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decapentaplegic and *wingless* are regulated by *eyes absent* and *eyegone* and interact to direct the pattern of retinal differentiation in the eye disc

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SUMMARY

Signaling by the secreted hedgehog, decapentaplegic and wingless proteins organizes the pattern of photoreceptor differentiation within the *Drosophila* eye imaginal disc; hedgehog and decapentaplegic are required for differentiation to initiate at the posterior margin and progress across the disc, while wingless prevents it from initiating at the lateral margins. Our analysis of these interactions has shown that initiation requires both the presence of decapentaplegic and the absence of wingless, which inhibits photoreceptor differentiation downstream of the reception of the decapentaplegic signal. However, wingless is unable to inhibit differentiation driven by activation of the epidermal growth factor receptor pathway. The effect of wingless is subject to regional variations in control, as the anterior margin of the disc is insensitive to wingless inhibition. The *eyes absent* and *eyegone* genes encode members of a group of nuclear proteins required to specify the fate of the eye imaginal disc. We show that both *eyes absent* and *eyegone* are required for normal activation of *decapentaplegic* expression at the posterior and lateral margins of the disc, and repression of *wingless* expression in presumptive retinal tissue. The requirement for *eyegone* can be alleviated by inhibition of the wingless signaling pathway, suggesting that *eyegone* promotes eye development primarily by repressing *wingless*. These results provide a link between the early specification and later differentiation of the eye disc.

Key words: *Drosophila*, Eye development, *decapentaplegic*, *wingless*, Pattern formation, Imaginal disc, *eyes absent*, *eyegone*

INTRODUCTION

A small number of signaling pathways is used repeatedly to direct developmental processes in both *Drosophila* and vertebrates. Members of the hedgehog (Fietz et al., 1994), TGF-β (Kingsley, 1994) and Wnt (Cadigan and Nusse, 1997) families, typified by hedgehog (hh), decapentaplegic (dpp) and wingless (wg) in *Drosophila*, establish patterns of growth and differentiation at multiple stages of development. However, the interactions between these pathways can vary, allowing each tissue and appendage to acquire its characteristic properties. In *Drosophila*, hh can activate the expression of either *wg* or *dpp* (Basler and Struhl, 1994; DiNardo et al., 1994; Heberlein et al., 1993; Ma et al., 1993), while dpp and wg signaling can interact antagonistically (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996; Treisman and Rubin, 1995) or cooperate to activate specific targets (Campbell et al., 1993; Lecuit and Cohen, 1997; Riese et al., 1997). Both the patterns of expression of these genes, and the rules governing their interactions, must be established for each tissue by specific regulators.

The spatial control of differentiation in the eye imaginal disc requires the coordination and regulation of these three signals. During the third larval instar, differentiation of photoreceptor clusters begins at the posterior margin of the eye disc and gradually spreads anteriorly (Ready et al., 1976). The initiation of differentiation requires *hh*, which is expressed at the posterior margin of second instar larval discs and activates *dpp* expression there (Dominguez and Hafen, 1997; Royet and Finkelstein, 1997). *dpp* function is also required for initiation, which does not occur in cells unable to receive the dpp signal (Burke and Basler, 1996; Chanut and Heberlein, 1997; Wiersdorff et al., 1996). Although *dpp* is expressed at the lateral margins as well as the posterior margin (Masucci et al., 1990), initiation from the lateral margins is prevented by the presence of wg (Ma and Moses, 1995; Treisman and Rubin, 1995). *wg* expression is absent from the posterior margin due to dpp signaling (Wiersdorff et al., 1996), and its ectopic expression there can block initiation (Treisman and Rubin, 1995). However, it is not clear how the expression domains of *dpp* and *wg* are first established. Because hh pathway activity leads to *dpp* expression in posterior regions of the eye disc and

wg expression in anterior regions (Dominguez and Hafen, 1997; Royet and Finkelstein, 1997; Heberlein et al., 1995), other factors must help to determine the specificity of the response to hh.

The anterior progression of differentiation is also driven by hh and dpp signaling, as inactivation of either protein using temperature-sensitive mutations arrests the process (Chanut and Heberlein, 1997; Ma et al., 1993). However, local loss of cell-autonomous downstream components of either signaling pathway does not have a dramatic effect on progression (Burke and Basler, 1996; Penton et al., 1997; Strutt and Mlodzik, 1997; Wiersdorff et al., 1996), suggesting that there is some redundancy between the two pathways, or that a third signal is involved. *hh*, now expressed in the differentiating photoreceptors, is required to activate a stripe of *dpp* expression in the morphogenetic furrow (Heberlein et al., 1993; Ma et al., 1993), the point at which cells undergo a shape change just prior to their differentiation (Ready et al., 1976). Ectopic expression of *hh*, but not *dpp*, is sufficient to trigger ectopic photoreceptor differentiation anterior to the normal furrow (Heberlein et al., 1995; Pignoni and Zipursky, 1997); however, ectopic *dpp* is effective in initiating a new morphogenetic furrow from the anterior margin of the disc, and can do so from a considerable distance (Pignoni and Zipursky, 1997; Chanut and Heberlein, 1997). The progression of differentiation through internal regions of the eye disc is still inhibited by ectopic *wg* (Treisman and Rubin, 1995).

The process by which hh or dpp signaling leads to photoreceptor differentiation is not entirely clear; it involves the expression of *atonal* (*ato*), a proneural gene encoding a helix-loop-helix (HLH) protein that is absolutely required for photoreceptor formation (Jarman et al., 1993, 1994, 1995), and the repression of *hairy*, encoding another HLH protein that inhibits premature photoreceptor formation in combination with extramacrochaetae (Brown et al., 1995; Heberlein et al., 1995). *ato* appears to specify R8, the first photoreceptor to form in each cluster; repeated activation of the epidermal growth factor (EGF) receptor signaling pathway by the spitz (spi) ligand then recruits the remaining cells of the cluster (Freeman, 1994, 1996; Tio et al., 1994; Tio and Moses, 1997).

Prior to the initiation of photoreceptor differentiation, a group of genes including *eyeless* (*ey*), *eyes absent* (*eya*), *sine oculis* (*so*), *eyegone* (*eyg*) and *dachshund* (*dac*) acts to determine the fate of cells in the eye disc, and is likely to control any differences in signaling mechanisms between the eye disc and other imaginal discs. All these genes are required for eye formation and have some ability to induce ectopic eye development in other imaginal discs (Bonini et al., 1993, 1997; Chen et al., 1997; Cheyette et al., 1994; Halder et al., 1995; Mardon et al., 1994; Pignoni et al., 1997; Quiring et al., 1994; Serikaku and O'Tousa, 1994; Shen and Mardon, 1997; C. Desplan and H. Sun, personal communication). They all encode nuclear proteins: *ey* encodes Pax-6 (Quiring et al., 1994) and *eyg* another Pax-like protein (C. Desplan and H. Sun, personal communication); *so* encodes a divergent homeodomain protein (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), and *eya* and *dac* encode novel nuclear factors (Bonini et al., 1993; Mardon et al., 1994). The eya protein has been demonstrated to interact molecularly with both so and dac (Chen et al., 1997; Pignoni et al., 1997), suggesting that complexes between these molecules may activate target genes required for eye development. *ey* is first expressed in the embryonic eye disc primordium (Quiring et al., 1994), while *so* is expressed and required in the entire visual system (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Later expression of *so* and *eya* in the eye disc in the second and early third instar stages, in gradients with their highest levels at the posterior margin (Bonini et al., 1993; Mardon et al., 1994), is dependent on *ey* (Halder et al., 1998); expression of *dac* in a similar pattern requires *eya* (Chen et al., 1997). While ectopic *ey* expression efficiently induces eye development on the legs, wings and antennae (Halder et al., 1995), the other genes have a weaker ability to induce ectopic eyes and only do so appreciably when *eya* is expressed in combination with either *so* or *dac* (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997).

