac* on either (data not shown), which reinforces the idea that PE re-specification as eye primordium occurs only if *tsh* is expressed.

The expression of *tsh* in PE makes cells respond to Dpp and Wg signals in an eye-specific manner

Expression of *tsh* activates *eya* expression mostly in the center and posterior half of the PE, but not in the anterior half (Fig.

Fig. 2. Overexpression of *ey* or *toy* is not capable of altering PE fate. Anterior is towards the left in all panels, and dorsal is upwards (except for *z*-sections in B and D). (A,B) *tsh*-expressing PE clones (arrows) upregulate Ey expression (A) and Eya (B). In some *tsh*-expressing clones, the upregulation of *ey* is not strictly cell-autonomous, as some cells adjacent to the expressing clone also increase Ey signal (A, arrowhead). (B) Confocal *z*-section through the white line is shown, demonstrating the PE location of the tsh+ clone (arrow), the cell-autonomous activation of Eya (green) and the repression of Hth (blue). Normal Eya expression is seen in the ME (me). (C) An *ey*-expressing clone in the peripodial epithelium (red), though, does not activate *eya* expression (green) or alter the morphology of the peripodial cells. (D,E) *toy*-expressing PE clones (toy+; arrowheads), marked with *lacZ* (blue). (D) *toy*-expressing PE cells (blue) upregulate Ey expression, while that of Eya remains absent. Separate channels of a *z*-section through the clone marked with the arrowhead are shown on the right. Ey and Eya are detected normally in the ME (me). The white line indicates the approximate location of the section. (E) In PE *toy*-expressing clones (arrowheads), Tsh is not upregulated.

3B). Clones in this anterior region retain the expression of *hth* (Fig. 3G), which is normally expressed in all PE cells (Pai et al., 1998; Pichaud and Casares, 2000). As *dpp* and *wg* are expressed in the domains of the posterior and anterior discs, respectively, we reasoned that these differences in the response of *tsh*-expressing cells could be the result of these signaling pathways acting differently in anterior and posterior domains of the PE.

To test this hypothesis, we first checked the response of normal PE cells to variations in both *wg* and *dpp* pathways. Clones where the *dpp* pathway was hyperactivated through the expression of a constitutively active *dpp*-receptor, *thick veins* (*tkvQD*; Fig. 3A; see Fig. 3D for comparison of the effects in the ME), or blocked by removing the signal transducer *Mothers against dpp* (*Mad*; not shown), showed no induction of *eya* expression or cell morphology changes. Neither did anterior clones expressing *Axin*, a negative regulator of the *wg* pathway (Fig. 3F) or overexpressing *wg* (not shown). Nevertheless, when alterations in the *dpp* and *wg* pathways were performed in the presence of ectopic *tsh*, PE cells showed gene expression responses characteristic of the ME. Thus, whereas posterior *tsh*-expressing PE cells induce *eya* expression (Fig. 2B, Fig. 3B), *tsh*-expressing cells in which the *dpp* pathway has been blocked by removing *Mad* no longer express *eya* (Fig. 3C). Again, this is the behavior exhibited by *tsh*+ ME cells deprived of *dpp* signaling (Fig. 3E) (Curtiss and Mlodzik, 2000). Similarly, while anterior *tsh*-expressing PE cells retain *hth* expression (Fig. 3G), most clones expressing both *tsh* and *Axin* lose *hth* expression (Fig. 3H), as they do if *Axin* is expressed in the ME within the *tsh* domain (Fig. 3I). PE tsh+ tkv+ clones still fail to activate eya in anterior dorsal and anterior ventral regions (not shown), suggesting that even in these clones *wg* signaling can prevent PE re-specification. Clones of PE cells expressing *tsh*, *tkvQD* and *Axin* now activate *eya* anywhere in the disc (Fig. 3J), indicating that, in the presence of *tsh*, *wg* and *dpp* antagonize each other to regulate *eya* expression. We note, however, that the squamous to columnar cell shape change induced by *tsh* is independent of the activity of the *wg* and *dpp* pathways (Fig. 3; not shown).

