Signaling by the TGF-β homolog decapentaplegic functions reiteratively within the network of genes controlling retinal cell fate determination in

*Drosophila*

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SUMMARY

Retinal cell fate determination in *Drosophila* is controlled by an interactive network of genes, including eyeless, eyes absent, sine oculis and dachshund. We have investigated the role of the TGF-β homolog decapentaplegic in this pathway. We demonstrate that, during eye development, while eyeless transcription does not depend on decapentaplegic activity, the expression of eyes absent, sine oculis and dachshund are greatly reduced in a decapentaplegic mutant background. We also show that decapentaplegic signaling acts synergistically with and at multiple levels of the retinal determination network to induce eyes absent, sine oculis and dachshund expression and ectopic eye formation. These results suggest a mechanism by which a general patterning signal such as Decapentaplegic cooperates reiteratively with tissue-specific factors to determine distinct cell fates during development.

Key words: dpp, eyeless, Retina, Determination, *Drosophila*, Cell fate, TGF-β

INTRODUCTION

Members of the transforming growth factor β (TGF-β) superfamily are secreted signaling molecules that are critical regulators of a wide range of developmental processes in metazoans (reviewed in Hogan et al., 1994; Hogan, 1996; Massague et al., 1997). In *Drosophila*, one of the best-characterized TGF-β homologs is decapentaplegic (dpp) (Padgett et al., 1987). dpp plays essential roles throughout fly development, controlling processes such as dorsal-ventral axis establishment, midgut morphogenesis and imaginal disc patterning (Ferguson and Anderson, 1992; Posakony et al., 1990; Royet and Finkelstein, 1997; Staehling-Hampton et al., 1994; Theisen et al., 1996; Wharton et al., 1993). How different groups of cells respond uniquely to the same extracellular signal and adopt distinct cell fates is an intriguing question. Recent studies have shown that dpp signaling functions cooperatively with tissue-specific transcription factors to regulate downstream gene expression and cell fate determination during embryonic development (Grieder et al., 1997; Xu et al., 1998). In this report, we demonstrate that dpp not only is required for the normal expression of eyes absent (eya), sine oculis (so) and dachshund (dac) prior to eye morphogenesis but also functions reiteratively with the retinal determination genes to control early steps of eye development in *Drosophila*.

The adult *Drosophila* compound eye is a precisely organized structure composed of about 750 repeated units, called ommatidia. Each ommatidium contains eight photoreceptor cells and a dozen of accessory cells (Tomlinson and Ready, 1987a,b). The adult eye is derived from a monolayer of cells in the larva, called the eye imaginal disc. Differentiation of all cell types occurs progressively from posterior to anterior across the eye disc (Wolff and Ready, 1993). At the beginning of the third instar larval stage, cells at the posterior margin begin to organize into ommatidial precursors. This developmental process is synchronized by a wave of changes termed the morphogenetic furrow (MF), characterized by alterations in cell shape, cell cycle and patterns of gene expression (Ma et al., 1993; Ready et al., 1976). As the MF sweeps across the eye disc, photoreceptor differentiation is left in its wake. Thus, the MF generates the highly organized pattern of ommatidia in the compound eye.

Several lines of evidence indicate that dpp patterns the eye imaginal disc by controlling MF initiation. First, dpp is expressed along the posterior and lateral margins of the second instar eye imaginal disc, well before the onset of MF movement (Blackman et al., 1991). Second, in flies carrying the hypomorphic eye-specific dpp<sup>blk</sup> mutation, the MF fails to initiate from the ventral margin of the eye disc (Chanut and Heberlein, 1997a,b; Treisman and Rubin, 1995). Third, loss-of-function mutations in mothers against dpp (mad), a...
downstream nuclear effector of the dpp signaling pathway, block MF initiation (Sekelsky et al., 1995; Wiers dorff et al., 1996). Finally, misexpression of dpp in the eye disc induces ectopic MF initiation from the anterior margin (Chanut and Heberlein, 1997a,b; Pignoni and Zipursky, 1997). While dpp signaling is clearly required for normal patterning of the eye imaginal disc, its role in retinal cell fate determination prior to MF initiation has not been explored.

