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Morphogenetic furrow initiation and progression during eye development in *Drosophila*: the roles of *decapentaplegic*, *hedgehog* and *eyes absent*

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SUMMARY

The *Drosophila* signaling factor *decapentaplegic* (*dpp*) mediates the effects of *hedgehog* (*hh*) in tissue patterning by regulating the expression of tissue-specific genes. In the eye disc, the transcription factors *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*) participate with these signaling molecules in a complex regulatory network that results in the initiation of eye development. Our analysis of functional relationships in the early eye disc indicates that *hh* and *dpp* play no role in regulating *ey*, but are required for *eya*, *so* and *dac* expression. We show that restoring expression of *eya* in loss-of-function *dpp* mutant backgrounds is sufficient to induce *so* and *dac* expression and to rescue eye development. Thus, once expressed, *eya* can carry out its functions in the absence of *dpp*. These experiments indicate that *dpp* functions downstream of or in parallel with *ey*, but upstream of *eya*, *so* and *dac*. Additional control is provided by a feedback loop that maintains expression of *eya* and *so* and includes *dpp*. The fact that exogenous overexpression of *ey*, *eya*, *so* and *dac* interferes with wild-type eye development demonstrates the importance of such a complicated mechanism for maintaining proper levels of these factors during early eye development. Whereas initiation of eye development fails in either Hh or Dpp signaling mutants, the subsequent progression of the morphogenetic furrow is only slowed down. However, we find that clones that are simultaneously mutant for Hh and Dpp signaling components completely block furrow progression and eye differentiation, suggesting that Hh and Dpp serve partially redundant functions in this process. Interestingly, furrow-associated expression of *eya*, *so* and *dac* is not affected by double mutant tissue, suggesting that some other factor(s) regulates their expression during furrow progression.

Key words: *Mad*, *smo*, *ey*, *eya*, *so*, *dac*, Regulatory interaction, *Drosophila*

INTRODUCTION

The *Drosophila* TGFβ homolog *decapentaplegic* (*dpp*) participates in the growth and patterning of many tissues in *Drosophila*, including the dorsoventral axis of the embryo (Ferguson and Anderson, 1992; Wharton et al., 1993), the proximodistal axis of the leg disc (Lecuit and Cohen, 1997) and the anteroposterior axis in the wing disc (Nellen et al., 1996; Lecuit et al., 1996). *dpp* function is also crucial to eye development, as demonstrated by loss-of-function alleles or allelic combinations that lead to loss of part or all of the eye (see, for instance, Spencer et al., 1982; Wharton et al., 1996; Chanut and Heberlein, 1997a). Eye differentiation in *Drosophila* is marked by a wave of cell division and differentiation, called the morphogenetic furrow (MF), that initiates from the posterior margin of the eye imaginal disc just prior to metamorphosis. The MF progresses through the unpatterned, dividing cells of the eye disc from posterior to anterior, leaving behind ordered cell clusters called ommatidia. Thus, the MF is a moving boundary that separates undifferentiated from differentiating tissue. Events associated with the MF include synchronization of the cell cycle, as well as striking changes in the expression and activity of many proteins (Wolff and Ready, 1993; Heberlein and Moses, 1995; Bonini and Choi, 1995; Treisman and Heberlein, 1998).

The phenotypes of mutations in Dpp signaling components indicate that Dpp signaling is essential for MF initiation (Wiersdorff et al., 1996; Burke and Basler, 1996). Consistent with this role, *dpp* is expressed along the posterior margin of the eye disc prior to MF initiation (Masucci et al., 1990). Furthermore, *dpp* can ectopically initiate a MF when expressed at the anterior margin of the eye disc (Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997). These ectopic MFs can lead to outgrowth and patterning of a complete duplicate eye disc, demonstrating the capability of the MF, and ultimately *dpp*, to pattern the eye disc.

dpp exerts its diverse effects in various tissues by regulating the expression of tissue-specific downstream genes. What are the downstream mediators of *dpp* function in MF initiation, and what factors interact with *dpp* to promote eye development? Several proteins that function during eye development are good candidates, including the Pax-6 homolog Eyeless (Ey), the homeodomain protein Sine oculis (So), and the novel nuclear proteins Eyes absent (Eya) and Dachshund (Dac). All four

genes, subsequently referred to as the early eye genes, are essential for eye development, as demonstrated by the fact that loss-of-function mutations lead to an eyeless phenotype (Quiring et al., 1994; Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994). Furthermore, ectopic expression of *ey*, *eya* and *dac* in other imaginal discs can lead to the formation of ectopic eyes (Halder et al., 1995; Bonini et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997; Chen et al., 1997, 1999).

The exact relationships between these four proteins are as yet unclear. However, they are likely to interact in at least two ways: they regulate each other's expression and they may interact physically (reviewed in Desplan, 1997). In terms of expression, *ey* lies at the top of an early eye gene hierarchy, and is responsible for inducing expression of *eya* and *so* (Quiring et al., 1994; Halder et al., 1995, 1998; Bonini et al., 1997; Niimi et al., 1999). *eya* and *so* may participate in initiating *dac* expression (Pignoni et al., 1997; Chen et al., 1997). However, many results suggest that feedback loops are likely to play a role in regulating expression of these genes and/or that these molecules function in one or more complexes to induce each other's expression and initiate eye development. Strikingly, ectopic expression of combinations of the early eye genes is much better at inducing ectopic eyes than any one individually, and two-hybrid and in vitro studies suggest that Eya and So and Eya and Dac, are capable of interacting physically (Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997, 1999).

dpp is likely to participate in this complex regulatory network. Although *ey* is expressed strongly in *dpp* mutant eye discs, *eya*, *so* and *dac* are absent (Chen et al., 1999). Furthermore, *dpp* can initiate ectopic expression of *so* and *dac* when expressed at the anterior margin of the eye disc (Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997). These results suggest that *dpp* is required for *eya*, *so* and *dac* expression. Consistent with a role as a *dpp* target, *dac* is not required for *dpp* expression (Mardon et al., 1994). However, *dpp* expression is patchy in eye discs from *eya* and *so* loss-of-function mutants, suggesting that *eya* and *so* are required for either initiation or maintenance of *dpp* at the posterior margin prior to MF initiation (Pignoni et al., 1997; Hazelett et al., 1998). Furthermore, ectopic eyes are only produced by the early eye genes if they are expressed in parts of the imaginal discs that have a source of *dpp* (Halder et al., 1998; Chen et al., 1999), suggesting that *dpp* activity is necessary in conjunction with the early eye genes. However, the exact relationship between Dpp and the early eye genes remains unclear.

We have carried out an analysis of functional relationships between *dpp* and the early eye genes in the early eye disc, at stages prior to MF initiation, by blocking the *dpp* signal autonomously in loss-of-function *Mad* clones. Our data indicate that *dpp* functions downstream of or in parallel with *ey*, but upstream of *eya*, *so* and *dac* prior to MF initiation. In contrast to the results of Chen et al. (1999), where *dpp* appeared to function both upstream and downstream of *eya* in the formation of ectopic eyes, we have found that restoring expression of *eya* to the posterior margin of the eye disc in the absence of *dpp* function is sufficient to induce *so* and *dac* expression and to largely rescue eye development. Thus, during wild-type eye development *eya* clearly functions downstream of *dpp*, although feedback loops that include *eya*, *so* and *dpp* may participate in regulating expression of these genes. Unlike *eya*, *so* and *dac* enhance the effects of loss-of-function *dpp* mutations on eye development. Furthermore, exogenous *ey*, *eya*, *so* and *dac* are all able to interfere with wild-type eye development, suggesting that regulating the amounts of these proteins relative to one another is crucial. Thus, *dpp* plays a critical role in helping to regulate a delicate balance of protein levels essential for eye development.

Although Dpp is required for MF initiation, its role during MF progression is less clear. Loss-of-function clones in Dpp signaling components have little effect on MF progression (Wiersdorff et al., 1996; Burke and Basler, 1996). Nevertheless, MF progression is slowed in some cases (Burke and Basler, 1996), and *dpp* is expressed in the MF as it traverses across the eye disc, where it is required for cell cycle regulation (Penton et al., 1997; Horsfield et al., 1998). Thus, a role for *dpp* during MF progression has not been ruled out.