These results suggest that *tsh*, when expressed in the PE, can reprogram this epithelial layer to respond to *wg* and *dpp* signals such that it develops in an eye primordium-specific manner.

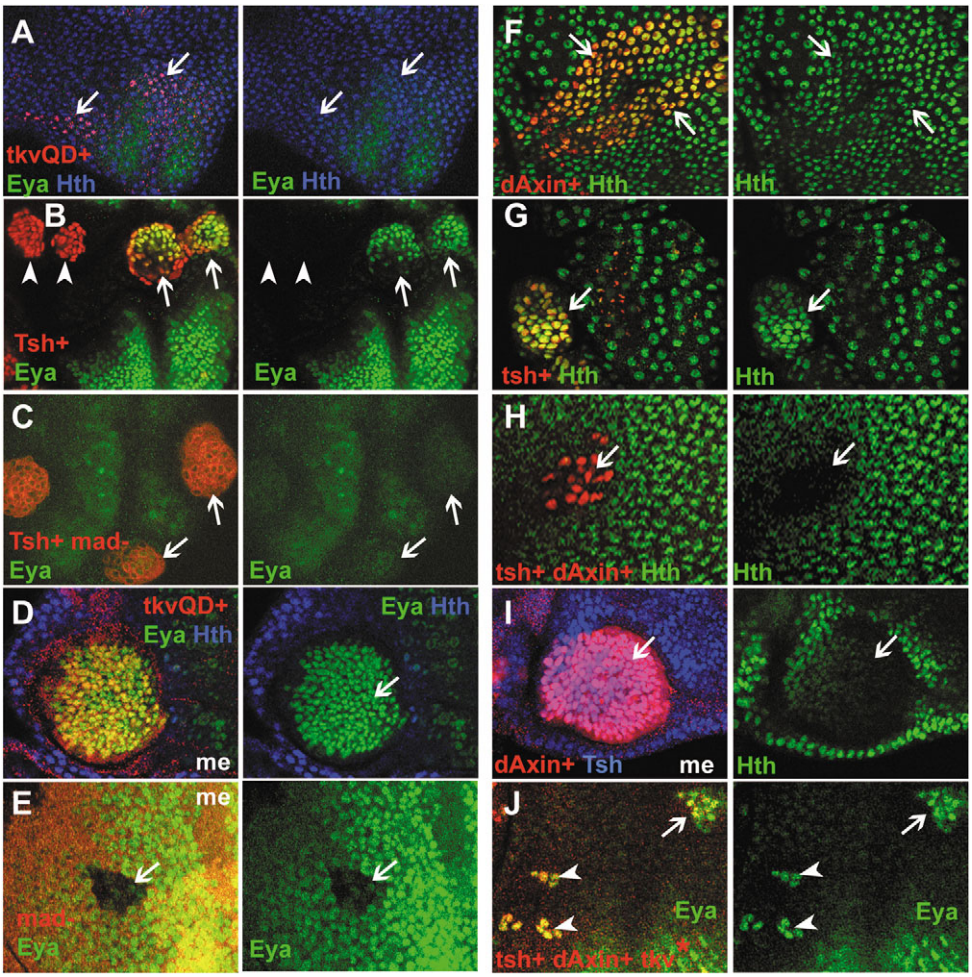


Fig. 3. *tsh* induces competence to respond to *dpp* and *wg* in peripodial cells. (A-C,F-H,J) PE clones. Anterior is towards the left, and dorsal upwards. (A) Clones expressing an activated *tkv* receptor (tkvQD+, red) do not activate Eya expression (green) or induce cell-morphological changes (arrows); in addition, Hth expression remains unchanged (blue). (B) Posterior *tsh*-expressing peripodial clones (Tsh+) induce Eya expression (arrows), but anterior ones do not (arrowheads). (C) In *tsh*-expressing clones simultaneously mutant for the *dpp* signal transducer *Mad* (Tsh+ Mad-; arrows) *eya* is never induced. (D,E) ME clones (arrows): (D) *tkvQD*-expressing clone (red) in anterior regions of the disc de-represses Eya (green; the co-expression is seen in yellow). These clones lose Hth expression (blue). (E) Conversely, a *Mad*– clone (marked by the absence of *lacZ*, in red) shows a strong reduction of Eya signal. (F) *Axin*-expressing clones (*lacZ*, red; arrows) grow normally in the PE, and do not affect Hth expression (green; overlap in yellow). (G) A *tsh*-expressing PE clone (tsh+; marked with Tsh in red; arrow) shows overgrowth, with more compact nuclei that strongly express Hth (overlap in yellow). (H) Cells in a clone co-expressing *Axin* and *tsh* (tsh+ Axin+, marked with Tsh in red; arrow) lose Hth expression and do not overproliferate. (I) An anterior ME *Axin*-expressing clone (*lacZ* in red; arrow) lying within the *tsh* domain (blue) downregulates Hth (green). Hth levels decrease towards the posterior of the clone, as Axin+ cells are farther away from the anterior *wg* expression domain. In these Axin+ clones, Tsh expression is maintained (co-expression seen in magenta). (J) PE clones expressing simultaneously *tsh*, *Axin* and *tkvQD* (marked with Tsh, red) activate Eya expression both in posterior (arrow) and in anterior (arrowheads) locations.

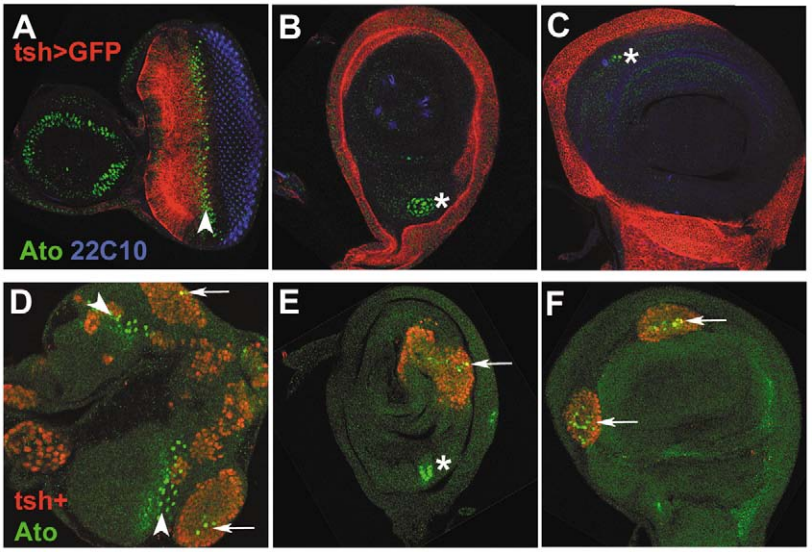


Fig. 4. *tsh* expression induces ato. (A-C) *tsh*-GAL4; UAS-GFP L3 discs (*tsh*>GFP, red) stained with anti-Ato (green) and 22C10 (neural marker, blue). (A) In the eye disc, *tsh*>GFP expression extends up to the MF (arrowhead) and overlaps *ato*. Posterior to the MF, 22C10 marks differentiating photoreceptors. (B,C) *tsh*-GAL4; UAS-GFP is expressed in proximal domains in both leg (B) and wing (C) discs. (B) Leg disc showing Ato expression in a cluster of chordotonal organs (asterisk in B and E). (C) Wing disc showing a small cluster of Ato+ cells in the ventral anterior hinge region (asterisk). (D-F) In *tsh*-expressing clones (tsh+, red) some cells turn on Ato expression in the eye disc (D), leg (E) and wing (F) discs (arrows). The arrowheads in D indicate the *ato* expression at the endogenous MF.