If dpp signaling contributes to the determination of retinal cell fates, this must occur by the combined action of dpp and other genes that are more specific to eye development. A group of four genes that may provide specificity to dpp signaling during eye development are eyeless (ey), eya, so and dac. Recent experiments have shown that these four genes are likely to function together in a complex regulatory network to control early eye development. First, these genes all encode conserved, nuclear proteins that are essential for eye development (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Pignoni et al., 1997; Quiring et al., 1994). Second, targeted expression of either ey, eya or dac alone is sufficient to induce ectopic eye formation in many tissues (Bonini et al., 1997; Chen et al., 1997; Halder et al., 1995; Shen and Mardon, 1997). Third, the proteins encoded by these genes are likely to form one or more complexes that control the transcription of each other and presumably other downstream targets required for normal eye development (Chen et al., 1997; Pignoni et al., 1997). We will refer to this group as the ‘RD’ (Retinal Determination) genes.

To directly address the role of dpp signaling during early eye development, we have studied the relationship between dpp and the RD genes. Using the GAL4-UAS ectopic expression system, we show that ectopic eye induction by ey is observed only at places where endogenous dpp is expressed. Moreover, synergistic induction of ectopic eye formation is observed as a result of dpp and ey coexpression. We further demonstrate that ey and dpp function cooperatively to induce the expression of eya, so and dac; raising the possibility that dpp is involved in regulating the expression of these genes during normal eye development. We provide several lines of evidence to support this model. First, expression of eya, so and dac in the eye disc are greatly reduced in a dpp mutant background. Second, synergy is also observed between ey and other RD genes, consistent with a model where cooperative regulation of eya, so and dac is the molecular basis of the synergy between ey and dpp. Finally, eya and so also function cooperatively with dpp to induce dac transcription, suggesting that dpp interacts with the RD genes at multiple levels. Based on these results, we propose that dpp signaling acts synergistically with retinal determination genes to control gene expression in the eye and is therefore essential for the establishment of retinal cell fates in Drosophila.

MATERIALS AND METHODS

Drosophila genetics

All Drosophila crosses were carried out at 25°C on standard media. The dpp12 and dpp14 lines were obtained from Ulrike Heberlein and balanced over SM6-Tm6B. dpp12/14 transheterozygotes, which lack most or all dpp imaginal disc-specific function (Lecuit et al., 1996), were selected as non-Tb larvae. Eye imaginal discs were dissected from larvae 72-78 hours (second instar) and 96-102 hours (third instar) after egg laying. Sibling SM6-Tm6B larvae were dissected at the same time as controls. UAS-ey, UAS-dac21m5m4, UAS-eya and UAS-so transgenic flies were previously described (Halder et al., 1995; Pignoni et al., 1997; Shen and Mardon, 1997). UAS-eya and UAS-so were obtained from Francesca Pignoni and Larry Zipursky. The UAS-dpp line used in this study was a generous gift of Denise Nellen and Konrad Basler (Nellen et al., 1996). UAS-lacZ flies were obtained from the Bloomington Stock Center. Flies carrying multiple combinations of these transgenes were generated through chromosome recombination using eye color as an initial selection. Genotypes were later confirmed using the polymerase chain reaction (PCR) and primers specific for each gene as described previously (Chen et al., 1997).

Scanning electron microscopy

Samples for scanning electron microscopy and histological sections were prepared as previously described (Shen and Mardon, 1997).

Immunohistochemistry

Imaginal discs were dissected and stained with anti-Elav, anti-Dac, anti-So and anti-Eya as previously described (Chen et al., 1997; Halder et al., 1998; Mardon et al., 1994). dpp expression was assayed using the BS3.0 lacZ reporter (Blackman et al., 1991). In all cases, at least 17 discs were examined for each genotype. All discs were mounted in 80% glycerol in PBS.