During MF progression, another signaling molecule, Hedgehog (Hh), is expressed in the developing photoreceptors (Lee et al., 1992). Since loss of *hh* function produces a "furrow-stop" phenotype (Heberlein et al., 1993; Ma et al., 1993), and ectopic Hh expression anterior to the MF gives rise to a progressing ectopic MF (Heberlein et al., 1995), it has been proposed that Hh plays an important role in MF progression. As with Dpp, the exact role Hh plays during eye development is obscured by the fact that Hh is a diffusible molecule. Although MF progression is halted in eye discs entirely mutant for *hh*, clones of *hh* mutant cells, or clones that block reception of the Hh signal merely slow, but do not block, MF progression (Strutt and Mlodzik, 1997; Domínguez, 1999; Greenwood and Struhl, 1999). Photoreceptor development appears to be rescued in these clones by more posterior and lateral tissue, suggesting that Hh is responsible for the production of a secondary signal that mediates its effects.

To clarify the roles of *dpp* and *hh* during MF progression, we have analyzed the effects of clones simultaneously mutant for Mad^{1-2} and smo^{3} on photoreceptor development during stages following MF initiation. In contrast to single mutant Mad^{1-2} or smo^{3} clones, the doubly mutant clones block MF progression, suggesting that *dpp* and *hh* play redundant roles during this process. Interestingly, early eye gene expression is unaffected in the double mutant clones, suggesting that factor(s) other than *dpp* and *hh* regulate their expression during MF progression.

MATERIALS AND METHODS

Drosophila genetics

The following fly strains were used:

Mutant alleles: dpp^{blk} (Masucci et al., 1990), dpp^{12} (Segal and Gelbart, 1985), smo^{3} (Chen and Struhl, 1996) and Mad^{1-2} (Wiersdorff et al., 1996).

Transgenes: *dpp-GAL4* (Chanut and Heberlein, 1997b), *ey-GAL4* (gift from U. Walldorf, GAL4 expression is controlled by the *ey* regulatory region, Hauck et al., 1999), *UAS-ey* (Halder et al., 1995), *UAS-eya* (Bonini et al., 1997), *UAS-so* (Pignoni et al., 1997), *UAS-*dac^{7c4} (Chen et al., 1997), *eyFLP* (gift from B. Dickson, FLP expression is controlled by the *ey* regulatory region, Hauck et al., 1999) and *arm-lacZ* (Vincent et al., 1994).

Enhancer trap strain: so^{7} (Cheyette et al., 1994).

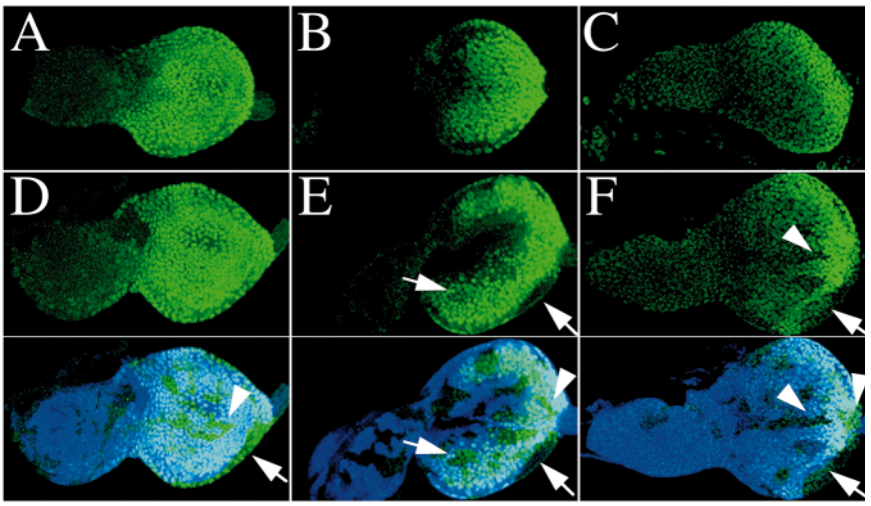

Fig. 1. Dpp signaling is required for Eya and Dac, but not Ey expression prior to MF initiation. All panels show confocal images of early third instar eye discs; anterior is to the left and dorsal is up. (A-C) Wild-type eye discs; (D-F) eye discs containing clones homozygous for the Mad^{1-2} allele. The two panels in D-F show the same disc. Wild-type and mosaic eye discs are stained (green) with anti-Ey (A,D) anti-Eya (B,E) and anti-Dac (C,F). Mad^{1-2} clones are marked by the absence of the clonal marker *arm-lacZ*, visualized by staining with anti-β-galactosidase (blue) in bottom panels of D-F; green and blue staining appear turquoise in areas where they overlap. Ey is expressed normally in Mad^{1-2} clones (D). Eya (E) and Dac (F) are not expressed in Mad^{1-2} clones that touch the margin or in the parts of internal clones that are a few cell diameters anterior of the margin (arrows). Internal clones that are close to the margin express Eya and Dac (arrowheads).

Homozygous Mad^{1-2} or smo^{3} clones were generated via the FLP/FRT system (Xu and Rubin, 1993). Experimental genotypes used were:

eyFLP/+ or *Y*; *arm-lacZ,FRT40/ Mad*$^{1-2}$*,FRT40*.

eyFLP/+ or *Y*; *arm-lacZ,FRT40/ smo*^{3}*,FRT40*.

eyFLP/+ or *Y*; *arm-lacZ,FRT40/ smo*^{3}*,Mad*$^{1-2}$*,FRT40*.

eyFLP/+ or *Y*; *arm-lacZ,FRT40/ Mad*$^{1-2}$*,FRT40*; *dpp-GAL4/UAS-eya*.

Histology

For immunocytochemistry, fixation and treatment of eye discs was performed essentially as described by Tomlinson and Ready (1987), using rat monoclonal anti-ELAV (gift from G. Rubin), rabbit (Cappel) and mouse anti-β-galactosidase (Promega), mouse anti-Eya (Bonini et al., 1993), rabbit anti-Ey (Halder et al., 1998; gift from U. Walldorf), mouse monoclonal anti-Dac (Mardon et al., 1994; gift from S. Cohen) and rabbit anti-Ato (Jarman et al., 1994; gift from A. Jarman).

For in situ hybridization, treatment of eye discs was performed essentially as described by Lehmann and Tautz (1994) for embryos, except that eye discs were dissected in 1× PBS and fixed in 9:1 PP/DMSO (PP is 1× PBS, 4% paraformaldehyde, 0.1% Tween-20, and 0.1% Triton X-100) for 30 minutes, rather than in the standard heptane/formaldehyde embryo fix. For double labeling eye discs for β-galactosidase expression followed by in situ hybridization, eye discs were dissected in 1× PBS, fixed in 9:1 PP/DMSO for 2 minutes, washed briefly in 1× PBS, and incubated in staining solution (Simon et al., 1985) at 37°C until staining was observed (approximately 5 minutes for *arm-lacZ*). Discs were then treated for in situ hybridizations as described above, starting with fixation in 9:1 PP/DMSO for 30 minutes.

For scanning electron microscopy, adult flies were dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, 90%, 100%), dried in a Balzers critical point dryer, and mounted on EM stubs. Samples were coated with gold using a Balzers sputter coater, and analyzed using a Zeiss Novascan-30 microscope.

RESULTS

dpp controls *eya*, *so* and *dac*, but not *ey*, expression prior to MF initiation

Mad^{1-2} homozygous mutant tissue touching the posterior margin fails to initiate a MF, demonstrating that Dpp signaling is essential for MF initiation (Wiersdorff et al., 1996). To determine whether Dpp signaling regulates Ey, Eya and Dac expression as part of that function, we examined their expression patterns in clones of homozygous Mad^{1-2} tissue prior to MF initiation.

Ey is expressed throughout the eye portion of the wild-type eye disc during early larval stages, prior to MF initiation (Quiring et al., 1994; Halder et al., 1998; Fig. 1A). Eya and Dac are expressed throughout the posterior half of the eye imaginal disc, with stronger expression at the posterior margin (Bonini et al., 1993; Mardon et al., 1994; Fig. 1B,C). Ey is expressed normally in homozygous Mad^{1-2} clones that touch the posterior margin (arrow in Fig. 1D) and in clones that are positioned internally in the disc (arrowhead in Fig. 1D), indicating that Dpp signaling is not required for Ey expression prior to MF initiation. In contrast, neither Eya nor Dac is expressed in homozygous Mad^{1-2} clones that touch the margin of the eye disc (arrows in Figs 1E,F). In addition, Eya and Dac are not expressed, or are expressed weakly, in internal clones that lie well anterior of the posterior margin (arrows in Fig. 1E,F). However, strong Eya and Dac expression is observed in internal clones that lie within a few cell diameters of the posterior margin (arrowheads in Fig. 1E,F).