tsh overexpression induces *ato*, a retinal proneural gene

Retinal differentiation starts at the posterior of the eye primordium, and depends on the expression of *hh* in the adjacent posterior margin cells. A key step in the retinal 'triggering' is the induction of *ato* by *hh* (Dominguez and Hafen, 1997). *ato* is expressed in a stripe of cells just abutting the MF (Fig. 4A). In the MF, *ato* expression becomes restricted first to evenly spaced proneural clusters and then to individual R8 photoreceptor (Jarman et al., 1994). Although the two available antisera to detect Ato and Tsh are both made in the same species, precluding a direct co-expression analysis, two lines of evidence indicate that *tsh* and *ato* expressions overlap at the MF. First, *tsh* and *ey* expression strictly coincide, and *ey* and *ato* overlap at the MF (data not shown); therefore, by correlation, *tsh* and *ato* overlap. Second, a *tsh*-GAL4 reporter also overlaps *ato* at the MF (Fig. 4A). To test whether *tsh* was also able to convey an eye-specific proneural competence, we analyzed the expression of *ato* in *tsh*-expressing eye disc cells. Some *tsh*-expressing clones show autonomous *ato* expression in a salt-and-pepper pattern (Fig. 4D). Surprisingly, this *ato* induction is not disc specific (Fig. 4B,E,C,F). This might be explained if *tsh* endows cells with a proneural potential.

tsh expression can re-specify PE as eye primordium, but retinal differentiation proceeds only if this expression is transient

Despite the fact that sustained expression of *tsh* in clones of PE cells leads to gene expression and morphological changes that are characteristic of eye primordium cells, these cells failed to differentiate into retina (Bessa et al., 2002). We reasoned that this failure might be related to sustained expression of *tsh*, as in our clones, *tsh* expression is driven by an ubiquitous and constitutive tubulin-GAL4 promoter. This does not recapitulate the normal situation, where *tsh* expression is dynamically turned off as retinal differentiation commences at the ME (Bessa et al., 2002; Fasano et al., 1991). To test this point, we induced a pulse of *tsh* expression by giving a heat shock to L2 larvae of the genotype *hs-Gal4; UAS-tsh*. We estimated that this treatment induces Tsh levels that are between two to three times the endogenous ones (data not shown). This transient *tsh* expression now results in the development of distinct clusters of 22C10-positive photoreceptors in the PE (Fig. 5A,B). This PE-eye development is autonomously induced by *tsh*, as we verified in marked clones in which the expression of *tsh* was modulated during development by the use of a temperature-sensitive form of the GAL4 repressor GAL80 (data not shown).

In addition, using the MS1096 and MD705 GAL4 promoters, both expressed in the margin and PE of the L3 eye discs (Fig. 5C,E), ectopic eyes are induced (Fig. 5D,F,G; see Fig. 5I for a description of the adult derivatives of the MS1096-expressing cells). Accordingly, in these discs, the clusters of 22C10-positive photoreceptors develop in regions in which the expression of the GAL4 source has been turned-off as the result of fate re-specification, and therefore where *tsh* (and *hth*) is no longer expressed (Fig. 5F, insets; not shown). Similarly driven *ey* expression in the PE, as expected, does not result in ectopic eye development (Fig. 5H), even if it is very efficient in eye-induction in other body places (see Fig. 5J). Therefore, these results indicate that *tsh* expression can induce eye primordium identity, but terminal differentiation proceeds only if *tsh* expression is subsequently turned off.

Knocking-down *tsh* function results in reduced eyes

To analyze an early role of *tsh* during eye development, we reduced *tsh* function by expressing an UAS-tshRNAi transgene in early discs. To drive this tshRNAi construct, we used a combination of GAL4 drivers: *ey*-GAL4, which is expressed in eye discs from L1, and the ubiquitous *arm*-GAL4, to globally increase the levels of expression of the RNAi construct (see Materials and methods). *ey*-GAL4; *arm*-GAL4/UAS-tshRNAi flies show a variable degree of eye reduction (up to 75%; Fig. 6A,B). To check for the efficiency of the UAS-tshRNAi, we drove its expression in clones, which allow the comparison of Tsh levels between RNAi-expressing cells and wild-type surrounding ones. In these clones, we detect a consistent, but variable, reduction of Tsh immunoreactivity (Fig. 6C,D), ranging from strongly reduced to almost normal levels (Fig.