We have examined the mechanism by which wg prevents photoreceptor development; we show that wg acts downstream of the dpp receptor thick veins and thus its inhibitory effect is not mediated by repression of the expression or activity of the dpp protein. One of the consequences of dpp function is the induction of photoreceptor development; this also requires activation of the GTP-binding protein ras by EGF receptor signaling (Simon et al., 1991; Xu and Rubin, 1993; Freeman, 1996). We show here that wg acts upstream of ras activation. To determine how the expression patterns of *dpp* and *wg* are regulated, we have tested the effects of *eyg* and *eya*, two genes essential for eye development. We show that both of these genes contribute to the activation of *dpp* expression and the inhibition of *wg* expression. The absence of photoreceptor development observed in *eyg* mutants can be rescued by inhibition of wg signaling, suggesting that repression of *wg* expression is a critical function of *eyg*.

MATERIALS AND METHODS

Fly strains

Alleles used were eyg^{1} (Lindsley and Zimm, 1992), $Df(3L)iro^{2}$ (Gomez-Skarmeta et al. 1996), eya^{1} (Bonini et al., 1993), Mad^{B1} (Wiersdorff et al., 1996), wg^{CX2} (Baker, 1987), $punt^{l(3)10460}$ (Ruberte et al., 1995), sog^{Y506} (Ferguson and Anderson, 1992), omb^{282} (Lecuit et al., 1996), and $l(1)omb^{D4}$ (Grimm and Pflugfelder, 1996). The reporters were *dpp-lacZ* BS3.0 (Blackman et al., 1991) and wg^{P} (Kassis et al., 1992). Transgenic lines used were UAS-tkv^{QD} (Nellen et al., 1996), UAS-*dTCFΔN* (van de Wetering et al., 1997), UAS-*dpp* (Frasch, 1995), UAS-*wg*, UAS-*hh* (Azpiazu et al., 1996), UAS-*s-spi* (Schweitzer et al., 1995b), UAS-ras^{v12} (Karim and Rubin, 1998), UAS-*fluΔarm* (Zecca et al., 1996), UAS-*omb* (Grimm and Pflugfelder, 1996), and *Act>CD2>*GAL4 (Pignoni and Zipursky, 1997). *dpp*-GAL4 was constructed by cloning the 10 kb *Kpn*I-*Xba*I-fragment of the *dpp* 3′ region used to make BS3.0 (Blackman et al., 1991) upstream of a *Not*I-*Kpn*I fragment containing the minimal *hsp70* promoter (Hiromi and Gehring, 1987) including 60 bp upstream and 200 bp downstream of the transcription start site, and placing this upstream of a *Kpn*I-*Xba*I fragment containing the GAL4-coding region and the α-tubulin 3′UTR (Brand and Perrimon, 1993) in the pCasPeR4 vector (Pirrotta, 1988). Unlike the shorter enhancer previously used (Staehling-Hampton et al., 1994), this driver gives expression of *lacZ* at the posterior margin that is as strong as its expression at the lateral margins. However, expression does not move anteriorly with the morphogenetic furrow. UAS-sgg^{act} (also referred to as UAS-sgg^{S9A}) was constructed by site-directed mutagenesis of the cDNA coding for the most abundant sgg protein (SGG10) according

to the protocols of the pALT system (Promega). The codon corresponding to ser-9 was changed into ala and verified by sequencing. A mutagenised 2.0 kb *Bgl*II-*Xba*I fragment was then cloned into the pUAST vector (Brand and Perrimon, 1993). *ey*-GAL4 was constructed by cloning a 3.6 kb *Eco*RI fragment containing the eye-specific enhancer of the *ey* gene into the vector p221-4 (a gift of E. Knust). p221-4 contains the GAL4 gene with a hsp70 minimal promotor in front of it.

Mosaic analysis

To make loss-of-function clones, $punt^{l(3)10460}$, eyg^{1}, eya^{1}, Mad^{B1}, sog^{YS04}, omb^{282} and $l(1)omb^{D4}$ were recombined with FRT elements at positions 82, 80, 40 and 18, respectively (Xu and Rubin, 1993). An FRT element at position 40 was recombined successively with wg^{CX2} and Mad^{B1} to create a doubly mutant chromosome arm. Males of the resulting FRT lines were crossed with females carrying the same FRT element, an *arm-lacZ* (Vincent et al., 1994) P element on the same chromosome arm (except for the *punt* clone in Fig. 1B and the *eya* clone in Fig. 4B), and either *hsFLP1* (Xu and Rubin, 1993) or *eyFLP1* (a gift of B. Dickson). Crosses using *hsFLP1* were heat shocked for 1 hour at 38˚C in both first and second instar. To make gain-of-function clones, a stock carrying *hsFLP1*, *Act>CD2>*GAL4 and either UAS-*lacZ* or *wg-lacZ* was constructed and crossed to other UAS lines, either individually or in combinations. Larvae were heat shocked 30 minutes at 37˚C in either second instar (for combinations including UAS-*dpp*) or first instar (for all other lines and combinations).

Histology

Eye discs were stained as described by Treisman and Rubin (1995), except that the fix used was 4% formaldehyde in PEM. Rat anti-elav (Robinow and White, 1991) was diluted 1:1, mouse anti-wg (Brook and Cohen, 1996) was diluted 1:10, mouse anti-omb (Grimm and Pflugfelder, 1996) was diluted 1:100, mouse anti-dac (Mardon et al., 1994) was diluted 1:5 and rabbit anti-ato (Jarman et al., 1995) was diluted 1:5000.