Like Eya and Dac protein, *so* mRNA is expressed in the posterior region of the eye disc prior to MF initiation (Cheyette et al., 1994; Fig. 2A). Mad^{1-2} posterior margin clones fail to express *so* (arrow in Fig. 2B). These results suggest that *dpp* function is required to induce or maintain Eya, *so* and Dac expression, but not Ey expression, at the posterior margin prior to MF initiation. This function is consistent with the pattern of *dpp* mRNA expression along the posterior and lateral margins at this stage of eye disc development (Masucci et al., 1990; Fig. 2C). Whereas *dpp* is not necessary for Eya and Dac expression in internal, posterior regions of the early eye disc, it does play a role in regulating Eya and Dac expression in internal, anterior regions of the disc. As shown in Fig. 2C, although *dpp* mRNA expression does not extend to the very center of the eye disc, it is expressed in a significant proportion of the interior of the disc. The possibility that *dpp* may regulate gene expression in more central regions may be attributed to the fact that it encodes a diffusible molecule.

Mad^{-} tissue is capable of sustaining MF-associated Eya, *so* and Dac expression

Although eye development and MF initiation are blocked in

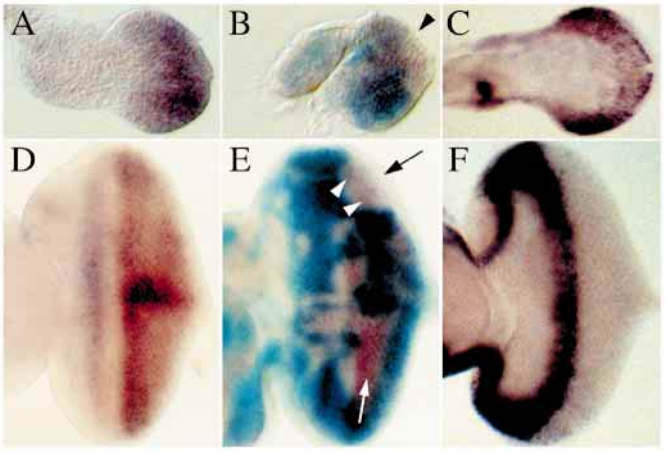

Fig. 2. Dpp signaling is required for *so* expression prior to but not after MF initiation. Anterior is to the left and dorsal is up in all panels. Early (A-C) and late (D-F) third instar eye discs. Wild-type eye discs subjected to in situ hybridization using a digoxigenin-labeled *so* (A,D) or *dpp* (C,F) cDNA probe (purple). Prior to MF initiation, *so* (A) is expressed in a graded fashion with strongest levels at the posterior margin, and *dpp* (C) is expressed along the entire margin of the eye disc. After MF initiation, *so* is expressed in the developing photoreceptors, and anterior to the MF (D); *dpp* expression is observed within the MF itself and continues along the lateral margins anterior to the MF. (B,E) Eye discs containing clones homozygous for the Mad^{1-2} allele (marked by the absence of the clonal marker *arm-lacZ*) subjected to β-galactosidase activity staining (blue), followed by in situ hybridization with the *so* cDNA probe (purple). *so* is not expressed in Mad^{1-2} clones prior to MF initiation (arrowhead in B), or in Mad^{1-2} clones that touch the posterior margin after MF initiation (black arrow in E). However, in Mad^{1-2} clones that lie within the interior of the disc (white arrow in E), *so* is expressed in photoreceptors. *so* is also expressed in regions of the posterior margin clone where eye differentiation has spread from surrounding Mad^{+} tissue (white arrowheads in E).

Mad^{-} tissue, the MF can pass normally through Mad^{1-2} internal clones. Furthermore, once initiated, the MF can spread laterally into Mad^{1-2} tissue (Wiersdorff et al., 1996). Thus, MF progression can be sustained even in the absence of Dpp signaling. To determine whether Dpp regulates expression of Ey, Eya, *so* and Dac during MF progression, we examined their expression in late third instar eye discs containing Mad^{1-2} clones.

In wild-type eye discs, *so* mRNA levels remain high both within the MF and in the photoreceptors that develop posterior to it (Fig. 2D). Expression of *so* is absent in Mad^{1-2} clones that touch the posterior margin and do not form eye tissue (black arrow in Fig. 2E), but present in internal, anterior clones where eye tissue develops (white arrow in Fig. 2E). Thus, although Dpp is required for *so* expression prior to MF initiation, it is not required for *so* expression when associated with a MF that has already initiated.

Like *so*, Eya is expressed anterior to the MF, within the MF, and in the developing photoreceptors posterior to the MF (Fig. 3A). Dac is expressed at high levels within, and at decreasing levels on either side, of the MF (Fig. 3B). Ey is expressed at high levels anterior to the MF, but is downregulated in the developing eye field (Fig. 3C). Eya and Dac are not detected in Mad^{1-2} marginal clones that fail to differentiate eye tissue (arrows in Fig. 3D,E). However, Eya and Dac are expressed normally as the MF passes through internal, anterior clones (Fig. 3D,E). Strikingly, wherever a MF has spread laterally into a Mad^{1-2} posterior margin clone and induced eye differentiation where it was initially blocked, Eya is expressed normally in the cells preceding the MF and in the developing photoreceptors (arrowheads in Fig. 3D); Dac is expressed in and around the MF (arrowheads in Fig. 3E). As with *so*, in late third instar eye discs Eya and Dac expression depend upon MF initiation, rather than the presence or absence of Dpp signaling.

As is the case prior to MF initiation, Dpp signaling does not regulate Ey expression after MF initiation. In the main eye field, as well as in Mad^{1-2} internal clones and parts of marginal clones into which the MF has spread resulting in eye tissue differentiation, Ey expression is downregulated, whereas it is maintained in other regions of marginal clones (Fig. 3F).

These experiments indicate first that Dpp signaling is not essential for Ey expression at any stage. Second, once the MF has initiated, expression of *so*, Eya and Dac does not require the function of Dpp. Instead, the major changes that occur in expression as a result of MF initiation and progression appear to be regulated by some other factor associated with the eye field (or MF) itself. Consistent with this idea, expression of Eya, *so* and Dac following MF initiation depends on the presence or absence of a MF, rather than the boundaries of Mad^{1-2} clones.

Exogenous Eya expression rescues a *dpp* loss-of-function eye phenotype

It is conceivable that the only role *dpp* plays during eye development is to induce expression of Eya, So and Dac at the posterior margin. If this is true, then forcing expression of these proteins at the posterior margin should allow eye development to proceed even in the absence of Dpp signaling. To test this hypothesis, we have used the GAL4 system to direct expression of these proteins during eye development (Brand and Perrimon, 1993), assaying for rescue of either loss of *dpp* function itself, or loss of the ability to transduce the Dpp signal.

dpp^{blk} is a regulatory loss-of-function allele that is adult viable and results in greatly reduced eyes (Chanut and Heberlein, 1997a). In dpp^{blk} eye discs, *dpp* expression at the posterior margin prior to MF initiation is restricted to the central area of the disc, possibly because the positive selfregulatory loops that contribute to the spread of *dpp* expression along the posterior margin fail in dpp^{blk} eye discs (Chanut and Heberlein, 1997a). Eye differentiation starts at the center of the posterior margin of the eye disc, where the dorsoventral axis intersects with the posterior margin. Because of the shape of the eye disc and the fact that the MF progresses through it as a straight line, formation of a complete eye requires continual reinitiation along the posterior and lateral margins of the disc (Ma et al., 1993). *dpp* is required all along the posterior and lateral margins for initiation at each point, such that the furrow can progress and spread along the anteroposterior axis (Wiersdorff et al., 1996; Chanut and Heberlein, 1997b). Consistent with the lack of *dpp* expression except in the center of the margin, a MF initiates only in the center of dpp^{blk} eye discs. Although it progresses anteriorly in a normal fashion, it fails to spread laterally. In accordance with the results described above for Mad^{1-2} clones, in dpp^{blk} third instar eye discs Ey expression is normal, but expression of Eya,