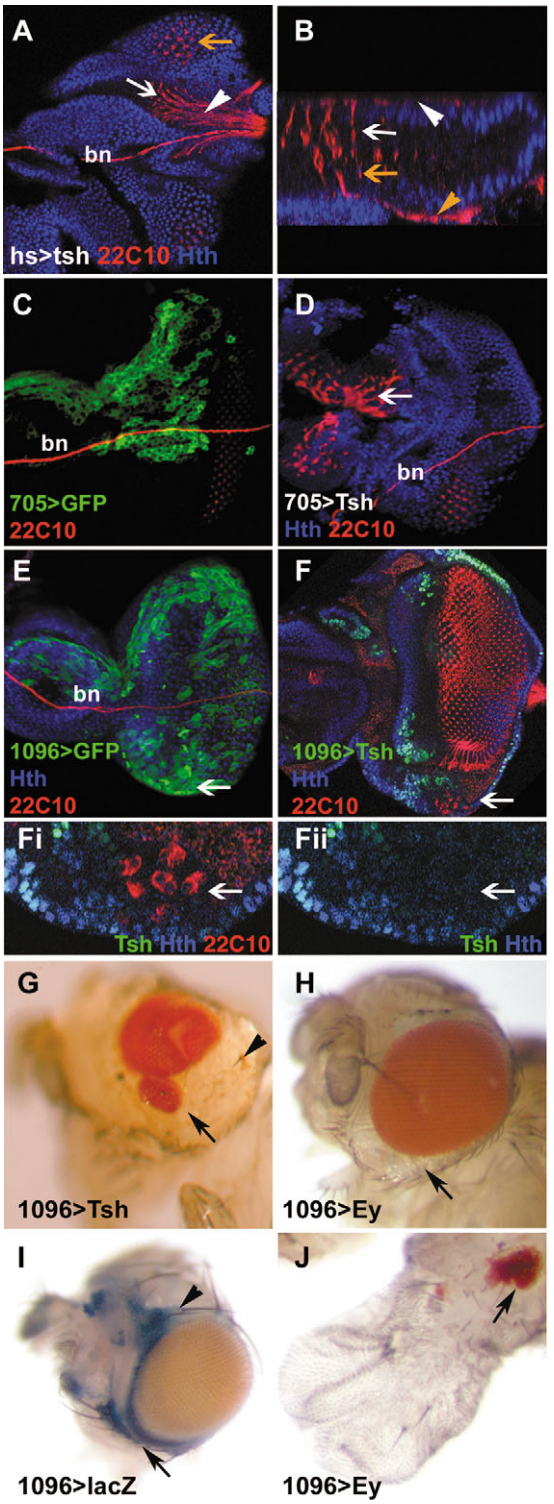


Fig. 5. Transient expression of *tsh* in the PE results in ectopic eye development. Anterior is towards the left in all images. Except for the cross-section in B, dorsal is upwards. (A,B) L3 eye disc of a *hs-Gal4; UAS-tsh* larvae, in which *tsh* was transiently induced during L2 through a heat-pulse, shows clusters of 22C10 (red) photoreceptors on the PE (marked by Hth nuclei, blue). (A) Focal plane through the PE showing the axons (white arrowhead) and cell bodies (white arrow); the ME photoreceptor cell bodies are also seen in this plane (orange arrow). (B) Confocal *z*-section through the same disc where the normal (orange) and the *tsh*-induced PE (white) photoreceptors (arrows) plus associated axons (arrowheads) can be distinctly seen. (C,E) Expression patterns of MD705-GAL4 (705>GFP) and MS-1096-GAL4 (1096>GFP) in late L3 eye discs. (C) MD705-GAL4 drives expression along the dorsoanterior margin and PE. (E) MS1096-GAL4 in both dorsal and ventral PE and margin of the eye disc, although more strongly ventrally. Fate maps (Haynie and Bryant, 1986) indicate that the dorsoanterior disc margin and PE give rise to dorsal anterior head capsule (including the frontal, vertical and post-vertical bristles) while anteroventral margin and PE develops into anteroventral head capsule (including the vibrissae, post-gena and lower post-occipital bristles). (D) MD705- and (F) MS1096-driven *tsh* expression results in PE photoreceptor differentiation (arrow). In 1096>Tsh discs, the region where these photoreceptors develop loses *tsh* and *hth* expression (close-up in Fi and Fii). (G) MS1096-driven *tsh* expression results in ventral ectopic eyes (arrow) that replace ventral head structures (loss of postgenal structures) and in small dorsal patches of photoreceptors normally accompanied by a bristle (arrowhead). (H) MS1096-driven expression of *ey* does not induce ectopic eyes in equivalent positions (arrow) in the head, although it does so in the wing hinge (J), where *tsh* is present (Bessa et al., 2002). (I) Expression of MS1096 detected by X-Gal histochemistry in MS1096-GAL4/UAS-lacZ adult heads. Signal is detected surrounding the eye in the dorsoanterior (arrowhead) and ventroanterior head capsule (arrow), in agreement with the fate map of the corresponding PE domains in eye discs. Bolwig's organ nerve (bn) runs along the peripodial layer of the eye disc and is detected by 22C10.