In situ hybridization

RD gene transcripts were detected by in situ hybridization using digoxigenin-labeled RNA probes. cDNA clones were generous gifts.
of W. Gehring (eya), N. Bonini (eya) and L. Zipursky (so). Imaginal discs were fixed with 4% formaldehyde in PBS for 20 minutes on ice and then 4% formaldehyde in PBS with 0.6% Triton X-100 for 15 minutes at room temperature. After washing in PBT (PBS + 0.1% Triton X-100), discs were treated with proteinase K (5 μg/ml in PBT) for 5 minutes and fixed in PBS containing 4% formaldehyde and 0.2% glutaraldehyde for 20 minutes at RT. Hybridization was carried out at 55°C in 5x SSC for 48 hours with the probe concentration at 1 ng/μl. After extensive washing, imaginal discs were incubated overnight with alkaline phosphatase-conjugated goat anti-digoxigenin antibody (1:1000, Boehringer) at 4°C. The color reaction followed standard protocols (Genius Kit, Boehringer). Discs were mounted in 80% glycerol in PBS. A more detailed protocol is available upon request.

RESULTS

Although previous studies have shown that dpp signaling is important for Drosophila eye development, the mechanism of its function during early stages of this process is not clear. During the course of our studies of ectopic photoreceptor induction, we noticed that there was a tight correlation between the location of ectopic eyes and the endogenous pattern of dpp expression. In particular, the dpp-GAL4 driver is the most efficient means of retinal induction by any of the RD genes (unpublished observations) and ubiquitous ey expression induces downstream genes only in the vicinity of the anteroposterior (AP) compartment boundary of discs where dpp is normally expressed (Halder et al., 1998). These results suggested that dpp signaling may be essential for the RD genes to specify retinal cell fates. We have now placed dpp in the pathway controlling early eye development using loss-of-function studies and by examining its relationship with the retinal determination genes, ey, eya, so and dac, employing the GAL4-UAS misexpression system (Brand and Perrimon, 1993) and using ectopic eye induction as an assay.

eya and dpp function synergistically to induce ectopic eye formation

dpp is normally expressed along the AP boundary of the larval wing disc (Fig. 1A). The GAL4 line 30A drives gene expression in a ring that surrounds the wing pouch, which will become the wing blade in the adult (Cohen, 1993). The 30A ring pattern corresponds to tissue that will form the hinge of the adult wing and overlaps endogenous dpp at only two spots (Fig. 1B). When ey is misexpressed using 30A-GAL4, ectopic eye formation is induced only at these two positions, dorsal (not shown) and ventral to the pouch at the AP boundary (Fig. 1C, arrow). One explanation for this phenomenon is that dpp activity is essential for ey to induce ectopic eye development. To test this idea, we asked whether coexpression of dpp and ey was sufficient to expand the domain of ectopic retinal development induced by ey alone. Consistent with its role as a general patterning factor, misexpression of dpp alone causes overproliferation of wing disc cells but no ectopic retinal tissue induction (Capdevila and Guerrero, 1994). However, when dpp and ey are coexpressed, synergistic induction of ectopic retinal tissue is observed: photoreceptor cells are induced in the wing disc along the entire posterior-ventral pouch margin where ectopic retinal tissue is never observed by misexpressing ey or dpp alone (Fig. 1D). Ectopic photoreceptor neurons are induced by dpp and ey coexpression at both the dorsal and ventral side of the wing pouch with 100% penetrance. In contrast, targeted expression of ey alone causes ectopic photoreceptor development with only 55% penetrance dorsally and 90% penetrance ventrally (Table 1). The average size of ectopic photoreceptor clusters is significantly increased when ey and dpp are coexpressed (Fig. 1C,D). Synergy is also observed in haltere discs when ey and dpp are coexpressed (Table 1 and data not shown). These results demonstrate that
dpp is sufficient to greatly expand the domain of photoreceptor development induced by ey misexpression.

**dpp regulates retinal determination gene expression**

Since dpp and ey act synergistically to induce ectopic eye development and dpp signaling is known to directly regulate transcription of downstream genes in other tissues, we suspected that dpp regulates RD gene expression. To test this hypothesis, we examined mRNA levels of ey, eya, so and dac in a dpp loss-of-function background. ey is normally expressed throughout the entire eye disc prior to MF initiation and anterior to the furrow during MF progression (Fig. 2A,C; Halder et al., 1998; Quiring et al., 1994). In dpp12/dpp14 transheterozygotes, the eye-antennal disc is much smaller than in wild-type due to a proliferation defect and MF initiation and photoreceptor development does not occur (Brook and Cohen, 1996; Spencer et al., 1982). Nevertheless, ey mRNA is still detectable in dpp12/dpp14 mutant eye discs throughout second and third instar larval development (Fig. 2B,D). In contrast, although eya is still expressed in the ocellar region (Fig. 2F, arrow), almost no eya, so or dac mRNA is detected in dpp12/dpp14 mutant eye discs prepared from second or third instar larvae (Fig. 2E-J and data not shown). These data indicate that dpp is not essential for ey expression but is required upstream of eya, so and dac in the eye disc.