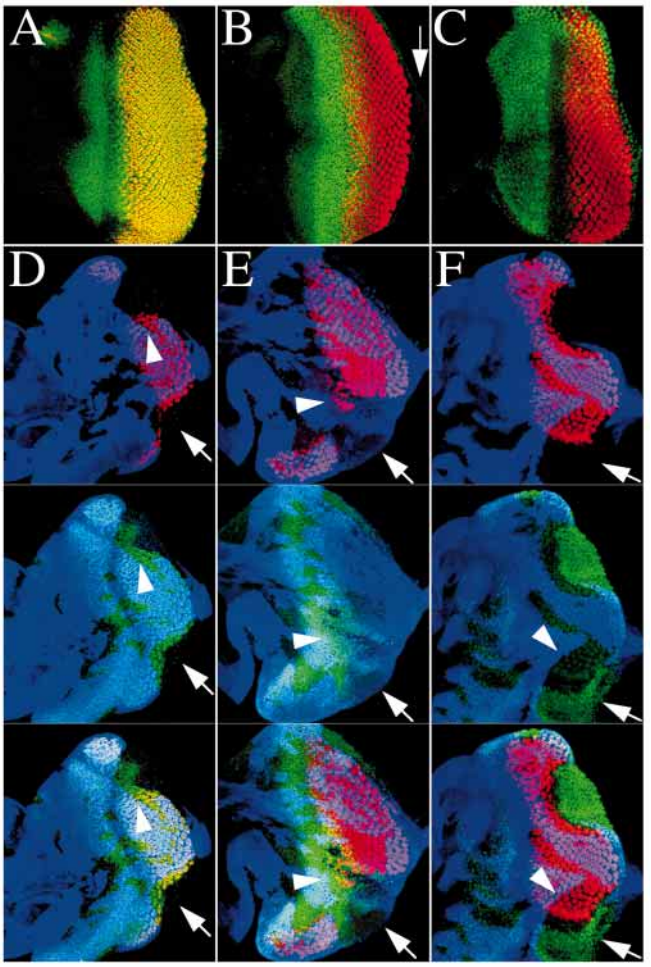

Fig. 3. After MF initiation Dpp signaling is not required for Eya, Dac or Ey expression. All panels show confocal images of late third instar eye discs. (A-C) Wild-type eye discs; (D-F) eye discs containing clones homozygous for the Mad^{1-2} allele. All three vertical panels in D-F show the same disc. Wild-type and mosaic eye discs are stained (green) with anti-Eya (A,D) anti-Dac (B,E) and anti-Ey (C,F) and with anti-ELAV (red) to mark developing photoreceptors (A-F). Mad^{1-2} clones are marked by the absence of the clonal marker *arm-lacZ*, visualized by staining with anti-β-galactosidase (blue) in all panels of D-F. Middle, bottom and top panels of D-F show merge of red and blue channels, green and blue channels, and all three channels, respectively. Red and green staining appear yellow, red and blue staining appear purple, and green and blue staining appear turquoise in areas where they overlap. Eye differentiation, marked by the presence of developing photoreceptors (red) fails in Mad^{1-2} clones that touch the posterior margins (arrows in D-F). These clones show an absence of either Eya (green, D) or Dac (green, E) expression, and strong Ey expression (green, F). However, in Mad^{1-2} clones that lie within the interior of the disc, and in regions of Mad^{1-2} posterior margin clones where eye differentiation has spread from surrounding Mad^{+} tissue (arrowheads in D-F), Eya is expressed in photoreceptors, Dac is expressed around the MF (marked by the anterior edge of the photoreceptors), and Ey expression is downregulated in the developing photoreceptors.

so and Dac is largely restricted to regions where a MF has initiated, giving rise to very small eyes (Fig. 4A-E).

We have generated fly strains in which expression of Eya, So or Dac is directed to the eye disc prior to MF initiation by heterologous promoters using *ey-GAL4* and *dpp-GAL4* drivers with the respective UAS target transgenes. The regulatory region used to construct *ey-GAL4* directs expression from embryonic stages through early larval stages throughout the eye disc. Following MF initiation, it drives strong expression anterior to, and weaker expression posterior to, the MF (Hauck et al., 1999, and not shown). *dpp-GAL4* directs expression to the posterior margin of the eye disc both prior to and after MF initiation (Chanut and Heberlein, 1997b, and not shown). Strikingly, when Eya expression is forced at the posterior margin of dpp^{blk} eye discs, the small-eyed dpp^{blk} phenotype is partially rescued (compare Fig. 4F-J with 4A-E). Specifically, the presence of Eya allows the MF to spread in a normal fashion dorsally along the lateral margin to form an almost complete dorsal half of an eye. In contrast, the MF does not spread ventrally along the margin, probably reflecting an unrelated role of *dpp* in the ventral half of the eye disc (see Discussion). Given the possibility that *eya* and *dpp* participate in regulatory loops (see Introduction) and that dpp^{blk} is a regulatory mutation and/or may not completely remove *dpp* function, it is possible that exogenous *eya* rescues dpp^{blk} by inducing *dpp* expression. To test this, we performed similar experiments in two other genetic backgrounds: in dpp^{12}/dpp^{blk} transheterozygotes (Fig. 4K,L) (dpp^{12} is a strong loss-of-function allele specific to imaginal discs, Brook and Cohen, 1996; Chen et al., 1999), and in Mad^{1-2} clones (Fig. 4M-O). As with dpp^{blk} homozygotes, in both cases, exogenous *eya* expression was capable of rescuing loss of photoreceptor development.

To test whether this effect is due to expanded expression of Ey, *so* and/or Dac, we monitored their expression in these genetic backgrounds. Examples of dpp^{blk};*dpp-GAL4/UAS-eya* eye discs are shown in Fig. 4G-J. These eye discs display a significant expansion of the Dac and *so* expression domains; expression levels are comparable to those in wild type and overlap with the regions where Eya expression is directed by *dpp-GAL4* (arrows in Fig. 4I,J). Comparable, albeit slightly weaker, results were obtained with dpp^{blk};*ey-GAL4/UAS-eya* (not shown). These observations suggest that, in the eye disc, Eya can induce *so* and Dac in the absence of Dpp signaling and lead to the initiation of the MF.

So and Dac cannot rescue the dpp^{blk} phenotype

In contrast to Eya, exogenous expression of So or Dac cannot rescue the dpp^{blk} phenotype. Instead, *dpp-GAL4/UAS-so* and *ey-GAL4/UAS-so* both cause a strong enhancement of the small-eye dpp^{blk} phenotype in 100% of the animals, which often leads to a complete lack of eye development (Fig. 5). No increase of Eya or Dac expression is detected in dpp^{blk}; *dpp-GAL4/UAS-so* or dpp^{blk}; *ey-GAL4/UAS-so* eye discs (Fig. 5C,D,H,I). Although in our hands dpp^{blk};*dpp-GAL4/UAS-dac* animals die at stages too early to be analyzed, *ey-GAL4/UAS-dac* results in a variable decrease in the size of dpp^{blk} eyes. In extreme cases, these animals develop few or no ommatidia. Expansion of Eya or *so* expression is not observed in dpp^{blk}; *ey-GAL4/UAS-dac* eye discs (Fig. 5M,O).

ey is incapable of inducing ectopic eyes in the absence of *dpp* (Chen et al., 1999), suggesting that it functions upstream of or in parallel with *dpp* in eye development. If so, exogenous Ey expression should have no effect on the dpp^{blk} phenotype. Consistent with its position near the top of the eye gene