RESULTS

Furrow initiation requires dpp signaling even in the absence of the inhibitory wg signal

The initiation of photoreceptor development at the posterior margin of the eye disc requires dpp signaling (Burke and Basler, 1996; Chanut and Heberlein, 1997; Heberlein et al., 1993; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996; Fig. 1A), and loss-of-function of components of the dpp pathway at the posterior margin results in the ectopic expression of *wg* in the mutant cells (Wiersdorff et al., 1996; Fig. 1B). Since the presence of wg at this position is sufficient to prevent morphogenetic furrow initiation (Treisman and Rubin, 1995), it is possible that the only requirement for dpp in initiation is to repress *wg*. This hypothesis has been proposed by Dominguez and Hafen (1997), based on their observation that clones of cells mutant for *protein kinase A* (*PKA*), in which the hh pathway is ectopically activated, can develop as photoreceptors in the anterior of the disc even when they lack both *dpp* and *wg*. However, it is not clear that the mechanism of normal furrow initiation can be inferred from the effects of loss of *PKA* in anterior regions. As a more direct test, we examined clones of cells mutant for both *Mothers against dpp* (*Mad*), which encodes an intracellular component required to transduce the dpp signal (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996), and a null allele of *wg*. Cells in these clones are unable to respond to dpp, but are also unable to produce wg. When such clones of cells occur at the posterior margin of the eye disc, they autonomously fail to initiate photoreceptor development (Fig. 1C). Clones of cells singly mutant for *Mad* also fail to differentiate as photoreceptors, but often have an additional non-autonomous inhibitory effect on photoreceptor differentiation by surrounding cells, which is likely to be mediated by wg (Fig. 1A). Thus dpp signaling is required not only to repress *wg* expression, but also independently for morphogenetic furrow initiation.

wg inhibits photoreceptor formation downstream of the dpp receptors

wg is required to prevent ectopic morphogenetic furrow initiation from the lateral margins of the eye disc (Ma and Moses, 1995; Treisman and Rubin, 1995). However, the mechanism by which wg inhibits photoreceptor differentiation is not well understood. It has been suggested that wg acts by preventing *dpp* expression, as *dpp* expression is lost in clones of cells lacking the kinase encoded by *shaggy/zeste-white 3* (*sgg*) (Heslip et al., 1997), which normally functions to inhibit the wg pathway. However, a low level of ectopic *wg* can inhibit photoreceptor differentiation without reducing *dpp* expression (Treisman and Rubin, 1995). As dpp positively autoregulates its own expression (Wiersdorff et al., 1996), inhibition of dpp function may result in a loss of *dpp* expression. The ability of wg to inhibit differentiation in the presence of dpp is illustrated in Fig. 2B, in which an eye-specific enhancer from the *eyeless* (*ey*) gene (Quiring et al., 1994) drives GAL4 to express a UAS-*wg* transgene throughout the eye disc beginning before the stage of furrow initiation (insets in Fig. 2B show the pattern of UAS-*lacZ* expression driven by *ey*-GAL4 in early and late third instar discs). If wg acted by inhibiting *dpp* expression, it should be possible to overcome its effects by expressing *dpp* from a heterologous promoter. However, co-expression of *dpp* and *wg* either under the control of the *ey*-GAL4 driver or using *hsFLP*-induced recombination to fuse GAL4 to the constitutive *Actin5C* promoter (*flp-out*-GAL4; Pignoni and Zipursky, 1997) did not allow initiation of photoreceptor development at the posterior margin (Fig. 2D,F).

Ectopic expression of *dpp* in the eye disc has been shown to specifically induce initiation of photoreceptor differentiation from the anterior margin of the disc in a non-autonomous fashion (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Fig. 2C,E). Surprisingly, we observed that this ectopic differentiation was not inhibited by wg signaling. Co-expression of *dpp* and *wg* throughout the disc under *ey*-GAL4 control resulted in initiation from the anterior margin at a much higher frequency than from the posterior margin (Fig. 2D). Furthermore, clones of cells co-expressing *dpp* and *wg* under the control of the *flp-out*-GAL4 system were still able to induce anterior morphogenetic furrow initiation, although clones at the posterior margin blocked photoreceptor differentiation (Fig. 2F). Thus initiation from the anterior margin must be able to overcome the inhibition normally caused by wg.

Another possible way for wg to inhibit dpp-induced photoreceptor differentiation would be by reducing the activity of the dpp protein. For example, wg might act on *short gastrulation* (*sog*), which encodes a secreted molecule thought, by analogy to its *Xenopus* homolog chordin, to bind the dpp protein in the ventral region of the embryo and prevent it from

binding to its receptors (Holley et al., 1995; Piccolo et al., 1996; Schmidt et al., 1995). However, loss of *sog* function at the lateral margins of the eye disc, unlike loss of *wg*, did not induce premature photoreceptor differentiation (data not shown). To test directly whether wg acts on or downstream of the dpp protein, we co-expressed *wg* with a constitutively active form of the dpp type I receptor thick veins (tkv^{QD}; Nellen et al., 1996; Lecuit et al., 1996) in the eye disc using *ey*-GAL4. Activated *tkv* was unable to overcome the inhibition caused by *wg* (Fig. 2H; compare to Fig. 2G for tkv^{QD} alone), suggesting that wg acts downstream of or in parallel to this receptor.

wg inhibits photoreceptor differentiation upstream of ras activation

Rather than affecting the dpp pathway directly, wg might block photoreceptor differentiation at a stage subsequent to dpp signaling. Formation of all photoreceptors is known to depend on the EGF receptor and its downstream component ras (Simon et al., 1991; Xu and Rubin, 1993). Furthermore, wg has recently been shown to antagonize EGF receptor signaling during the specification of the cuticle pattern in the embryo (O'Keefe et al., 1997; Szuts et al., 1997). To determine whether wg also acts on this pathway in the eye, we tested whether a secreted and active form of the ligand spitz (s-spi; Schweitzer et al., 1995b) or a constitutively active form of ras (Fortini et al., 1992; Karim and Rubin, 1998) could bypass the block caused by wg. In discs expressing both *wg* and activated *ras* ubiquitously, we observed extensive photoreceptor differentiation and growth (Fig. 2L), as in discs expressing activated *ras* alone (Karim and Rubin, 1998; Fig. 2K). Thus wg must act upstream of ras activation to block differentiation. Expression of *s-spi* also rescues photoreceptor differentiation in discs expressing *wg* ectopically (Fig. 2J; compare to Fig. 2I for *s-spi* alone). The rescue is less robust than that caused by ras activation, either because expression of *s-spi* is not sufficient to fully activate ras, or because wg blocks stages both upstream and downstream of spi activity.

To determine whether the inhibition of photoreceptor differentiation is mediated by the conventional wg signal transduction pathway, we tested the ability of a constitutively active form of armadillo (Δarm; Zecca et al., 1996), the β-catenin homolog thought to participate in transcriptional activation of wg target genes (Peifer and Wieschaus, 1990; van de Wetering et al., 1997), to block photoreceptor differentiation. When we expressed activated *arm* in clones of cells using *flp-out*-GAL4, we indeed observed a block of photoreceptor differentiation within the clone (Fig. 2M). This block was not rescued by co-expression of activated *tkv* (Fig. 2N), but was overcome by co-expression of activated *ras* (Fig. 2P), consistent with the results for ectopic *wg* expression. We also tested whether a transcription factor known to be induced by wg at the lateral margins, optomotor-blind (omb; Pflugfelder et al., 1992; Zecca et al., 1996), could mediate the inhibition. We found, however, that ectopic expression of *omb* inhibited cell growth, making it difficult to evaluate its effect on differentiation (data not shown). Loss of *omb* function at the lateral margins did not lead to ectopic photoreceptor differentiation (data not shown), so it is likely that other target genes contribute to this effect of wg. Another known target gene in the eye disc, which we have not tested, is *orthodenticle* (Royet and Finkelstein, 1997).