We further examined the role of dpp in the regulation of the RD genes using ectopic expression studies. dpp is not only required for eya, so and dac expression in wild-type eye discs, but targeted ey expression induces dac, eya and so only in those areas where dpp is already present (Fig. 3A-C, arrows). In contrast, although no ectopic Dac, Eya or So protein is induced by dpp alone, ectopic expression of these proteins is induced around the posterior half of the wing pouch when ey and dpp are coexpressed (Fig. 3D-F and data not shown). In addition, weak induction of so expression is also observed in the ventral anterior quadrant of the wing disc (Fig. 3F, arrow). Thus, dpp signaling enables ey to positively regulate downstream target gene expression and this may account for the synergistic induction of ectopic retinal development by ey and dpp. Although coexpression of ey and dpp synergistically induces strong expression of dac, so and eya around most of the wing pouch in the posterior compartment (Fig. 3D-F), photoreceptor cells are induced away from the AP boundary only in the ventral half (Fig. 1D). We reasoned that the failure of ey and dpp to drive photoreceptor development in the dorsal half of the wing pouch may be due to insufficient dac gene induction in that area (Fig. 3D). Indeed, when ey, dpp and dac are coexpressed, ectopic retinal tissue is induced all the way around the posterior wing pouch (Fig. 4H). This result further supports the hypothesis that induction of dac is a critical component of ectopic photoreceptor induction by ey and dpp.

**ey, eya and dac act synergistically to induce ectopic eye formation**

If eya and dac are the primary downstream targets of dpp during eye development, then it should be possible to bypass the requirement for dpp and induce ectopic eye formation by overexpressing ey with eya or dac. While targeted expression of eya or dac alone driven by 30A-GAL4 is unable to induce photoreceptor development, strong synergistic induction of ectopic eye formation is observed when ey is coexpressed with either dac or eya (Fig. 4A,D and data not shown). Specifically, domains of ectopic photoreceptor induction are significantly expanded and appear with complete penetrance both dorsal and ventral to the wing pouch. Similarly, strong synergy between ey and dac is also observed in the haltere disc (Fig. 4B,E). Ectopic photoreceptor induction at the dorsal side of the haltere disc increases from 5% of discs examined with ey alone to 71% when ey and dac are coexpressed (Table 1).

The phenotypes observed in adults as a result of ey and dac or eya coexpression are consistent with those observed in imaginal discs. Specifically, no ectopic eye formation is induced by either dac or eya alone driven by 30A-GAL4 (data not shown). When ey alone is misexpressed, ectopic eyes are never found on the haltere and only small patches of retinal tissue appear on the ventral side of the wing hinge at 20% penetrance (Fig. 4C). In contrast, when ey and dac are coexpressed, large ectopic eyes are observed on the wing and haltere hinges with complete penetrance (Fig. 4F). Clear ommatidial structures are observed not only on the ventral side but also on the dorsal side of the wing hinge and the structure of the lens and interommatidial bristles of these ectopic eyes is similar to wild type (Fig. 4I and data not shown). Similar results are also observed when ey and eya are coexpressed (data not shown). Thus, although there is clear synergy between ey and dac or eya, ectopic photoreceptor induction in both imaginal discs and adults is still limited to the vicinity of the AP boundary and the source of dpp signaling. Moreover, photoreceptor differentiation is still restricted to the vicinity of the AP boundary when ey, dac, eya and so are simultaneously induced by 30A-GAL4, indicating that dpp and ey must regulate other essential targets in this process (data not shown).