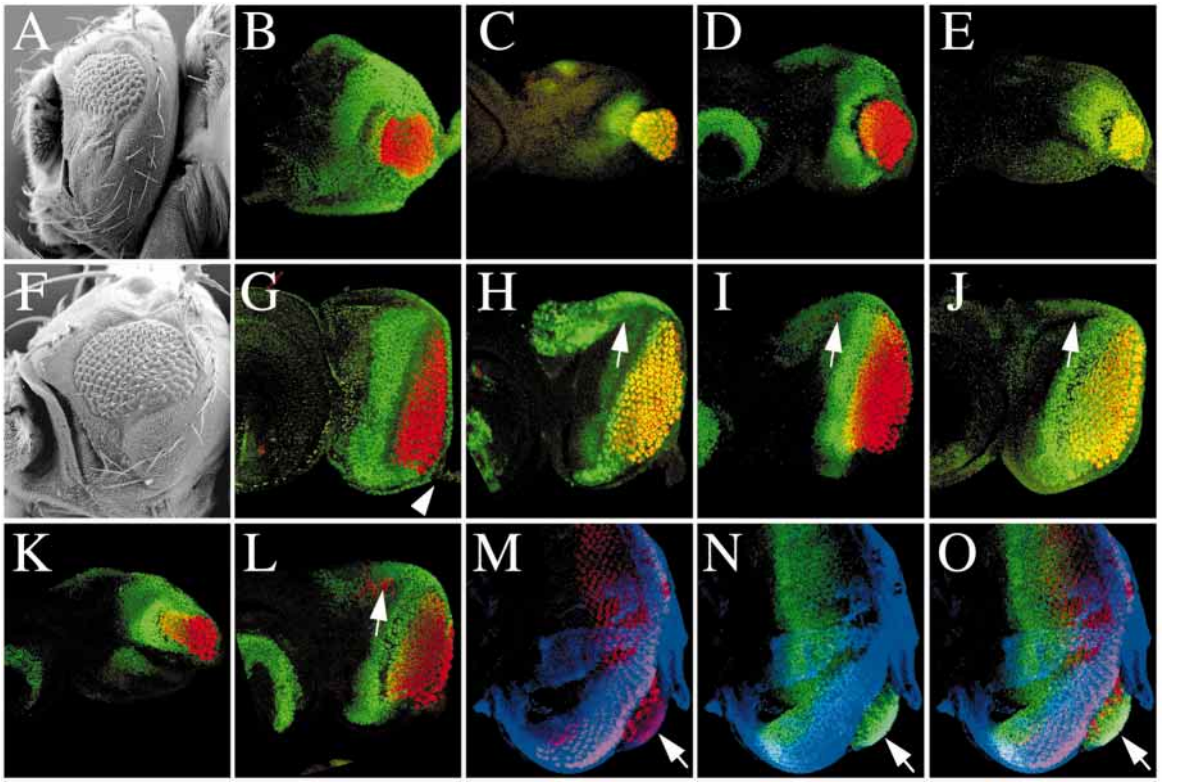

Fig. 4. Exogenous Eya expression rescues loss *dpp* function. (A-D) dpp^{blk}/dpp^{blk}. (E) dpp^{blk},*so-lacZ*/dpp^{blk},*so-lacZ*. (F-I) dpp^{blk}/dpp^{blk};*dpp-GAL4/UAS-eya*. (J) dpp^{blk},*so-lacZ*/dpp^{blk},*so-lacZ*;*dpp-GAL4/UAS-eya*. (K) dpp^{12}/dpp^{blk}. (L) dpp^{12}/dpp^{blk};*dpp-GAL4/UAS-eya*. (A,F) Scanning electron micrographs of adult fly heads (see Fig. 6A for comparison to wild-type). (B-E,G-L). Late third instar eye discs stained in red with anti-ELAV to mark developing photoreceptors, and in green with anti-Ey (B,G), anti-Eya (C,H), anti-Dac (D,I,K,L) or anti-β-galactosidase (E,J) (see Figs 2C, 3A-C for comparison to wild type). Adult dpp^{blk}/dpp^{blk};*dpp-GAL4/UAS-eya* eyes contain greater numbers of facets than adult dpp^{blk}/dpp^{blk} eyes (compare F with A) and are therefore larger. dpp^{blk}/dpp^{blk};*dpp-GAL4/UAS-eya* and dpp^{12}/dpp^{blk};*dpp-GAL4/UAS* eye discs are larger than dpp^{blk}/dpp^{blk} and dpp^{12}/dpp^{blk} eye discs, respectively (compare B-E with G-J, and K with L), contain more developing ommatidia (red staining), and express Dac (green, arrow in I,L) and *so* (green, arrow in J) in expanded domains that overlap with the areas where Eya (green, arrow in H) is exogenously expressed. Whereas the dorsal half of the eye is almost completely rescued, the position of the optic stalk at the ventral/posterior margin of the dpp^{blk}/dpp^{blk};*dpp-GAL4/UAS-eya* and dpp^{12}/dpp^{blk};*dpp-GAL4/UAS* eye discs (arrowhead in G) indicates that exogenous Eya has no effect on the ventral half. (M-O). Single eye disc containing clones homozygous for the Mad^{1-2} allele, marked by the absence of the clonal marker *arm-lacZ* (blue) as in Fig. 3; *UAS-eya* was expressed in this disc under the control of *dpp-GAL4*. The disc was stained with anti-Dac (green), and with anti-ELAV (red) to mark developing photoreceptors. (M-O) merge of red and blue channels, green and blue channels, and all three channels, respectively. Eye differentiation, marked by the presence of developing photoreceptors (red) is rescued in Mad^{1-2} clones that touch the posterior margins (arrow in M,O, compare with Fig. 3D-F). These clones express Dac strongly (arrow in N,O, compare with Fig. 3E).

hierarchy, *ey-GAL4/UAS-ey* does not induce detectable levels of Eya, *so* or Dac expression or rescue eye development in dpp^{blk} discs. Surprisingly, however, *ey-GAL4/UAS-ey* also interferes with dpp^{blk} eye development to a variable extent, often resulting in smaller eyes (not shown).

These data indicate that Ey, So and Dac are incapable of inducing sufficient levels of the other early eye genes in the absence of normal Dpp signaling, or of rescuing eye development. Finally, rather than rescuing eye development, excess So in a dpp^{blk} background strongly interferes with eye development. Excess Ey and Dac also interfere with eye development, but to a lesser extent.

Ey, Eya, So and Dac overexpression interferes with wild-type eye development

The observation that exogenous expression of Ey, So or Dac leads to eye reduction or eye loss in dpp^{blk} animals suggests that the presence of excess amounts of these proteins might interfere with MF induction and eye development. To test this further, we analyzed the effects of *ey-GAL4/UAS-ey*, *ey-GAL4/UAS-eya*, *ey-GAL4/UAS-so* and *ey-GAL4/UAS-dac* in an otherwise wild-type background. As with dpp^{blk}, overexpression of each of these molecules in a wild-type background causes a reduction of the eye (Fig. 6), with So resulting in the most significant reduction and Eya the least significant reduction. Similar results were obtained using a *dpp-GAL4* driver (Fig. 6E), suggesting that the effects are due to expression at the posterior margin, not to expression throughout the developing eye disc. With the exception of *UAS-so*, which has never been shown to induce ectopic eyes, all of these UAS constructs in combination with *dpp-GAL4* induce ectopic eyes similar to those described previously (not shown), demonstrating that these proteins are produced and are functional. These observations indicate that the amount of Ey, Eya, So and Dac, as well as perhaps the