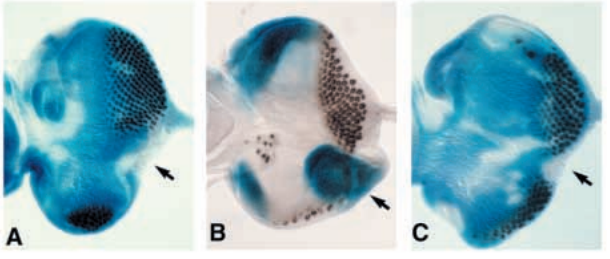

Fig. 1. Repressing *wg* expression is not the only function of dpp. All panels show third instar eye discs stained with the neuronal-specific antibody anti-elav (brown). Clones in A and C are marked by the absence of *arm-lacZ* staining (blue). (A) Posterior margin clone (arrow) mutant for Mad^{B1}. Photoreceptors do not differentiate within the clone. (B) Posterior margin clone (arrow) mutant for $punt^{l(3)10460}$, marked by lack of photoreceptor differentiation. The disc carries the wg^{P} enhancer trap, and *wg* expression is revealed by blue X-gal staining. *wg* is ectopically expressed within the clone. (C) Posterior margin clone (arrow) mutant for both Mad^{B1} and wg^{CX2}. Photoreceptors again fail to differentiate within the clone.

The expression patterns of *dpp* and *wg* are regulated by *eyes absent* and *eyegone*

The normal restriction of morphogenetic furrow initiation to the posterior margin of the eye disc is due to the presence of *hh* and *dpp* and the absence of *wg* at this position (Dominguez and Hafen, 1997; Baker, 1988; Masucci et al., 1990). Although negative regulation of *wg* expression by dpp, and dpp function by wg, provides a mechanism for the maintenance of their expression domains, it does not explain how they are established. We therefore examined whether genes implicated in early events of eye development are involved in the regulation of *dpp* and *wg* expression. One such gene is *eyes absent* (*eya*), which encodes a novel nuclear protein required for eye formation (Bonini et al., 1993). No photoreceptors form in eya^{1} mutant eye discs and extensive cell death reduces the size of the third instar disc (Bonini et al., 1993). However, prevention of cell death does not appear to be the primary function of *eya*, as clones of *eya* mutant cells proliferate extensively prior to the third instar stage (Pignoni et al., 1997), although they are replaced by wild-type head cuticle in the adult eye (data not shown). *eya* mutant cells fail to differentiate as photoreceptors, resembling *sgg* mutant cells, in which the wg pathway is over-active (Heslip et al., 1997; Treisman and Rubin, 1995). We examined the expression of *dpp* and *wg* in *eya* mutant eye discs and in clones of *eya* mutant cells. *dpp-lacZ* expression was greatly reduced in early third instar *eya* mutant discs, prior to the initiation of the morphogenetic furrow (Fig. 3A,B), and was completely lost in *eya* mutant clones (Fig. 4B; Pignoni et al., 1997), suggesting that *eya* is required for *dpp* transcription. Although the initiation of *wg* expression in early *eya* mutant eye discs appeared to be normal (Fig. 3D,E), ectopic wg protein was observed in *eya* mutant clones in late third instar discs (Fig. 4C). This wg protein appears to be active, as *omb*, a target of wg in the eye disc (Zecca et al., 1996), was also expressed in *eya* clones (Fig. 4D).

The phenotypes of eye-specific mutations in *eya* and *so* are very similar, suggesting that these genes act at the same level

in the hierarchy leading to eye disc specification (Pignoni et al., 1997). *ey* appears to act upstream of *eya* and *so* in both normal and ectopic eye development (Halder et al., 1998). We have therefore not examined the effects of mutations in *so* or *ey*. Another gene required for eye formation that has not been placed within this hierarchy is *eyegone* (*eyg*; Hunt, 1970); in its absence, no photoreceptors differentiate and the eye disc does not reach its normal size and shape (Figs 3C,F, 5A). We examined the expression patterns of *dpp* and *wg* in early third instar *eyg* mutant discs. *dpp* expression was restricted to the posterior margin of *eyg* mutant discs, in contrast to its expression around the posterior and lateral margins of wild-type discs (Fig. 3A,C). On the contrary, *wg* expression was expanded, especially on the dorsal side of the disc, where it extended to the posterior margin (Fig. 3D,F). *eyg* thus acts to delimit the domains of *dpp* and *wg* expression; since it encodes a Pax-like transcription factor (C. Desplan and H. Sun, personal communication), it is possible that this regulation is direct. Most clones of *eyg* mutant cells develop normally (Fig. 4E) and do not affect *dpp* or *wg* expression (data not shown). Possibly *eyg* mutant clones are rescued by dpp, hh or another secreted factor diffusing in from surrounding wild-type cells. Alternatively, *eyg* may act on a localized region or during a restricted time period in development.

The *eyg* phenotype results from its effect on *wg* expression

To determine whether these effects on *dpp* and *wg* expression were the basis for the effects of *eya* and *eyg* on photoreceptor development, we tested whether their mutant phenotypes could be rescued by restoration of dpp signaling or by inhibition of wg signaling. To specifically target initiation of the morphogenetic furrow in discs transheterozygous for eyg^{1} and a deficiency removing *eyg*, which completely lack photoreceptors (Fig. 5A), and to avoid early effects on growth of the eye disc, we used a *dpp*-GAL4 driver (see Materials and Methods), which directed expression at the posterior margin of wild-type and *eyg* mutant discs (Fig. 5B and data not shown). Activation of dpp signaling by expression of tkv^{act} or dpp itself at this position failed to rescue photoreceptor formation (Fig. 5C and data not shown). To inhibit the wg pathway, we expressed a constitutively active form of the protein kinase encoded by *sgg*, made by mutating serine-9, a site for inhibitory phosphorylation of the mammalian homolog glycogen synthase kinase-3β (Cross et al., 1995; Stambolic and Woodgett, 1994). sgg negatively regulates the activity of arm (Peifer et al., 1994), so a hyperactive form of sgg should block