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**Table 1. ey, dpp and dac act synergistically to direct ectopic retinal development**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>UAS-ey</th>
<th>UAS-ey, dpp</th>
<th>UAS-ey, dac</th>
<th>UAS-ey, dpp, dac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc type</td>
<td>Wing</td>
<td>Haltere</td>
<td>Wing</td>
<td>Haltere</td>
</tr>
<tr>
<td>Total scored</td>
<td>51</td>
<td>19</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>No neurons</td>
<td>10%</td>
<td>47%</td>
<td>0%</td>
<td>24%</td>
</tr>
<tr>
<td>Ventral only</td>
<td>35%</td>
<td>5%</td>
<td>100%</td>
<td>17%</td>
</tr>
</tbody>
</table>

Wing and haltere imaginal discs were dissected from late third instar animals carrying the GAL4 driver 30A (Brand and Perrimon, 1993) and all seven combinations of UAS-ey, UAS-dpp and UAS-dac transgenes. No ectopic neural development, as judged by anti-Elav staining was observed with UAS-dac or UAS-dpp, either alone or together (data not shown). Ectopic neurons were observed with the other four combinations of transgenes. No disc was found to contain ectopic neurons only at the dorsal position. The percentage of imaginal discs of each type (wing or haltere) with one of three phenotypes observed (no ectopic neurons, ventral neurons only, or both ventral and dorsal ectopic neurons) are shown.
**dpp functions reiteratively to regulate RD gene expression**

The results presented above demonstrate that dpp can cooperate with ey to regulate RD gene expression and photoreceptor induction. Previous studies have suggested that the initiation of transcription of these genes can be fitted into a primarily linear pathway with ey most upstream, eya and so in the middle and dac further downstream (Chen et al., 1997; Halder et al., 1998). Thus, it is possible that dpp signaling might cooperate directly and exclusively with ey. Alternatively, dpp could interact at multiple levels within this pathway. To distinguish these two models, we tested whether dpp functions synergistically with eya and so to regulate the expression of dac. No ectopic dac expression is induced by so alone (Fig. 5A) and targeted expression of eya induces ectopic dac expression only at a single ventral spot on the AP boundary of the wing disc when driven by 30A-GAL4 (Fig. 5B, arrow). Consistent with the idea that the Eya and So proteins function cooperatively as a complex (Pignoni et al., 1997), strong synergistic induction of dac is observed when eya and so are coexpressed (Fig. 5C, arrows). However, dac expression is still restricted mainly to places where endogenous dpp is present. In contrast, when dpp is coexpressed with eya, strong dac expression is induced all along the ventral-posterior pouch margin (Fig. 5D). Moreover, ectopic Dac is detected around the entire circumference of the wing pouch as a result of dpp, eya and so coexpression (Fig. 5E). A similar result is also observed in the haltere disc (Fig. 5F). However, it should be noted that targeted expression of dpp, eya and so with 30A-GAL4 is unable to induce ectopic photoreceptor development (data not shown). Since coexpression of dpp, eya and so is sufficient to induce dac expression in places where dpp and ey cannot, we conclude that dpp interacts with the network at multiple levels to control the expression of retinal determination genes. Consistent with this interpretation, we were unable to detect induction of ey expression in response to misexpression of dpp, eya and so with 30A-GAL4 (data not shown).

**DISCUSSION**

The TGF-β homolog dpp plays critical roles during many developmental processes in Drosophila. How cells respond to the same signal in a tissue-specific manner is a fundamental question in developmental biology. During fly eye development, dpp is involved in the control of several processes, including cell proliferation, pattern formation and MF movement (Chanut and Heberlein, 1997a,b; Heberlein et al., 1993; Masucci et al., 1990; Penton et al., 1997; Pignoni and Zipursky, 1997; Royet and Finkelstein, 1997; Spencer et al., 1982). However, what role dpp plays during early eye development is not clear. In this paper, we have studied the relationship between dpp and a group of retinal determination (‘RD’) genes using both loss- and gain-of-function experiments. We demonstrate that dpp signaling interacts with the retinal determination network at multiple levels to control gene expression and retinal development in Drosophila.

