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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^{blk} 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; Wiersdorff 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 dpp^{12} and dpp^{14} lines were obtained from Ulrike Heberlein and balanced over SM6-Tm6B. $dpp^{12/14}$ 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-*dac^{21m5m4}, *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

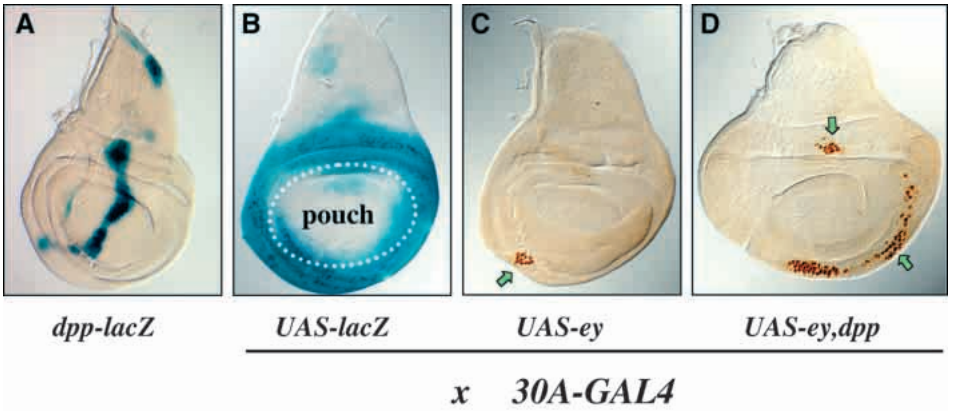

Fig. 1. *dpp* functions synergistically with *ey* to induce photoreceptor development. (A) *dpp* is expressed along the AP boundary in wild-type wing discs (shown in blue). (B) β-galactosidase activity in *30A-GAL4/UAS-lacZ* larval wing discs shows that GAL4 is expressed around the pouch (a dotted line demarcates the pouch). (C,D) Third instar larval wing discs were stained for the neuron-specific Elav protein. Normally, there are no Elav-positive cells in the wing disc (not shown). Driven by *30A-GAL4*, *UAS-dpp* alone does not induce ectopic retinal cells (not shown) and *UAS-ey* alone induces neurons only at the AP boundary (C, arrow). Dorsal staining is not present in the sample shown. In contrast, in *UAS-ey* plus *UAS-dpp* larvae (D), ectopic photoreceptors extend far into the posterior compartment of the wing disc along the ventral pouch margin and at the dorsal side of the pouch at the AP boundary (arrows). Posterior is to the right and dorsal is up for all wing and haltere discs in all figures.

of W. Gehring (*ey*), 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 5× 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.