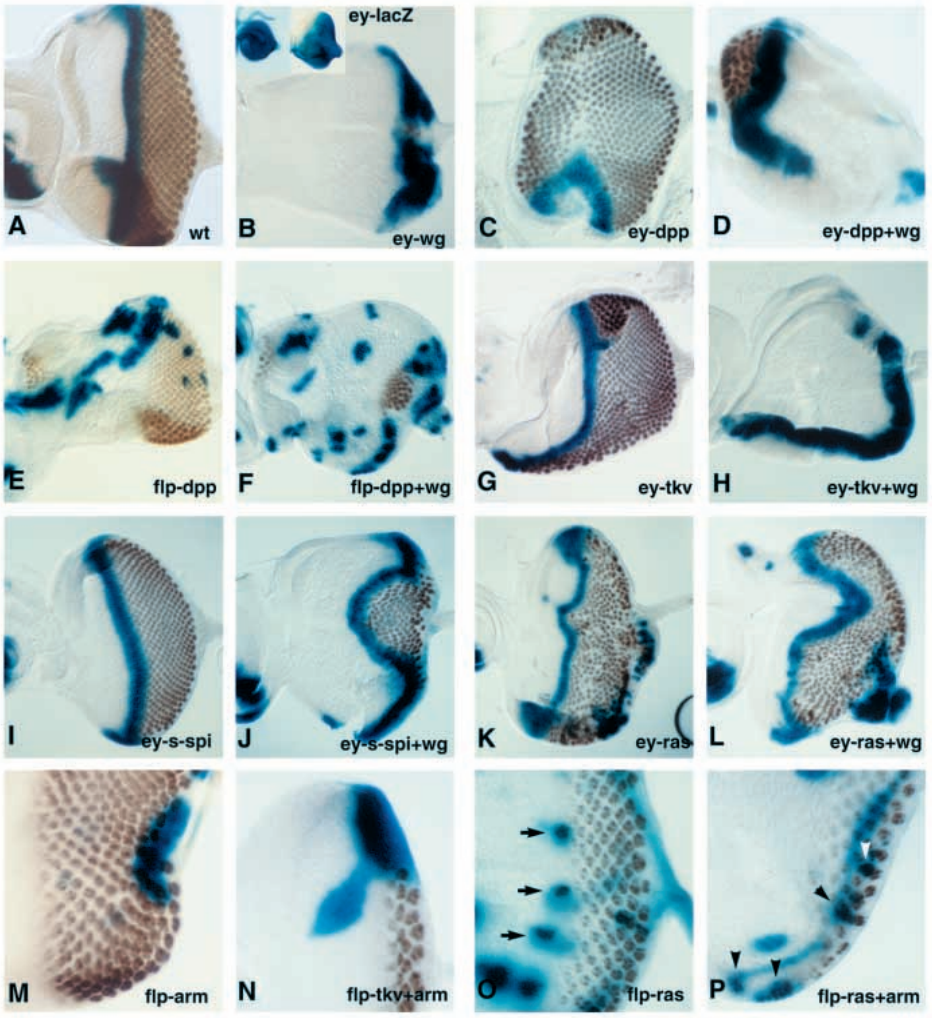

Fig. 2. wg inhibits photoreceptor differentiation downstream of tkv and upstream of ras. (A-D, G-L) Third instar eye discs carrying *ey*-GAL4 and the reporter construct *dpp-lacZ*, stained with anti-elav (brown) and X-gal (blue). (E-F, M-P) Third instar eye discs with clones of cells expressing GAL4 under the control of the *Actin5C* promoter, induced by removal of a stop signal by hs-FLP-mediated recombination (see Materials and Methods), and also carrying UAS-*lacZ*, stained with anti-elav (brown) and with X-gal (blue) to mark the clone. (A) Wild type; (B) UAS-*wg*; insets show X-gal staining of early (left) and late (right) third instar eye discs carrying *ey*-GAL4 and UAS-*lacZ*. (C, E) UAS-*dpp*; (D, F) UAS-*dpp*, UAS-*wg*; (G) UAS-tkv^{QD}; (H) UAS-tkv^{QD}, UAS-*wg*; (I) UAS-*s-spi*; (J) UAS-*s-spi*; UAS-*wg*; (K) UAS-ras^{v12} ; (L) *UAS-*ras^{v12}; *UAS-wg*. Photoreceptor differentiation is inhibited by wg at the posterior but not the anterior margin; it is not rescued by co-expression of *dpp* or activated *tkv*, but is rescued weakly by secreted *spi* and strongly by activated *ras*. (M) UAS-*Δarm*; (N) UAS-*Δarm*; UAS-tkv^{QD}; (O) UAS-ras^{v12}; (P) UAS-ras^{v12}, UAS-*Δarm*. Photoreceptor differentiation is autonomously inhibited by activated *arm*, even when activated *tkv* is co-expressed. Activated *ras* can induce ectopic photoreceptor formation anterior or posterior to the furrow (arrows in O; note that *lacZ* expression in the ectopic photoreceptors fills their axons). This photoreceptor formation is not prevented by co-expression of activated *arm* (arrowheads in P).

transmission of the wg signal. Indeed, expression of this form of *sgg* at the wing margin prevented differentiation of the *wg*-dependent margin bristles (data not shown). We also used a dominant negative form of the wg-responsive *dTCF* transcription factor (van de Wetering et al., 1997). Expression of either of these molecules led to the initiation of a morphogenetic furrow in the *eyg* mutant discs (Fig. 5E,F) and formation of small adult eyes (data not shown). Since inhibition of the wg pathway at the posterior margin of *eyg* mutant discs is sufficient to allow photoreceptor formation, we conclude that the misexpression of *wg* observed at the posterior of the *eyg* mutant discs is a major cause of the absence of photoreceptor development. As expected since it can overcome the effect of ectopic wg (Fig. 2L), activated ras was also able to rescue photoreceptor differentiation in *eyg* mutant discs (Fig. 5G,H). Interestingly, expression of *hh*, which is sufficient to induce photoreceptor differentiation in the eye disc (Heberlein et al., 1995; Dominguez and Hafen, 1997), was unable to do this in the absence of *eyg* (Fig. 5D), showing that *hh* cannot overcome the inhibition of initiation caused by *wg*; the same conclusion was reached by co-expression of *hh* and *wg* in wild-type discs (data not shown).

eya has multiple functions in promoting eye development

We attempted to rescue the *eya* mutant phenotype by expressing the same molecules under the control of *ey*-GAL4, as *dpp* expression requires *eya* and *ey* expression does not (Figs 3B, 6A; Bonini et al., 1997; Halder et al., 1998). The *ey*-GAL4 driver induces sufficient target gene expression to rescue the *eyg* phenotype, although early effects on eye disc growth are also observed (Fig. 5H and data not shown). However, neither tkv^{act}, sgg^{act}, *dpp*, *hh*, nor pairwise combinations of these factors were able to induce photoreceptor formation in *eya* mutant discs (Fig. 6B,C,E and data not shown). Expression of sgg^{act} did result in a reduction in size of the *eya* discs (Fig. 6C), as it does in wild-type discs (Fig. 6D); thus its effect on growth is not secondary to premature differentiation. The lack of *dpp* expression in *eya* mutant discs was not rescued by ectopic *hh* (Fig. 6E), suggesting that *eya* is required downstream of or in conjunction with *hh* to direct *dpp* expression; *eya* must also regulate additional factors required downstream of *dpp* for photoreceptor differentiation. Finally, we tested the ability of activated *ras* to rescue the *eya* phenotype when expressed under *ey*-GAL4 control, and found that it was also usually insufficient to allow photoreceptor differentiation (Fig. 6F); thus *eya*-regulated factors are still required downstream of activation of the EGF receptor pathway. The effects of *eya* and *eyg* on *dpp* and *wg* and the interactions between *dpp* and *wg* signaling are summarized in Fig. 7.

DISCUSSION

Inhibition of retinal differentiation by wg is not due to loss of *dpp* expression or activity

In the leg disc, *dpp* and *wg* have been shown to maintain their complementary domains of expression by mutual repression (Brook and Cohen, 1996; Heslip et al., 1997; Jiang and Struhl, 1996), and it has been suggested that this interaction also occurs in the eye disc (Heslip et al., 1997). dpp signaling is indeed required to repress *wg* expression at the posterior margin of the eye, as *wg* is ectopically expressed in cells unable to receive the dpp signal (Wiersdorff et al., 1996; Fig. 1B). However, this is not the only requirement for dpp signaling, as clones doubly mutant for *Mad* and *wg* still fail to initiate photoreceptor differentiation. Consistent with this finding, ectopic *wg* expression is only observed in clones mutant for strong loss-of-function *Mad* alleles, suggesting that *wg* repression requires only a low level of dpp signaling (Wiersdorff et al., 1996). The ability of dpp to induce anterior initiation, like its requirement for posterior initiation, cannot be attributed to its repression of *wg*, as wg does not prevent anterior initiation. Indeed, we have observed that dpp can induce the ectopic expression of *wg* at the anterior margin when it is misexpressed using either *flp-out*-GAL4 or *ey*-GAL4 (data not shown), suggesting that other factors determine the effect of dpp on *wg* transcription.