**dpp functions cooperatively with ey to control Drosophila eye development**

We have shown that ey and dpp function synergistically to induce ectopic eye formation in the wing disc. Targeted expression of dpp alone is not sufficient to induce ectopic eye formation and ey alone causes ectopic photoreceptor cells only where dpp is normally expressed. In contrast, when dpp and ey are coexpressed, the domain of ectopic retinal tissue not only increases in size, but extends far away from the source of endogenous dpp. This suggests that the synergy between ey and dpp cannot simply result from overproliferation of wing disc cells caused by misexpression of dpp but must involve cooperative induction of retinal cell fates.

Several lines of evidence suggest that upregulation of RD gene expression by dpp and ey is likely to account for the synergy that we have observed. First, dpp acts upstream of dac, so and eya during normal eye development: while the initiation of dpp expression does not depend on eya, so or dac function (Mardon et al., 1994; Pignoni et al., 1997), eya, so and dac transcription is greatly reduced in a dpp loss-of-function background. Second, ectopic induction of eya, so and dac by ey alone is found only at positions where dpp is normally expressed (Halder et al., 1998 and this paper). Third, ectopic expression of dpp is sufficient to enable ey to induce eya, so and dac expression far away from the source of endogenous dpp. Finally, in the presence of high levels of Eya or Dac, ey induction of ectopic photoreceptor is expanded but still limited to the vicinity of endogenous dpp. Therefore, eya or dac can either partially bypass the requirement for dpp or broaden the sensitivity of cells to ey induction of retinal development. These data suggest a model where Dpp, a general signaling factor, is essential during early eye development to cooperate with the homeoselector protein Ey to initiate downstream gene expression and determine retinal cell fates (Fig. 6). Whether dpp signaling cooperates directly with ey or indirectly through another factor is not known.

Synergistic induction of eya, so and dac by dpp and ey is likely to account for dpp function in the control of MF initiation. During normal eye development, dpp expression is tightly regulated and is restricted to the posterior margin of the early eye disc where MF initiation takes place. This localized expression of dpp is important for determining the pattern of MF initiation: ectopic furrow initiation from the anterior margin of the eye disc is induced by misexpressing dpp (Chanut and Heberlein, 1997a,b; Pignoni and Zipursky, 1997). Moreover, misexpression of dpp at the anterior margin also upregulates the expression of all four RD genes (Pignoni and Zipursky, 1997). While ey is expressed throughout the eye disc prior to MF initiation, eya, so and dac are strongly transcribed only along the posterior margin of the eye disc where endogenous dpp is expressed. Since eya, so and dac are each required for MF initiation, we propose that a primary function of dpp signaling during early eye development is to positively regulate these genes in cooperation with ey and thus localize MF initiation to the posterior margin.

**dpp regulates RD gene expression at multiple levels**

Previous work has suggested that dpp acts as a morphogen to control development by regulating multiple transcription factors in a concentration-dependent manner (Nellen et al., 1996). For example, dpp regulates optomotor-blind (omb) and spalt in the wing disc and Distalless (Dll) and dac in the leg disc, each in spatially distinct domains (Lecuit et al., 1996; Lecuit and Cohen, 1997). dpp also controls multiple processes and presumably multiple downstream targets during normal development.
eye development. Reiterative utilization of one signaling pathway in the same tissue to determine different cell fates is likely to be a common mechanism throughout development. For example, the repeated activation of the ras pathway is required for the differentiation of each cell type in the Drosophila eye (Freeman, 1997). It is believed that ras

![Fig. 3. ey and dpp act synergistically to induce the expression of dac, eya and so. Wing imaginal discs were stained with antibodies specific for Dac (A,D), Eya (B,E) and So (C,F). When ey alone is driven by 30A-GAL4, ectopic RD gene expression is detected only at the AP boundary (A-C, arrows). In contrast, RD gene expression is induced in the posterior compartment around most of the pouch in UAS-ey, UAS-dpp/30A-GAL4 wing discs (D-F). In addition, so expression is also weakly induced in the anterior-ventral wing disc in UAS-ey, UAS-dpp/30A-GAL4 larvae (F, arrow). The wild type pattern of dac expression in the wing disc (see Fig. 5A) is largely out of the plane of focus in all panels.