ey 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

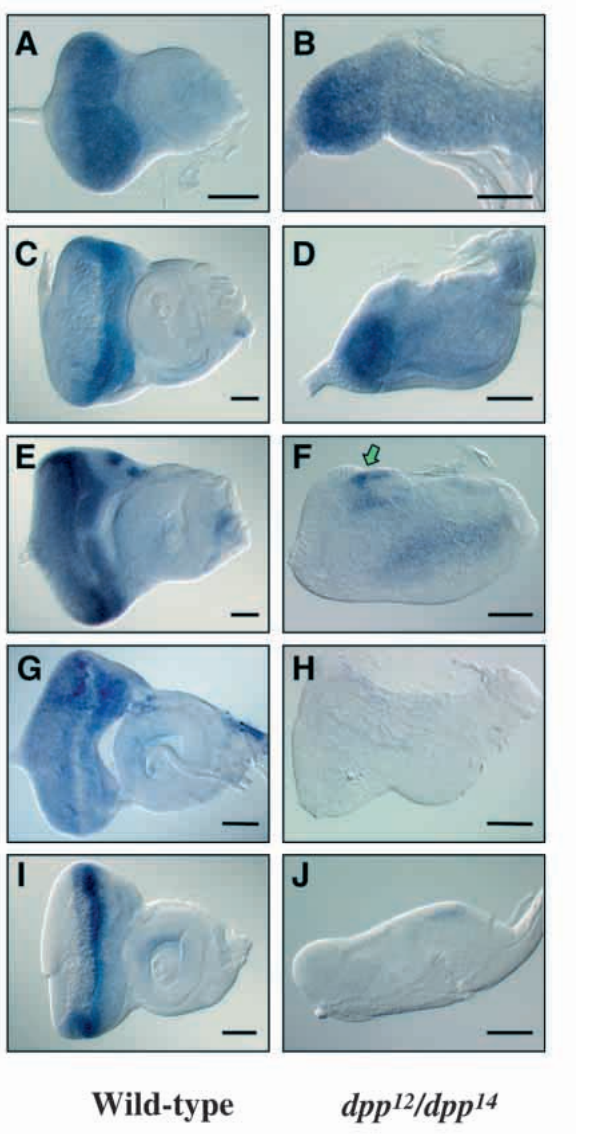

Fig. 2. *dpp* is required for *eya*, *so* and *dac* but not *ey* expression in eye imaginal discs. Antisense RNA probes were used to detect *ey*, *eya*, *so* and *dac* transcripts in both wild-type and *dpp* mutant eye discs. (A-D) *ey* expression is normal in $dpp^{12/14}$ mutants. (A,C) Wild-type eye-antennal discs from late second or late third instar larvae, respectively. In $dpp^{12/14}$ mutants, *ey* is still transcribed in both late second (B) and late third (D) instar eye discs. (E-J) Transcription of *eya*, *so* and *dac* is greatly reduced in $dpp^{12/14}$ mutant eye discs. Late third instar wild-type (E,G,I) or $dpp^{12/14}$ mutant (F,H,J) eye discs were stained for *eya* (E,F), *so* (G,H) or *dac* (I,J) mRNA expression. Posterior is to the left and dorsal is up for all eye discs. The eye disc is to the left and the antennal disc to the right. Scale bars in all panels are 50 μm.

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

Genotype	*UAS-ey*		*UAS-ey, dpp*		*UAS-ey, dac*		*UAS-ey, dpp, dac*	
Disc type	Wing	Haltere	Wing	Haltere	Wing	Haltere	Wing	Haltere
Total scored	51	19	30	17	40	21	45	46
No neurons	10%	47%	0%	24%	0%	0%	0%	2%
Ventral only	35%	47%	0%	59%	0%	29%	0%	33%
Ventral+dorsal	55%	5%	100%	17%	100%	71%	100%	65%

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 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 dpp^{12}/dpp^{14} 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 dpp^{12}/dpp^{14} 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 dpp^{12}/dpp^{14} 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).

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* transcription 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