The primary effect of wg does not appear to be the regulation of *dpp* expression. Even though *dpp* expression is lost when the wg pathway is activated in *sgg* mutant clones (Heslip et al., 1997), we found that ectopic expression of *wg* can inhibit photoreceptor differentiation without reducing *dpp* expression (Treisman and Rubin, 1995; Fig. 2B). It is possible that a level of wg signaling too low to completely antagonize sgg or abolish *dpp* expression is still able to prevent photoreceptor formation. Our results show that expression of dpp (Fig. 2D,F) or constitutive activation of the dpp pathway using an activated tkv receptor (Fig. 2H,N) does not rescue the block caused by wg, as would have been expected if wg acted solely by altering the level of *dpp* expression or activity. Unlike *dpp*, the activated tkv construct is not sufficient to promote anterior initiation, although it does induce ectopic ventral initiation (Fig. 2C,G); this could be due to its lack of non-autonomous activity, as expression driven by *ey*-GAL4 is lost from the anterior margin during the third larval instar (Fig. 2B). Another explanation might be that, in this case, dpp signaling requires the combined action of tkv and the other type I receptor encoded by *saxophone* (*sax*; Brummel et al., 1994; Xie et al., 1994), although *sax* is clearly not sufficient for signaling as cells mutant for *tkv* fail to initiate

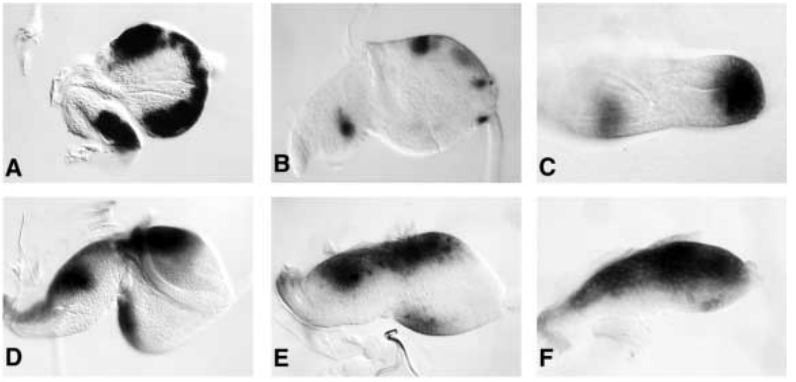

Fig. 3. Regulation of *dpp* and *wg* expression by *eya* and *eyg*. The expression pattern of a *dpp-lacZ* reporter construct (A-C) and a *wg-lacZ* enhancer trap (D-F) is shown in early third instar wild-type (A,D), eya^1 (B,E) and eyg^1 (C,F) eye discs. Very little *dpp* is present in *eya* mutant discs, while *wg* expression appears normal. Expression of *dpp* is lost from the lateral margins in *eyg* mutants and expression of *wg* is expanded to the posterior margin.

a furrow (Burke and Basler, 1996). We have also found that ectopic wg does not prevent the expression of the proneural gene *ato* (Jarman et al., 1994; data not shown). It is not clear whether *ato* acts upstream or downstream of *dpp*, as mutations in either gene result in loss of expression of the other (Jarman et al., 1995; Dominguez and Hafen, 1997) and the two are probably involved in a feedback loop.

On the contrary, we have found that the phenotype caused by ectopic wg is rescued by expressing activated forms of spi or ras, raising the possibility that wg interferes with EGF receptor signaling upstream of ras. Recently, it has been shown that, in the embryonic segments, wg and secreted spi emanate from distinct sources and promote opposing cell fates. This led to the proposal that wg antagonizes signaling by spi through the EGF receptor and the ras/MAPK cascade (O'Keefe et al., 1997; Szuts et al., 1997). Since EGF receptor signaling is required for the formation of all photoreceptors (Freeman, 1996; Tio and Moses, 1997; Xu and Rubin, 1993), it is a possible target for wg inhibition in the eye disc. However, it does not appear that the effects of ectopic wg can be completely explained by antagonism of spi signaling, as mutations in *spi* allow the specification of R8 and the progression of the furrow (Freeman, 1994, 1996; Tio et al., 1994; Tio and Moses, 1997), while the presence of ectopic *wg* does not. It is possible that another ligand, such as vein (Simcox et al., 1996; Schnepp et al., 1996), normally activates the EGF receptor in R8 and that this ligand is also antagonized by wg. Another possibility is that ras activation in R8 is mediated by another tyrosine kinase receptor; one of the identified FGF receptors is expressed in the morphogenetic furrow (Emori and Saigo, 1993). The lower effectiveness of rescue by s-spi than by ras^{v12} could also suggest that wg has effects both upstream of spi expression or processing, and downstream of these events. Some factors known to be required between spi and ras that could be targets of wg inhibition are daughter of sevenless (Herbst et al., 1996; Raabe et al., 1996), downstream of receptor kinases (Olivier et al., 1993; Simon et al., 1993) and son of sevenless (Rogge et al., 1991; Simon et al., 1991). Alternatively, wg could act by stimulating the expression or function of argos, a secreted antagonist of spi (Schweitzer et al., 1995a).

Interestingly, expression of activated *ras* alone is sufficient to induce photoreceptor development in regions anterior to the morphogenetic furrow, as well as extra photoreceptor cells posterior to it (Fig. 2O). Such ectopic development appears to be restricted to a 'zone of competence' near the furrow, as the presence of activated ras in more anterior regions leads to *dpp* expression but not photoreceptor differentiation (data not shown). Such a zone has been described before as responsive to ectopic *hh* expression or to the loss of *PKA* (Heberlein et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Thus, it appears that the effects of ras activation and *hh* expression are very similar, and most of the functions of hh could be achieved by activating ras.

dpp and wg can cooperate to induce ectopic furrow initiation at the anterior margin

Since wg appears to inhibit photoreceptor differentiation downstream of dpp signaling, our observation that it does not inhibit dpp-induced initiation from the anterior margin is surprising. This does not seem simply to be due to dpp diffusing further and stimulating differentiation beyond the range of wg inhibition, as co-expression of the cell-autonomous components tkv^{act} and arm^{act} can also induce anterior initiation, even though tkv^{act} alone does not (data not shown). One possible explanation is that a cofactor required for wg inhibition is absent from the anterior margin. The anterior margin is likely to have some molecular differences from the rest of the disc; for example, the *homothorax* gene is expressed at the anterior margin and is required there to inhibit ectopic furrow initiation (Pai et al., 1998). Loss of *PKA* also induces *wg* expression only in this region (Dominguez and Hafen, 1997), and it is the only part of the disc able to respond to ectopic dpp (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). The anterior margin may require special characteristics because it forms the boundary between the eye disc and the antennal disc, two imaginal fields with quite different modes of development.