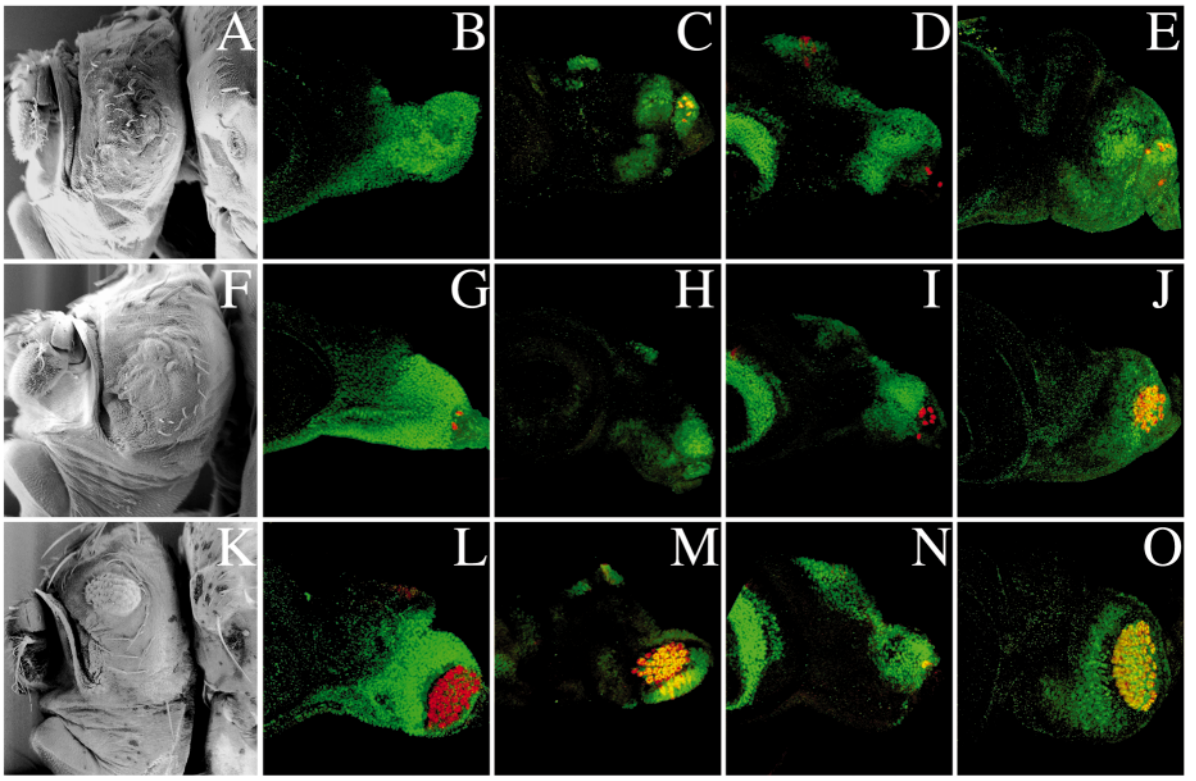

Fig. 5. Exogenous So and Dac expression fails to rescue dpp^{blk}. (A-D) dpp^{blk}/dpp^{blk};*dpp-GAL4/UAS-so*. (E) dpp^{blk},*so-lacZ*/dpp^{blk},*so-lacZ*;*dpp-GAL4/UAS-so*. (F-I) dpp^{blk}/dpp^{blk};*ey-GAL4/UAS-so*. (J) dpp^{blk},*so-lacZ*/dpp^{blk},*so-lacZ*; *ey-GAL4/UAS-so*. (K-N) dpp^{blk}/dpp^{blk};*ey-GAL4/UAS-dac*. (O) dpp^{blk},*so-lacZ*/dpp^{blk},*so-lacZ*; *ey-GAL4/UAS-dac*. (A,F,K) Scanning electron micrographs of adult fly heads. (B-E, G-J, L-O) Late third instar eye discs stained in red with anti-ELAV to mark developing photoreceptors, and in green with anti-Ey (B,G,L), anti-Eya (C,H,M), anti-Dac (D,I,N) or anti-β-galactosidase (E,J,O). Exogenous expression of So directed by either *dpp-GAL4* (A-E) or *ey-GAL4* (F-J) results in a strong enhancement of the small-eyed dpp^{blk} phenotype, leading to a complete or almost complete absence of photoreceptor development (red staining). Exogenous expression of Dac directed by *ey-GAL4* (K-L) results in a variable enhancement of the dpp^{blk} phenotype, sometimes leading to the complete absence of photoreceptor development. The eye disc in N has developed only a single ommatidium.

relative amounts of these molecules, is critical to the proper development of the eye.

MF progression does not occur in *smo*$^{-}$,*Mad*$^{-}$ clones

The results presented above demonstrate the important role *dpp* plays in eye development by regulating expression of the early eye genes such that MF initiation can occur. In contrast, neither MF progression nor the expression patterns of the early eye genes during MF progression depend on *dpp*. Since *hedgehog* (*hh*) has been shown to be important for MF progression (reviewed in Treisman and Heberlein, 1998), it could be the factor that regulates expression of the early eye genes during this stage of eye development. To test this hypothesis, we examined the expression patterns of Ey, Eya and Dac in late third instar larval eye discs containing loss-of-function clones in *smoothened* (*smo*), which functions as part of the *hh* receptor; loss of *smo* function interferes autonomously with the reception of the *hh* signal (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996).

It has been previously reported that marginal smo^{3} clones fail to develop photoreceptors, suggesting that inability to transduce Hh signaling prevents MF initiation (Dominguez and Hafen, 1997). Furthermore, internal smo^{3} clones slow, but do not stop, MF progression (Strutt and Mlodzik, 1997;

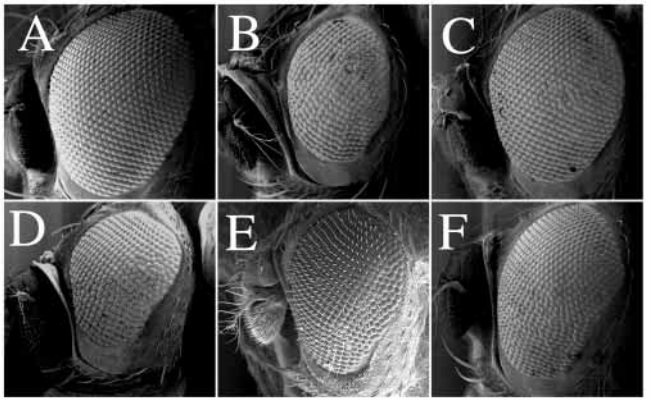

Fig. 6. Exogenous Ey, Eya, So and Dac expression interfere with wild-type eye development. Scanning electron micrographs of adult fly heads. (A) Wild-type, (B) *ey-GAL4/UAS-ey*, (C) *ey-GAL4/UAS-eya*, (D) *ey-GAL4/UAS-so*, (E) *dpp-GAL4/UAS-so*, (F) *ey-GAL4/UAS-dac*. Adult eyes that develop from otherwise wild-type eye discs in which the early eye proteins were exogenously expressed during larval stages contain fewer facets than wild type (compare B-E with A). In addition, these eyes show a loss of the regular arrangement of facets that is characteristic of wild-type eyes. Exogenous expression of Ey (B) and So (D,E) show the greatest effects; exogenous expression of Eya (C) shows the weakest effects.

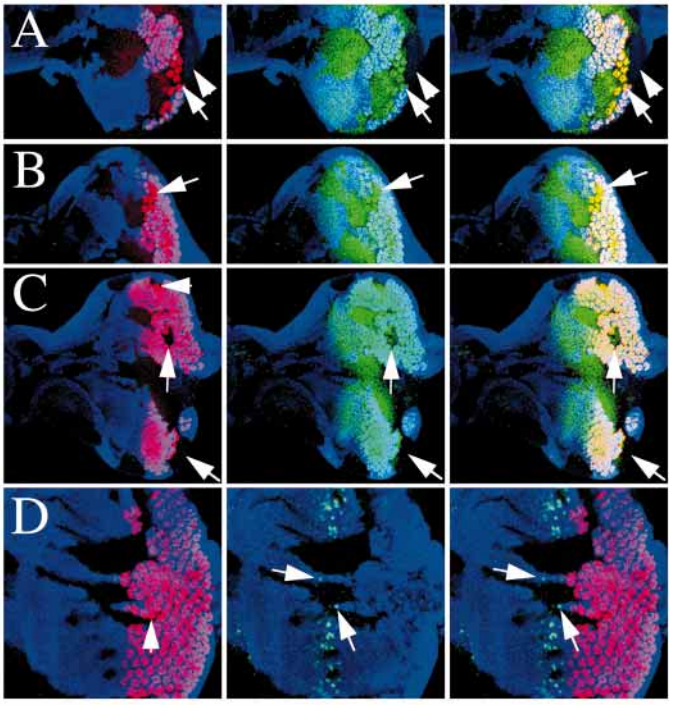

Fig. 7. Dpp and Hh are redundantly required for MF progression. All panels show confocal images of late third instar eye discs. (A,B) Eye discs containing clones homozygous for the *smo*^{3} allele; (C,D) eye disc containing clones homozygous for both *smo*^{3} and *Mad*$^{1\text{-}2}$. The three panels in A-D show the same disc. Mosaic eye discs are stained (green) with anti-Eya (A-C) or anti-Ato (D), and with anti-ELAV (red) to mark developing photoreceptors (A-D). Clones are marked by the absence of the clonal marker *arm-lacZ* (blue) in all panels. Left, middle and right panels show merge of red and blue channels, green and blue channels, and all three channels, respectively. Photoreceptors (red) fail to form and Eya (green) is not expressed in *smo*^{3} clones that touch the margins (arrowhead in A). However, in *smo*^{3} clones that lie within the interior of the disc, and in regions of *smo*^{3} posterior margin clones where eye differentiation has spread from surrounding *smo*$^{+}$ tissue (arrows in A,B), Eya is expressed normally and photoreceptors develop. In contrast, although Eya is expressed normally, photoreceptor development fails in both marginal and interior *smo*^{3},*Mad*$^{1\text{-}2}$ clones (arrows C). Occasionally, part of an ommatidium develops along the edge of a *smo*^{3},*Mad*$^{1\text{-}2}$ clone (red, arrowheads in left panels of C,D). In *smo*^{3},*Mad*$^{1\text{-}2}$ clones, Ato expression in presumptive R8 cells fails in an autonomous fashion. However, Ato expression, and therefore R8 development, can occur in even narrow regions of *smo*$^{+}$,*Mad*$^{+}$ tissue (arrows in D).

Domínguez, 1999; Greenwood and Struhl, 1999), suggesting an important role for Hh signaling in MF progression. Consistent with these reports, photoreceptor development is blocked in central regions of marginal *smo*^{3} clones. However, this effect is nonautonomous: photoreceptors develop along the outer edges of marginal clones and, eventually, throughout internal clones. In the regions of marginal clones that fail to develop photoreceptors, Eya (arrowhead, Fig. 7A) and Dac (not shown) are not expressed, and Ey (not shown) is expressed at high levels. In contrast, Eya (arrowheads, Fig. 7A,B) and Dac (not shown) are expressed, and Ey (not shown) expression is downregulated, along the edges of marginal clones where photoreceptors develop, and in internal clones. These results are very similar to those for *Mad*$^{1\text{-}2}$ clones (Fig. 3), and suggest that *hh* and *dpp* play similar roles in eye development. Both are essential for MF initiation, and are required for regulating MF initiation-associated expression of Eya and Dac, but not Ey. However, neither is absolutely required for MF progression, and neither regulates MF progression-associated expression of the early eye genes.