6E,F). Therefore, the overexpression of the tshRNAi is causing a hypomorphic condition for *tsh*. Flies containing tshRNAi-clones, which were unmarked in the eyes, often showed reduced eyes with abnormal morphology (not shown). These data indicate that *tsh* is required for normal eye development, as the early reduction of its function seriously compromises eye development, in agreement with previous results (Singh et al., 2002).

Discussion

The integration of selector genes and signaling information leads to the activation of organ-specific genetic programs. In some cases, the expression domains of such selector genes are larger than the final organ primordium whose identity they define. This is the case during the specification of the eye primordium in *Drosophila*, which is singled out as part of a larger territory of *ey*- and *toy*-expressing cells (Pappu and Mardon, 2004). Therefore other factors must exist helping to refine which cells ultimately comprise the final organ primordium. In this paper, we present evidence that, during the development of the eye disc, *tsh* contributes to specifying the eye primordium versus the PE. Thus, tsh is expressed only in the ME and, if misexpressed, re-specifies PE by inducing cell morphology and gene expression changes, and reducing *tsh* function results in impaired eye development. *tsh* might accomplish its function by making the eye disc cells respond to *wg* and *dpp* signaling in an eye-specific manner.

Asymmetric expression of *tsh* underlies the specification of one epithelial layer of the eye disc as eye primordium, which alters the response of eye disc cells to Wg and Dpp

During the development of the eye disc, only cells of the ME will be specified as eye primordium. Although Wg and Dpp

signals play essential roles during eye development (reviewed by Treisman and Heberlein, 1998), PE cells are relatively insensitive to these signaling pathways, as measured by cell survival, morphology, proliferation or gene expression changes (this work) (Baena-Lopez et al., 2003). Here, we show that *tsh* starts being expressed in the ME around the time when the eye primordium is specified, and that *tsh* has the potential to re-direct eye disc PE cells towards eye development, an ability the eye selector genes *toy* and *ey* do not have on their own. Our results indicate that the PE can be re-specified by *tsh* throughout most of the life of the larva. Thus, *tsh*-expressing clones induced during L1 and L2 induce *eya* and *Dac* expression (Fig. 2; data not shown). The transient expression of *tsh* during L2 (Fig. 5; data not shown), or its induction by Gal4 drivers active during late-L2/L3, results in ectopic PE eyes.

We propose that one way in which *tsh* might be involved in eye fate specification is by altering the response of eye disc cells to Dpp and Wg signals. The molecular mechanisms by which *tsh* might achieve this during eye development remain to be further investigated, but they might be similar to those already described during embryogenesis, where Tsh modulates *wg* and *dpp* pathways directly interacting with Armadillo, the *wg* signaling transducer, and with Brinker, a transcriptional repressor of the *dpp* pathway (Gallet et al., 1999; Gallet et al., 1998; Jazwinska et al., 1999; Rushlow et al., 2001; Saller et al., 2002).