Another possibility is that initiation from the anterior margin actually results from a duplication of the eye field. Outgrowths and complete duplications of the eye disc are often seen when anterior initiation is induced by ectopic dpp (Pignoni and Zipursky, 1997). Proximal-distal growth of the leg requires adjacent cells expressing *dpp* and *wg*, and distally complete duplications of the leg can result from ectopic expression of one near the normal expression domain of the other (Basler and Struhl, 1994; Campbell et al., 1993; Diaz-Benjumea et al., 1994). Interestingly, one target gene activated by the combined presence of dpp and wg in the leg disc is *dac* (Lecuit and Cohen, 1997), which is sufficient to induce eye development in the antennal disc and other tissues (Shen and Mardon, 1997). It is possible that both *dac* expression and duplicating outgrowth can also be activated by the combination of dpp and wg in the eye disc; ectopic *dac* expression is indeed observed in conjunction with anterior initiation induced by clones of cells expressing *dpp* and *wg* (data not shown). Early expression of *wg* throughout the eye disc (Royet and Finkelstein, 1997) might cooperate with *hh*-induced *dpp* at the posterior margin to induce normal *dac* expression and eye development; our observation that expression of activated *sgg* dramatically reduces the size of the eye disc even in the absence of photoreceptor differentiation (Fig. 6C,D) supports a role for wg signaling in early growth of the disc.

Genes required to specify the eye disc regulate the expression patterns of *dpp* and *wg*

Regional differences in the response to dpp, wg and hh are likely to be due to preexisting differences in the distribution or function of earlier acting genes. In the case of the leg disc, *wg* expression in ventral anterior cells is already present when cells are recruited to form the primordium and imposes an asymmetric response to the hh signal, restricting *dpp* expression to the dorsal region (Cohen et al., 1993). However, in the eye disc primordium, neither *dpp* nor *wg* expression has been shown to be inherited from embryonic expression. Both *eya* and *eyg* appear to be required for the normal activation of *dpp* expression and repression of *wg* expression in the eye disc, although *eya* has a stronger effect on *dpp* and *eyg* on *wg*. As *eya* is first expressed in the eye disc in a gradient with its high point at the posterior margin (Bonini et al., 1993), it is a good candidate to promote posterior *dpp* expression and prevent *wg* expression upon *hh* induction. However, in *eya* mutants, a low level of *dpp* expression can be initiated (Fig. 3B; Pignoni et al., 1997) and early *wg* expression is restricted to its normal domain. The effects of loss of *eya* appear stronger in late third

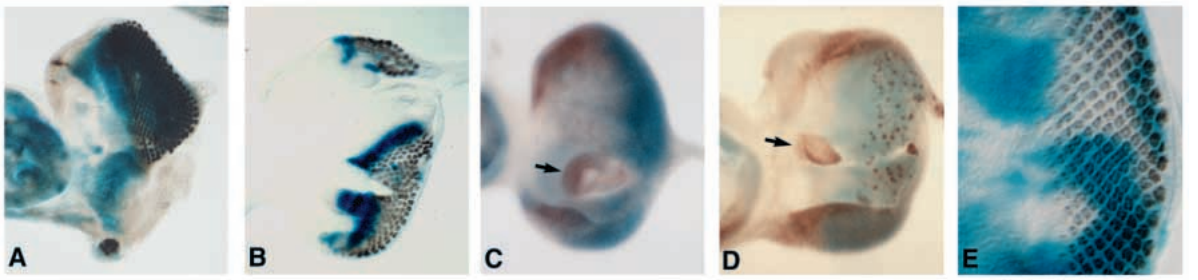

Fig. 4. Expression of *dpp* and *wg* in clones of *eya* mutant cells. (A-D) Third instar eye discs containing *eya* mutant clones. (A,C,D) The clone is marked by the absence of *arm-lacZ* staining (blue). (A,B) Stained for anti-elav (brown) and (B) is stained for *dpp-lacZ* (blue). Photoreceptors do not differentiate within the *eya* mutant clones and *dpp* is not expressed within the clones (marked by the absence of elav staining). (C) Stained with anti-wg (brown) and (D) with anti-omb (brown). *wg* and *omb* are ectopically expressed within the clones (arrows). (E) Third instar eye disc containing *eyg* mutant clones marked by the absence of arm-lacZ staining (blue), and stained for anti-elav (brown). Photoreceptor development proceeds normally in the *eyg* mutant clones.

instar homozygous mutant discs or clones of cells (Fig. 4 and data not shown). *eya* might therefore be required for the maintenance of these expression patterns by the autoregulatory and *wg*-repressing functions of dpp, as well as for the induction of *dpp* by hh (Fig. 6E). The effects of ectopic *eya* expression have only been examined using a *dpp*-GAL4 driver (Bonini et al., 1997; Pignoni et al., 1997), making it difficult to evaluate whether *eya* is sufficient for *dpp* expression or *wg* repression.

Our results support those of Pignoni et al. (1997), who showed that even very late loss of *eya* function, posterior to the morphogenetic furrow, results in the absence of photoreceptors, and proposed that *eya* acts at multiple stages of photoreceptor development. Similarly, we show that the *eya* phenotype cannot be rescued by altering the activity of the dpp, wg or hh pathways; expression of the proneural gene *ato* is not restored to *eya* mutant discs by overexpression of activated *tkv*,

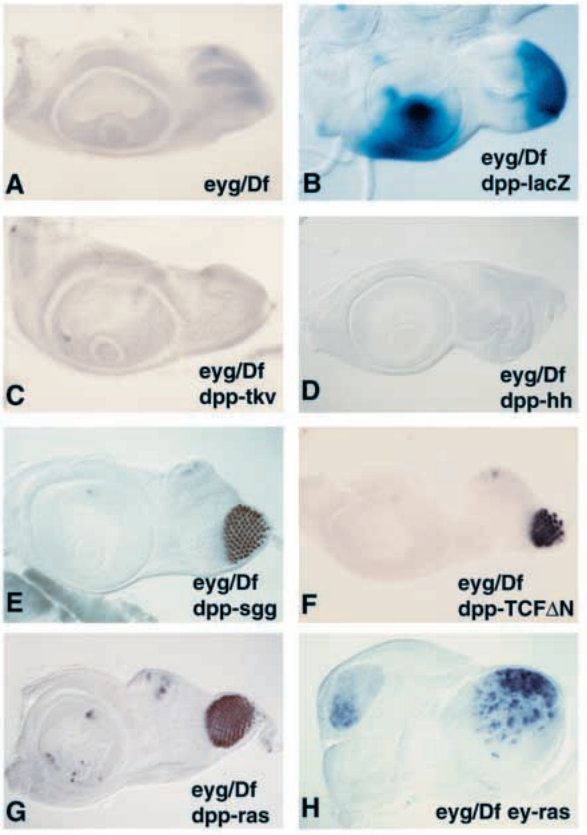

Fig. 5. Rescue of *eyg* by inhibition of wg signaling. (A,C-H) Late third instar eye discs stained with anti-elav; (B) a late third instar eye disc stained with X-gal. (A) *eyg*^{1}/*Df(3L)iro*^{2}; (B) *eyg*^{1}, UAS-*lacZ*/*Df(3L)iro*^{2}; *dpp*-GAL4/+; (C) *eyg*^{1}, UAS-*tkv*^{QD}/*Df(3L)iro*^{2}; *dpp*-GAL4/+; (D) *eyg*^{1}, UAS-*hh*/*Df(3L)iro*^{2}; *dpp*-GAL4/+; (E) *eyg*^{1}/*Df(3L)iro*^{2}; *dpp*-GAL4/UAS-*sgg*^{act}; (F) *eyg*^{1}/*Df(3L)iro*^{2}; *dpp*-GAL4/UAS-*dTCFΔN*. (G) *eyg*^{1}/*Df(3L)iro*^{2}; *dpp*-GAL4/UAS-*ras*^{act}; (H) *eyg*^{1}/*Df(3L)iro*^{2}; *ey*-GAL4/UAS-*ras*^{act}. Photoreceptors develop in *eyg* mutant discs when wg signaling is inhibited by expression of activated *sgg* or dominant negative *dTCF* or when activated *ras* is expressed, but not when dpp signaling is stimulated by expression of activated *tkv* or when *hh* is expressed.