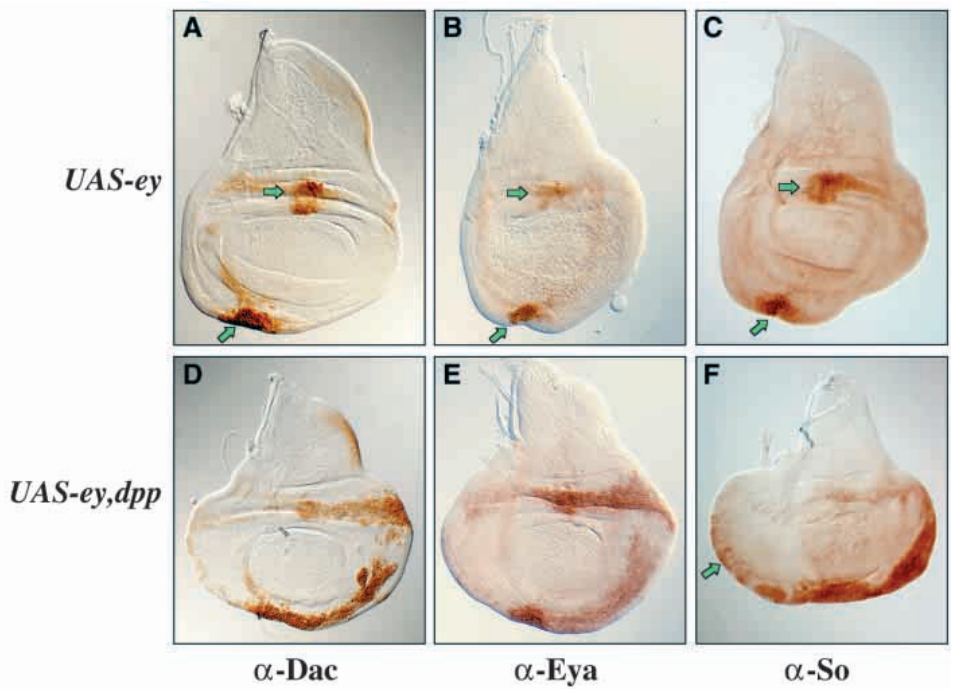

Fig. 3. *ey* and *dpp* act synergistically to induce the expression of *dac*, *eya* and *so*. Wing imaginal discs were stained with antibodies specific for Dac (A,D), Eya (B,E) and So (C,F). When *ey* alone is driven by *30A-GAL4*, ectopic RD gene expression is detected only at the AP boundary (A-C, 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.

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*

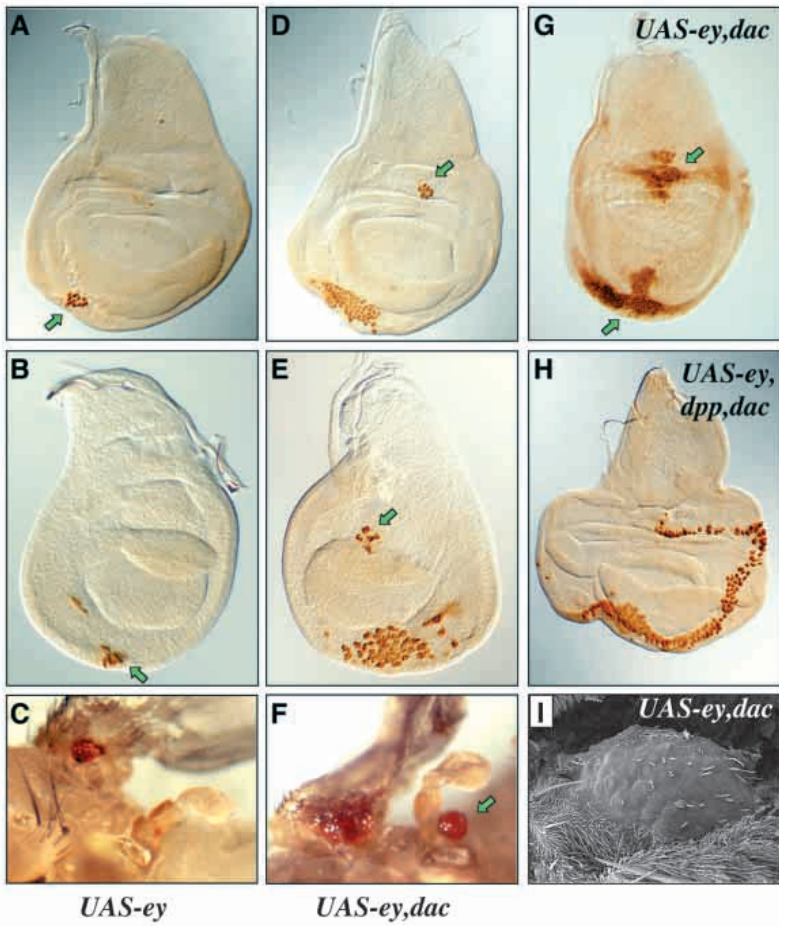

Fig. 4. *ey* functions synergistically with either *eya* or *dac* to induce ectopic eye formation. Only a small cluster of Elav-positive cells are observed at the ventral side of wing (A) or haltere (B) discs prepared from *UAS-ey/30A-GAL4* larvae (arrows). (C) In adults of this genotype, ectopic retinal 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.