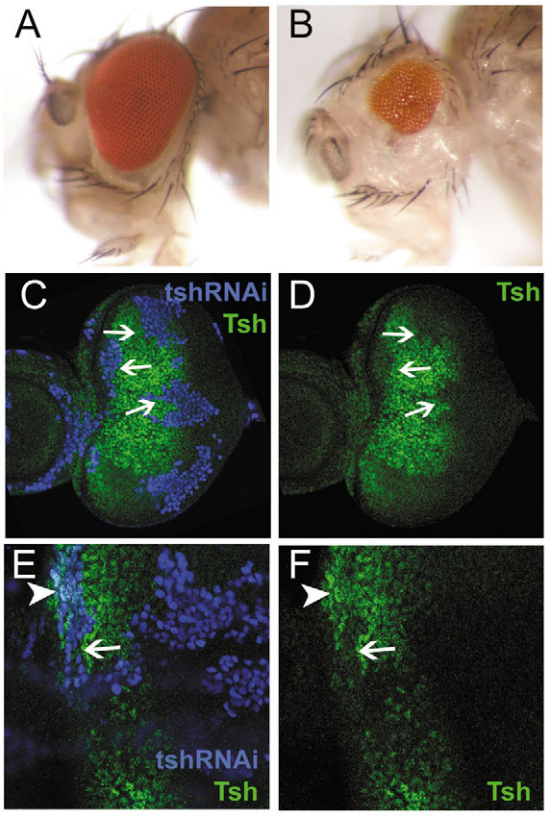


Fig. 6. Reduction of *tsh* function results in small eyes. (A,B) Lateral views of (A) wild-type and (B) *ey*-GAL4; *arm*-GAL4/UAS-tshRNAi adult heads. (C-F) Clones expressing tshRNAi, positively marked with *lacZ* (blue) and Tsh (green). (C,D) Late L3 disc showing autonomous loss of Tsh-immunoreactivity (arrows). (E,F) Close up of a dorsal clone in which cells have different levels of Tsh immunoreactivity: none (arrow) or normal (arrowhead).

In the eye disc, the cells specific response to *wg* and *dpp* enabled by *tsh* is superimposed onto the expression of eye-selector genes. Such combination of factors in turn would specify the eye primordium. The fact that Tsh and Ey have the potential to interact directly (Bessa et al., 2002) makes it possible for Ey to tether Tsh-containing transcriptional complexes to eye-specific targets genes.

We have also observed that *ato* expression is induced in some of the *tsh*-overexpressing eye-disc cells. Therefore, *tsh* has the potential not only to sensitize eye disc cells to *wg* and *dpp* signals, but also to make them prone to neural differentiation. Niwa and co-workers (Niwa et al., 2004) have recently shown that *dpp* and *wg* regulate the spatial activation of *ato* to position several adult sensory organs, including the eye, within the corresponding imaginal discs. This mechanism for positioning *ato* would define, according to these authors, a sensory organ prototype upon which selector genes, such as *ey*, would specify the final sensory type. Interestingly, the ectopic *ato* expression induced by *tsh* is not disc specific and, if *tsh* induction is transient, results in ectopic neurons (Fig. 5E,F). This *ato* induction might be mediated by *tsh* enabling cells to respond to *dpp* and *wg*.

Temporal regulation of *tsh* in establishing competence and allowing differentiation

Our results underlie the importance of the precise and dynamic spatiotemporal pattern of expression of *tsh*: on the one hand, *tsh* expression must be confined to the ME layer of the eye disc; on the other, and in order for eye development to proceed, *tsh* has to be first expressed in undifferentiated cells to be later turned off to allow retinal differentiation. The earlier paradox of *tsh* acting both as eye repressor and inductor, depending on the Gal4 promoters used, can now be explained as follows: Gal4 promoters that are not repressible by the gene expression changes induced upon *tsh* overexpression, such as *ey*-GAL4, will lead to sustained expression of *tsh* and, therefore, to a blockage of eye development (Manfroid et al., 2004; Singh et al., 2002). Other drivers that are turned off after *tsh* expression (i.e. MS1096, MD705, this study) will mimic the situation found in the ME (that is, on/off), and in these cases, eye development will proceed. We note that in experiments where *ey* is ectopically expressed, eyes tend to develop in the proximal parts of appendages (Bessa et al., 2002; Chen et al., 1999; Halder et al., 1998) (J.B. and F.C., unpublished) which derive from *tsh*-expressing domains in their respective imaginal discs (Azpiazu and Morata, 2000; Casares and Mann, 2000; Wu and Cohen, 2002). This correlation reinforces the idea of *tsh* as a potential eye-competence factor.