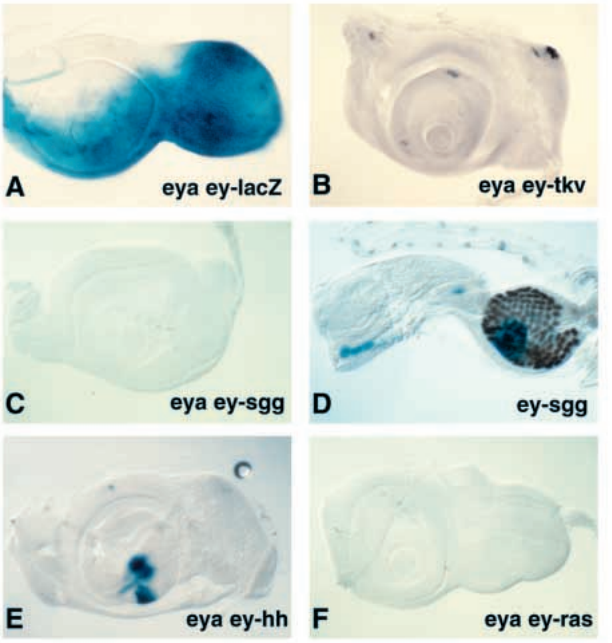

Fig. 6. (A-C, E-F) Third instar eye discs homozygous for *eya*^{1}. (A) *eya*^{1}; *ey*-GAL4/UAS-*lacZ*, stained with X-gal; (B-F) stained with anti-elav. (B) *eya*^{1}; *ey*-GAL4/UAS-*tkv*^{QD}; (C) *eya*^{1}; *ey*-GAL4/UAS-*sgg*^{act}; (D) *ey*-GAL4/UAS-*sgg*^{act}; *dpp-lacZ* is stained in blue; (E) *eya*^{1}; *ey*-GAL4/UAS-*hh*; *dpp-lacZ* is stained in blue; (F)*eya*^{1}; *ey*-GAL4/UAS-*ras*$^{v12.}$ No photoreceptors develop in *eya* mutant discs on expression of *hh*, activated *tkv*, *sgg* or *ras* and *dpp* expression is not induced by *hh*.

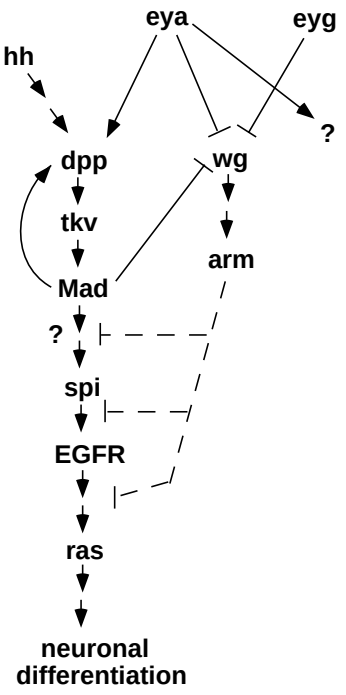

Fig. 7. Cascade of genetic requirements for photoreceptor development. Selected components of the pathway leading to photoreceptor differentiation are shown. *wg* expression is inhibited by dpp signaling and *Mad* is required for this regulation (Wiersdorff et al., 1996). wg, acting through arm, inhibits differentiation somewhere upstream of the activation of ras by spi signaling through the EGF receptor, and at least part of this effect is upstream of spi secretion. *eya* is required for *dpp* expression as well as for late *wg* repression, and independently for photoreceptor differentiation. *eyg* seems to influence photoreceptor differentiation primarily by inhibiting *wg* expression.

activated *sgg* or *hh* (data not shown), confirming that *eya* blocks differentiation upstream of *ato* expression (Jarman et al., 1995). However, activation of ras is also insufficient to rescue *eya* mutant discs, although it does rescue the block caused by ectopic wg, which we show is downstream of *ato* expression. Thus *eya* must be required for photoreceptor development both upstream and downstream of *ato* expression.

Although the expression pattern of *eyg* has not been described, its requirement for neuronal differentiation can be rescued by inhibiting wg signaling at the posterior margin of the eye disc, showing that an important part of its function must be to repress *wg* in this region. As neither hh, dpp nor activated tkv can rescue the *eyg* phenotype, *eyg* must act downstream of, or in conjuction with, hh and dpp activity. If *eyg* acted solely to repress *wg*, it would be difficult to understand how its ectopic expression could induce ectopic eye development (H. Sun, personal communication). However, ectopic eyes induced by *eyg* are only observed ventral to the normal eyes, and may thus result from ectopic initiation at the ventral margin of the eye disc, as opposed to induction of eye development in another imaginal disc. Interestingly, ectopic *ey* is not able to induce eye development in *wg*-expressing domains of other discs (Halder et al., 1998), and coexpression of *eyg* enhances ectopic eye formation by *ey* (H. Sun, personal communication), perhaps through its ability to repress *wg*.

Although we do not know whether *eya* and *eyg* directly regulate *dpp* or *wg*, this is a possibility as *eyg* contains two DNA-binding domains (Jun and Desplan, 1996) and *eya* a transcriptional activation domain (Pignoni et al., 1997). *so* affects *dpp* and may affect *wg* in the same way as *eya*, since the *so* and *eya* mutant phenotypes are very similar and their encoded proteins can form a complex (Pignoni et al., 1997). As *ey* appears to act upstream of these genes (Halder et al., 1995; Bonini et al., 1997), it may affect *dpp* and *wg* indirectly by regulating the expression of *so* and *eya*. In contrast, *dac* is not required for *dpp* expression (Mardon et al., 1994), although it is required to prevent *wg* expression at the posterior margin (Treisman and Rubin, 1995). *dac* is not required for normal growth of the eye disc, suggesting that it functions later than *eyg*. It would be interesting to test whether its mutant phenotype in the eye disc is solely due to ectopic *wg* expression.

In summary, our results show that wg inhibits normal photoreceptor differentiation in a manner independent of *dpp* expression or activation. The expression patterns of both *dpp* and *wg*, and perhaps their cross-regulatory interactions, are determined during early eye development by genes including *eya* and *eyg*. Such tissue-specific regulators may explain how very different processes can be controlled by the same signals.

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