![Fig. 4. ey functions synergistically with either eya or dac to induce ectopic eye formation. Only a small cluster of Elav-positive cells are observed at the ventral side of wing (A) or haltere (B) discs prepared from UAS-eyJ30A-GAL4 larvae (arrows). (C) In adults of this genotype, ectopic retinal tissue is visible only at the ventral aspect of the wing hinge. In contrast, ectopic photoreceptor cells are observed both dorsally (arrows) and in a broader region ventrally in both wing (D) and haltere (E) discs prepared from UAS-ey, UAS-dac/30A-GAL4 larvae. In adults of this genotype, large ectopic eyes are visible on both the dorsal (not shown) and ventral wing hinge (F) and the haltere (F, arrow). (G) Strong eya expression is observed in UAS-ey, UAS-dac/30A-GAL4 wing discs. (H) Further synergy is observed when ey, dpp and dac are misexpressed with 30A-GAL4, resulting in ectopic Elav-positive cells around the entire posterior half of the wing pouch. (I) Scanning electron microscopy reveals that the external morphology of ectopic eyes is similar to wild-type. Haltere discs are shown at twice the magnification as wing discs.]
signaling functions together with distinct groups of transcription factors to determine different cell fates.

In this report, we demonstrate that dpp signaling is reiteratively used to regulate gene expression within the retinal cell fate determination pathway in Drosophila. Specifically, we have shown that dpp signaling enables eya to induce strong eya, so and dac expression in the posterior, but not anterior, wing disc compartment. In contrast, dpp functions synergistically with eya and so to activate the expression of dac in both compartments. This activation of dac expression by dpp, eya and so is unlikely to result from feedback induction of eya (see below) for two reasons. First, targeted expression of eya and dpp is unable to induce dac in the anterior wing disc compartment. Second, ectopic eya transcription is not detected in response to misexpression of dpp, eya and so driven by 30A-GAL4 in the wing disc. Thus, these data suggest that dpp signaling interacts with the retinal determination pathway at at least two levels to regulate RD gene expression (Fig. 6). Interestingly, while targeted expression of dpp, eya and so with 30A-GAL4 is unable to induce eya expression or ectopic photoreceptor development in the wing disc, coexpression of eya and so using dpp-GAL4 is sufficient to induce eya expression and photoreceptor development in the antennal disc (Pignoni et al., 1997). These differences most likely reflect the unique transcriptional environments present in the specific portions of each imaginal disc tested in these assays.

Recent studies of the homeobox gene Ultrabithorax (Ubx) present another example of one gene acting at multiple levels of a regulatory pathway during development. Ubx is expressed throughout the developing haltere disc and controls the choice between wing and haltere development by regulating the expression of genes that are differentially required for wing and haltere morphogenesis and differentiation (Lewis, 1978; Weatherbee et al., 1998). Interestingly, Ubx not only regulates multiple genes that are involved in distinct aspects of wing development, it also regulates multiple genes within the same regulatory pathway. Moreover, Ubx is thought to regulate each of its target genes in the haltere independently of one another. In contrast, dpp signaling activates RD gene expression through synergistic interactions with multiple RD genes. This cooperative interaction between dpp and the RD genes may

![Gene Regulation in the Retinal Determination Network](image)

**Fig. 5.** dpp signaling functions cooperatively with eya and so to induce dac expression. Wing imaginal discs were prepared from third instar larvae and stained for Dac protein expression. (A) No ectopic dac expression is induced by misexpression of so alone driven by 30A-GAL4; only endogenous Dac protein is observed. (B) Weak ectopic dac expression is induced at the AP boundary in UAS-eya/30A-GAL4 discs (arrow). (C) eya and so misexpression induces much higher levels of Dac protein, but still near the AP boundary. (D) dac expression is strongly induced throughout the posterior compartment along the wing pouch by UAS-eya, UAS-dpp misexpression. (E,F) Strong dac expression is induced around the entire wing (E) and haltere (F) pouch when dpp, eya and so are coexpressed.