Multiple roles of *tsh* during eye disc development

Several studies have uncovered at least three roles for *tsh* during eye development: promoting proliferation (Bessa et al., 2002; Singh et al., 2002), acting as an eye repressor (Bessa et al., 2002; Singh et al., 2002) and acting as an eye inducer (Pan and Rubin, 1998; Singh et al., 2002; Singh et al., 2004). The first two roles (proliferation and eye repression) are linked to the function of the transcription factor Hth (Singh et al., 2002; Bessa et al., 2002). Thus, Tsh and Hth (together with Ey)

maintain the eye disc cells in a proliferative, undifferentiated state, which is incompatible with eye differentiation (Bessa et al., 2002; Singh et al., 2002). This state is kept as long as cells express *hth,* which is positively regulated by *wg* (Pichaud and Casares, 2000; Baonza and Freeman, 2002; Lee and Treisman, 2001; Singh et al., 2002) and repressed by *dpp* (Bessa et al., 2002). As *tsh* keeps *hth* on, sustaining *tsh* expression artificially in the disc blocks further eye differentiation (Singh et al., 2002; Bessa et al., 2002) (this work). Once *hth* is repressed by Dpp signaling close to the MF, cells enter a preproneural state, that still maintains *tsh* expression, in which *dpp* activates the expression of retinal genes such as *eya.* Our results suggest that *tsh* is required for the eye-specific interpretation of Wg and Dpp signals, and therefore for both the maintenance of proliferation and the specification of the retina. This model thus predicts that removal of the earliest *tsh* function (which corresponds to the most anterior regions in older discs) should result in eye loss due to either lack of proliferation or to the incorrect specification of the primordium; removal of later *tsh* function (which corresponds to more posterior regions of older discs) should cause a premature derepression of the eye differentiation program and excess of eye. In fact, Singh and co-workers (Singh et al., 2002) have described both phenotypes in *tsh* loss-of-function clones: eye loss and eye overgrowths. Our experiments, in which *tsh* function is reduced uniformly from early stages of eye development, agrees with an early role of *tsh* in eye specification and/or proliferation. This model of *tsh* function is further complicated by the fact that the dorsoventral genes also impinge on *tsh* function (Singh et al., 2004). Still, some *tsh*– clones showed no phenotype (Pan and Rubin, 1998; Singh et al., 2002). This might be explained by perdurance of the Tsh product, local differences in the requirement of *tsh* within the eye disc or the existence of compensatory functions.

tsh acts in parallel to *ey* in the eye-specification gene network

toy and *ey* lay atop the eye specification genetic network in *Drosophila.* However, neither Toy nor Ey is able to activate the expression of *tsh* in the PE (Fig. 2E), and *tsh* expression in maintained in *ey* mutant discs (not shown). The reverse is also true, as *tsh* upregulates *ey* expression in the eye disc (Bessa et al., 2002; Pan and Rubin, 1998), but is unable to activate its expression de novo in any other disc. This indicates that *tsh* expression is regulated independently of the *Pax6* genes in the eye disc. This situation is analogous to that of *Optix,* a *Six3* homolog, which is expressed in the eye disc independently of *ey* with a pattern reminiscent of that of *tsh* (Seimiya and Gehring, 2000). Nevertheless, *Optix* does not seem to regulate *tsh,* as ectopic expression of *Optix* in the eye disc does not trigger *tsh* expression (J.B. and F.C., unpublished). Taking into account all these results, we propose that *tsh* functions in parallel to *ey* (and probably to *toy*) as an eye competence factor.

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