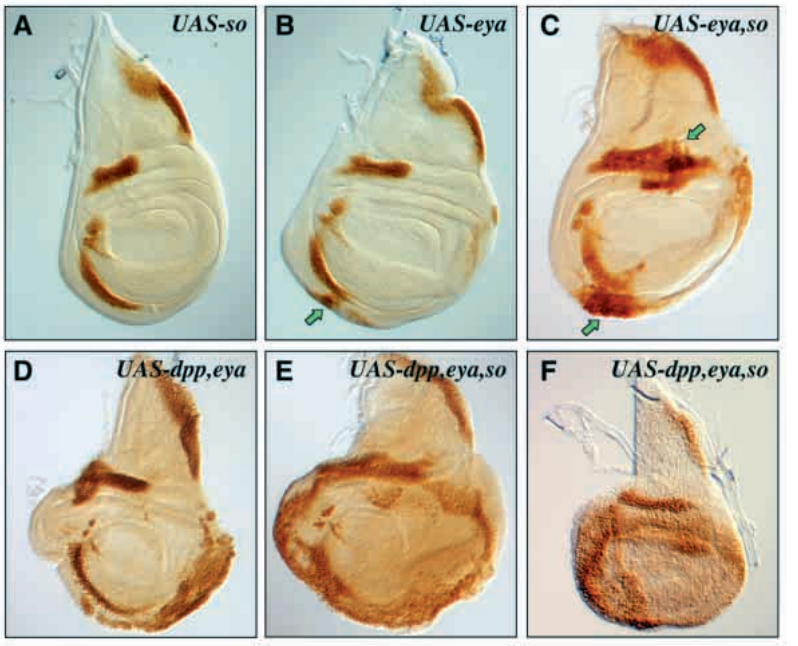

Fig. 5. *dpp* signaling functions cooperatively with *eya* and *so* to induce *dac* expression. Wing imaginal discs were prepared from third instar larvae and stained for Dac protein expression. (A) No ectopic *dac* expression is induced by misexpression of *so* alone driven by *30A-GAL4*; only endogenous 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.

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 *ey* 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 *ey* (see below) for two reasons. First, targeted expression of *ey* and *dpp* is unable to induce *dac* in the anterior wing disc compartment. Second, ectopic *ey* 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 *ey* expression or ectopic photoreceptor development in the wing disc, coexpression of *eya* and *so* using *dpp-GAL4* is sufficient to induce *ey* 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

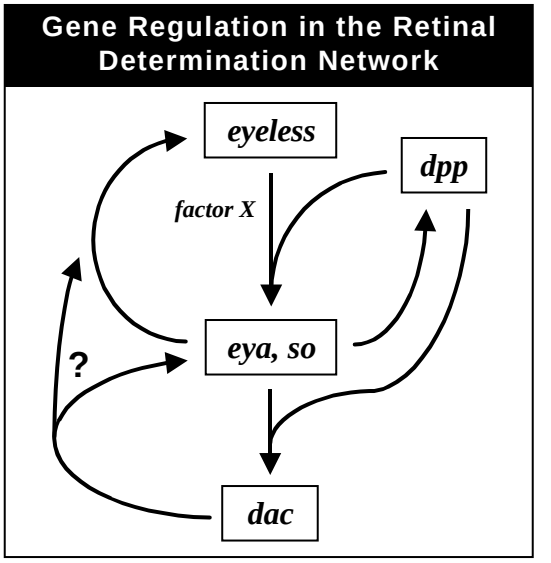

Fig. 6. A model for retinal cell fate determination in *Drosophila*. *dpp* functions cooperatively with the RD genes at multiple levels to control gene expression and retinal cell fate determination. *dpp* signaling interacts with both *ey* and *eya/so* to synergistically induce downstream gene expression. *ey* may also cooperate with other factors (*factor X*) to regulate *eya* and *so* (see text for details). In addition, there is extensive crossregulation 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, ectopic RD gene expression and photoreceptor differentiation are observed only in the posterior compartment of the wing disc. Therefore, some 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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