**Fig. 6.** A model for retinal cell fate determination in Drosophila. dpp functions cooperatively with the RD genes at multiple levels to control gene expression and retinal cell fate determination. dpp signaling interacts with both eya and eya/so to synergistically induce downstream gene expression. eya may also cooperate with other factors (factor X) to regulate eya and so (see text for details). In addition, there is extensive crosstalk between and within these pathways. Although the initiation of dpp does not depend on eya or so, the maintenance of dpp expression requires the activity of these genes. eya, so and dac are not required for either the initiation or maintenance of upstream gene expression but are likely to participate in the regulation of ey expression and function to lock in the retinal determination pathway.
provide specificity to dpp signaling. We propose that synergistic and reiterative use of dpp signaling within the retinal cell fate determination pathway restricts high levels of RD gene expression to the source of dpp expression. As a consequence, MF initiation only occurs at places where dpp is transcribed even though Dpp is a diffusible molecule that can regulate gene expression over many cell diameters (Lecuit et al., 1996; Lecuit and Cohen, 1997). Thus, the interaction between the RD genes and dpp signaling represents a novel example of how two distinct pathways can be actively integrated to control cell fate determination and morphogenesis.

**Retinal cell fate determination is controlled by a highly interactive network**

Our studies suggest that the pathway controlling retinal cell fate determination is complex. dpp not only interacts with the RD genes at multiple levels, but positive feedback loops also exist between dpp and the retinal determination network (Fig. 6). That is, dpp is required for the expression of eya and so and each of these genes, in turn, is essential for the maintenance of dpp expression during larval eye development (Pignoni et al., 1997). In addition, ey may interact with other factors besides dpp to initiate retinal development. When ey and dpp are coexpressed, ectoderm RD gene expression and photoreceptor differentiation are observed only in the posterior compartment of the wing disc. Therefore, other factor(s) that regulates the induction of RD gene expression by targeted ey must differ in its activity between the anterior and posterior compartments of the wing disc (Fig. 6, factor X). Such a factor is likely to be important for the regulation of eya but not dac since coexpression of eya, so and dpp is sufficient to induce dac around the entire wing pouch in both compartments. One obvious candidate for factor X is hedgehog (hh). hh, which encodes another secreted signaling molecule, is normally expressed at the posterior margin of the eye disc prior to MF initiation and is required for both dpp expression and furrow initiation (Borod and Heberlein, 1998; Dominguez and Hafen, 1997). hh is also expressed in the posterior compartment of the wing disc where ey and dpp misexpression is able to drive ectopic photoreceptor development. Whether hh regulates the RD genes remains to be determined.

Several lines of evidence suggest that retinal cell fate determination is controlled by a network that integrates a general signaling pathway with a group of tissue-specific transcription factors. First, ey, eya, so and dac are required for normal and ectopic eye development. Second, dpp is essential for MF initiation and our data suggests that dpp is very likely to be required for ectopic retinal induction as well. Third, genetic synergy is observed among nearly all pairwise combinations of these genes and greater synergistic eye induction is detected as more RD genes are coexpressed. Fourth, the encoded products of these genes are likely to function in one or more protein complexes to regulate gene expression. Finally, all members of this group of genes can cooperate to regulate the expression of each other in a complex series of positive feedback loops that may function to ‘lock-in’ retinal cell fates (Fig. 6). A prediction of this model is that the product encoded by mad, a downstream nuclear effector of Dpp (Sekelsky et al., 1995), may also participate in the regulatory complexes formed by the RD proteins.

Recent studies have shown that the RD genes are highly conserved in vertebrates (Hammond et al., 1998; Oliver et al., 1995; Quiring et al., 1994; Xu et al., 1997; Zimmerman et al., 1997). Homologs of ey, eya, so and dac are all expressed in the developing mammalian retina and the ey homolog, Pax6, is required for normal eye development in mice, rats and humans (Glaser et al., 1992; Hill et al., 1991; Oliver et al., 1995; Ton et al., 1991; Xu et al., 1997). Similarly, gene targeting in mice has shown that some members of the TGF-β family as well as other components of the signal transduction pathway are important for vertebrate eye development (Dudley et al., 1995; Nomura and Li, 1998). Finally, the expression of mouse Eya-1 and Eya-2 in the retina depend upon the activity of Pax6, suggesting that some of the regulatory relationships among the RD genes are also conserved (Xu et al., 1997). Thus, a mechanism that integrates both general patterning signals and tissue-specific factors, as shown here for Drosophila eye development, may specify cell fates throughout development and perhaps also phylogeny.

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