Both *Mad*$^{1\text{-}2}$ and *smo*^{3} clones interfere with eye development in a nonautonomous fashion, i.e. the effects of each can be partially "rescued" by factor(s) that diffuse into the clone from surrounding tissue. We therefore asked whether they might play redundant roles in this process, as well as in the regulation of early eye gene expression. To test this, we generated clones doubly mutant for *Mad*$^{1\text{-}2}$ and *smo*^{3}. Such clones never develop photoreceptors whether they lie at the margin or in the interior of the eye disc (arrows, Fig. 7C), suggesting that the absence of both genes interferes autonomously both with MF initiation and with MF progression. Similar results were obtained by Greenwood and Struhl (1999) using *tkv, smo* clones. *dpp* and *hh* therefore play redundant roles in eye development.

In contrast to the results of Greenwood and Struhl (1999), who found that no photoreceptors ever formed in *tkv, smo* double mutant tissue, we occasionally find one ommatidium that forms along the edge of the wild-type tissue, such that some of the component photoreceptors lie within the clone (arrowheads in Fig. 7C,D). One possible explanation is that as long as the founding R8 photoreceptor develops within the wild-type tissue, the other photoreceptors can be recruited from the surrounding *smo*^{3}, *Mad*$^{1\text{-}2}$ cells. If so, formation of R8 cells should behave absolutely autonomously with respect to the clonal boundary. Indeed, we find that Atonal, which is expressed in R8 cells and is required for their development (Jarman et al., 1994), is not expressed in *smo*^{3}, *Mad*$^{1\text{-}2}$ clones in an autonomous fashion (Fig. 7D).

Interestingly, Eya (Fig. 7C) and Dac (not shown) are still expressed in double mutant clones that lie in the interior of the disc, and expression also occurs in the regions of marginal clones that are close to developing eye fields outside of the clone. Downregulation of Ey expression closely follows the presence of developing photoreceptors, and thus the clonal boundaries (not shown). Thus, still another factor, other than *dpp* or *hh*, is required to regulate expression of at least Eya and Dac once the MF has initiated.

DISCUSSION

The coordination of expression of all the factors required for eye development is an immense task. However, many complex developmental processes require only one or a few transcription factors that initiate regulatory cascades controlling the expression of other necessary genes. Genes such as *ey*, *eya*, *so* and *dac* appear to be at the top of such a regulatory cascade in the eye. The fact that there are several genes acting at approximately the same point in the hierarchy raises questions about how they interact with each other, as well as how they coordinate with factors such as Dpp and Hh that are responsible for regulating growth and patterning.

ey and *dpp* cooperate to initiate eye development

The lack of *eya*, *so* and *dac* expression in *Mad*$^{1\text{-}2}$ clones that

lie at the margins of the eye disc prior to MF initiation reflects a role for *dpp* in controlling early eye gene expression at these stages of eye development. Evidence from several studies, including ours, suggests that *ey* acts together with *dpp* at or near the top of the hierarchy. First, *ey* expression is not regulated by *dpp* (Figs 1, 3; Chen et al., 1999). Second, *ey* and *dpp* are both required for *eya*, *so* and *dac* expression prior to MF initiation (Figs 1, 2; Halder et al., 1998; Chen et al., 1999). Third, *ey* is not capable of rescuing dpp^{blk} eye development (not shown) or of inducing ectopic eyes in regions of imaginal discs in which *dpp* is not already expressed (Halder et al., 1998; Chen et al., 1999). These observations suggest that *ey* functions upstream of or in parallel with *dpp*. The possibility that *ey* is responsible for *dpp* expression, leading indirectly to *eya*, *so* and *dac* expression, is unlikely. Since *ey* cannot induce ectopic eyes without a source of *dpp*, it probably cannot induce *dpp* expression, at least not in the absence of factors that are specific to the eye disc. Moreover, Ey protein binds to the regulatory region of *so* (Niimi et al., 1999), suggesting it is directly involved in *so* regulation. Thus, it is likely that *ey* and *dpp* cooperate to induce expression of the other early eye genes.

Such cooperation could achieve two ends. First, *ey* is expressed throughout the eye disc and from embryonic stages of development through MF initiation. However, induction of *eya*, *so* and *dac* expression and MF initiation occurs approximately 48 hours later, around the time of the transition between second and third instars. Moreover, *eya*, *so* and *dac* are not expressed throughout the eye disc as *ey* is, but have stronger levels of expression around the margins than in other regions. The initiation of *dpp* expression at the posterior margin at approximately the same time suggests that it could be the spatiotemporal signal that sets the MF in motion. Second, *dpp* induces expression of tissue-specific genes as part of its role in patterning many diverse structures in *Drosophila*. An interaction with *ey* could be essential to ensuring that in the eye imaginal disc *dpp* initiates factors that are appropriate to eye development, such as *eya*, *so* and *dac*. Similar interactions between tissue-specification factors and growth and patterning factors are likely to be common in development. For instance, Lab and Exd both bind to a *dpp*-responsive enhancer required for *labial* expression in the endoderm (Grieder et al., 1997).

eya functions downstream of *dpp*

Our data show that *dpp* is required for *eya*, *so* and *dac* expression, suggesting that *dpp* lies upstream of all three (Figs 1, 2). For *dac*, this conclusion is supported by the experiments of other groups. *dac* clearly lies farthest downstream of all the genes that we have considered: *dpp*, *eya* and *so* are all required for its expression (Figs 1, 2; Pignoni et al., 1997; Chen et al., 1997, 1999), but *dac* is not required for *dpp* or *eya* expression (Mardon et al., 1994; Chen et al., 1997). However, the relationship between *dpp*, *eya* and *so* is less clear. *eya* and *so* are apparently required for initiating or maintaining *dpp* expression (Pignoni et al., 1997; Hazelett et al., 1998), suggesting that positive feedback loops are important in regulating the expression of these three genes. Thus, it is difficult to determine, based on the expression analysis alone, whether *dpp* is required for initiating expression of *eya* and *so*, which maintain their own expression by maintaining that of *dpp*, or vice versa.

Our functional analysis in loss-of-function *dpp* backgrounds provides additional information. Interestingly, *eya* but not *so* or *dac*, is able to rescue loss-of-function *dpp* phenotypes. Thus, *eya* lies downstream of *dpp* in a functional sense: once *eya* is expressed, it can carry out all of its functions in the absence of *dpp*. In addition, exogenous *eya* initiates expression of both *so* and *dac*, suggesting that *eya* regulates expression of both of these genes during wild-type eye development.

The ability of *eya* to rescue loss of *dpp* function is at odds with the results of Chen et al. (1999), who found that *eya* is incapable of inducing ectopic eyes in the absence of *dpp*. One reason for this may be differences in the molecular environments between the eye disc and the other imaginal discs. There are likely to be eye-specific factors that are independent of the early eye gene heirarchy, but that interact with it to promote eye development. In support of this hypothesis, *eya* is more effective at inducing ectopic eyes in the antennal disc than in imaginal discs such as the wing and leg discs (Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997). Since the antennal disc is attached to the eye disc, the environment might be more similar to that of the eye disc than in other imaginal discs

Interestingly, *eya* is much more effective at rescuing the dorsal half of both the dpp^{blk} and dpp^{12}/dpp^{blk} eyes than the ventral half (Fig. 4). The small eye that develops in these animals lies almost entirely within the dorsal portion of the eye disc (Chanut and Heberlein, 1997a). Similarly, other mutations in *dpp*, or in components of the *dpp* signaling pathway, affect the ventral half of the eye more strongly than the dorsal half (Wiersdorff et al., 1996). Thus, besides its function in MF initiation along the entire extent of the posterior and lateral margins, *dpp* may play an additional role in the ventral half of the eye. Possibly *dpp* is required to antagonize an inhibitor of MF initiation in the ventral half of the eye disc.

eya induces *so* and *dac*, leading to MF initiation

If the hierarchy were a linear pathway in which *dpp* induces *eya*, which then initiates *so* and *dac*, then exogenous *so* or *dac* should be able to rescue the phenotype of mutations in upstream factors. However, neither is able to rescue the dpp^{blk} phenotype. For *dac*, since there is a great deal of evidence that *dac* lies downstream of the other early eye genes, the simplest explanation for this result is that *dpp* acts through *eya* to produce a factor with which *dac* must interact to promote MF initiation. Possibly the factor is *eya* itself, or *so*, neither of which can be induced by *dac* in the absence of *dpp* (Fig. 5).

