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 (eya) 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 (eya) 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 overlying 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, antagonist 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
Materials and methods

Genotypes and genetic manipulations

Larvae were raised at 25°C, unless otherwise indicated. tsh́ (called in this work tsh2) (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 (Haller et al., 1995), UAS-toy (Czerny et al., 1999), UAS-tyk-Glo1 (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 (Hazelet et al., 1998), Arm-GAL4 (Tolwinski and Wieschaus, 2001) and hs-GAL4 [P[w-php]=GAL4-63hsp70.PB][89-21; 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 induced 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; tsh>y+>GAL4, UAS-toy (Zecca and Struhl, 2002); and UAS-tsSM63/TM6B stock, or from the following crosses: yw hsFlp 122; tsh>y+>GAL4; UAS-tsSM63/TM6B females to UAS-Axin A2 or UAS-tyk-Glo1; UAS-Axin A2 males; and yw hs-flp122; act>y+y+>GAL4, UAS-lacZ/CyO (Ito et al., 1997) females to UAS-ey; UAS-toy; UAS-tyk-Glo1; +; 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.

Msd́ loss-of-function clones were induced in the eye disc in larvae of the genotype ey-Flp: FRT 40A Msd́ FRT 40A arm-Z (Hazelet 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 GGCgGTCCTCGTGATGGCGCGATGACGAAAG-GAGCGGATACACTTTGGAACACTGGCACAACAGGGTGTGGCCAGTTCGGTGTCCAGATGTGTGTGGTGGCAGCAGAGTTTTCCATCCCTGGGAACGCTGACACCCACATGAGACGAGAGCAGACATTGGCGCGTAAGTACACCTGTGTTTTGTTGTAATCTG which they were returned to 25°C for the rest of their development.

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.

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**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.
tsh in eye primordium specification

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. 3I), 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.
**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.
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 eye- 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 organ-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 redirect 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 signals 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).

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. MS1906, 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, ey 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 inductor (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 preneural 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 is 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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