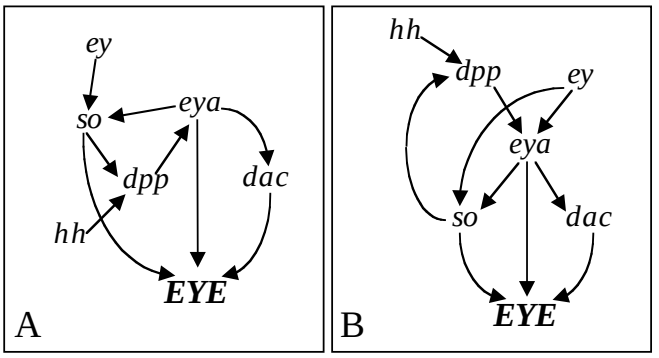

Fig. 8. (A,B) Schematic representations of two possible hierarchies including the early eye genes and *dpp*. See text for details.

The fact that *so* is unable to rescue dpp^{blk} suggests two possibilities. *so* may act upstream of *dpp* to initiate its expression, leading to *eya* expression and a feedback loop that maintains expression of *eya* and *so*. In this model, *eya* goes on to induce *dac* expression, possibly with *so*, and one or more combinations of these three proteins leads to the initiation of eye development (Fig. 8A). Alternatively, *so* expression could be initiated downstream of *dpp* by *eya*. In this scenario *so*, like *dac*, requires *eya* or additional factors that are induced by *eya*. Once present, the factors interact to initiate eye development (Fig. 8B). Although Ey is known to bind directly to the *so* regulatory region (Niimi et al., 1999), it may do so as part of a complex that regulates expression of the early eye genes and mediates MF initiation (see below). Moreover, *so* is unable by itself to induce ectopic expression of the other early eye genes and generate ectopic eyes (Pignoni et al., 1997). Finally, *eya* can induce expression of *so* in a dpp^{blk} eye disc, but *so* cannot induce expression of *eya* in a dpp^{blk} eye disc. We therefore favor the latter model.

As with Mad^{1-2} clones, Ey is expressed, and Eya and Dac are not expressed, at the center of marginal smo^{3} clones or smo^{3}, Mad^{1-2} clones from late third instar larvae (Fig. 7) This suggests that Hh is also required for regulating the expression of the early eye genes prior to MF initiation. Like *dpp*, *hh* is expressed at the posterior margin prior to MF initiation (Royet and Finkelstein, 1997; Dominguez and Hafen, 1997; Borod and Heberlein, 1998), and is required for MF initiation (Dominguez and Hafen, 1997). Dpp and Hh could play parallel roles in regulating early eye gene expression. However, since Hh is required for *dpp* expression at the posterior margin prior to MF initiation, (Borod and Heberlein, 1998), it seems more likely that Hh participates in regulating early eye gene expression through Dpp (Fig. 8).

Dpp and maintenance of the correct levels of the early eye genes

Several observations indicate that maintaining a balance of the relative amounts of the early eye proteins present at the posterior margin during MF initiation is crucial to the process. For instance, exogenous expression of Ey, Eya, So or Dac has deleterious effects on eye development. Strikingly, rather than rescuing the eye phenotype of dpp^{blk}, exogenous expression of Ey, So or Dac interferes to produce even smaller eyes (Fig. 5). Moreover, exogenous overexpression of any one of the four proteins can interfere with wild-type eye development as well (Fig. 6). Finally, although Eya alone can partially rescue dpp^{blk}, coexpression of Eya and So, or Eya and Dac, interferes with dpp^{blk} eye development nearly as well as So or Dac alone (our unpublished observations). One possible interpretation of these results is that overexpression of these proteins, which are likely to function as transcription factors, could overwhelm existing regulatory mechanisms.

Alternatively, the early eye proteins could function in a complex that participates in inducing and maintaining expression of the others, and in initiating the MF. In support of this hypothesis, it has been suggested that these proteins function in a complex, based on the fact that co-expression of various combinations of the early eye genes are much better at inducing formation of ectopic eyes than expression of any one alone, and that Eya and So, as well as Eya and Dac, can interact physically (Bonini et al., 1997; Pignoni et al., 1997; Chen et al., 1997, 1999). Our results could extend such a hypothesis to include Ey, since it interferes with wild-type eye development as well as the others. In addition, despite the fact that *ey-GAL4* directs expression throughout the region of the eye disc anterior to the MF, the actual detectable expression domain of the early eye protein being directed is more limited (in *ey-GAL4/UAS-dac* eye discs expression of Dac is detected only at the margins, Fig. 4). This suggests that Dac is unstable except at the margin, where it may be able to form complexes with additional factors.

As a signaling molecule involved in tissue patterning, *dpp* is in a good position to coordinate the regulation of protein expression, such that the proteins reach appropriate levels relative to one another. Our work indicates that *dpp* is a key player in coordinating the critical balance between the early eye proteins, possibly by a feedback loop in which *dpp* participates with *eya* and *so*. A positive feedback loop that includes *eya*, *so* and *dpp* could help reinforce or maintain expression of these factors at the posterior margin until MF initiation has occurred. In addition, since the balance of protein amounts appears to be critical, the feedback loop could ensure that each protein is expressed at the right level relative to the others.

Dpp and Hh play redundant roles during MF progression

Although Dpp and Hh are clearly required in MF initiation, their roles in MF progression are less clear. Photoreceptor development is "rescued" in Mad^{1-2} clones, apparently by a diffusible factor(s) that has a source in either the MF or the developing eye field itself: the eye fields generated in Mad^{1-2} tissue when a MF spreads into it are always contiguous with an existing eye field. Likewise, photoreceptors can develop in smo^{3} clones, probably also through the influence of factor(s) generated outside of the clone (Fig. 7; Strutt and Mlodzik, 1997; Domínguez, 1999; Greenwood and Struhl, 1999). In contrast, smo^{3},Mad^{1-2} double mutant clones are not "rescued" by surrounding wild-type tissue.

One possible explanation for these results is that Hh and Dpp play redundant roles during MF initiation, such that diffusion of Hh from surrounding wild-type tissue rescues Mad^{1-2} clones, and vice versa. It is not likely that the functions of the two genes are normally equivalent. *hh* and *dpp* are expressed in different cells during MF progression, and the effects on MF progression of loss-of-function mutations or ectopic expression of the two genes are not exactly the same. For instance, smo^{3} clones hinder MF progression to a greater extent than Mad^{1-2} clones do.

hh regulates expression of *dpp* during MF progression (Heberlein et al., 1993, 1995). Thus, it could be argued that Dpp cannot rescue the effects of smo^{3} because it is not expressed in the absence of Hh signal. However, Dpp is expressed in the cells surrounding smo^{3} clones, and cannot be ruled out as the "rescuing" factor. Moreover, Dpp has a known role in mediating the effects of Hh signal in other imaginal discs (Zecca et al., 1995; Lecuit et al., 1996; Nellen et al., 1996). What is perhaps more surprising is that Hh might rescue Mad^{1-2}. This might be attributable to additional functions Hh may have that are not mediated by Dpp, functions that have been proposed both in eye development and elsewhere (Treisman and Heberlein, 1998; Mullor et al., 1997; Strigini and Cohen, 1997).

Another potential problem with proposing redundant

functions for Dpp and Hh is that whereas Hh is able to initiate ectopic furrows anywhere in the eye disc anterior to the endogenous MF (Heberlein et al., 1995; Pignoni and Zipursky, 1997), ectopic MF formation by Dpp occurs only at the anterior margin (Burke and Basler, 1996; Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997). This could indicate that Dpp is not sufficient, in the absence of Hh, to promote MF progression. In this case it seems likely that additional, unknown, factors are involved. For instance, if some other diffusible molecule can "rescue" the effects of smo^3 but not Mad^{1-2}, and Hh can "rescue" the effects of Mad^{1-2}, then the single mutant but not the double mutant clones would be "rescued" as we have observed. A similar proposal has been put forth by Greenwood and Struhl (1999), in which an unidentified signal transduced by Raf is the other factor involved. However, it seems equally likely that Dpp and Hh can substitute for one another, and that the unidentified factor(s) play permissive roles.

Although the functions of Dpp and Hh during MF progression are still not entirely known, it is already clear that they include regulation of gene expression (Greenwood and Struhl, 1999). However, despite the importance of Dpp and Hh in regulating expression of the early eye genes prior to MF initiation, early eye gene expression during MF progression appears to be independent of the activity of these signaling molecules. *eya* and *so*, at least, are expressed within and posterior to the MF, and required for MF progression and subsequent photoreceptor development (Pignoni et al., 1997). Thus, other factors important for early eye gene expression, and therefore more generally for eye development, remain to be identified.

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