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The *Drosophila eyes absent* gene directs ectopic eye formation in a pathway conserved between flies and vertebrates

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SUMMARY

The fly *eyes absent* (*eya*) gene which is essential for compound eye development in *Drosophila,* was shown to be functionally replaceable in eye development by a vertebrate *Eya* homolog. The relationship between *eya* and that of the *eyeless* gene, a *Pax-6* homolog, critical for eye formation in both flies and man, was defined: *eya* was found to be essential for eye formation by *eyeless*. Moreover, *eya* could itself direct ectopic eye formation, indicating that *eya* has the capacity to function as a master control gene for eye formation. Finally, we show that *eya* and *eyeless* together were more effective in eye formation than either gene alone. These data indicate conservation of the pathway of *eya* function between flies and vertebrates; they suggest a model whereby *eya/Eya* gene function is essential for eye formation by *eyeless/Pax-6,* and that *eya/Eya* can in turn mediate, via a regulatory loop, the activity of *eyeless/Pax-6* in eye formation.

Key words: eye development, *eyes absent*, *eyeless*, *Drosophila*

INTRODUCTION

The *Drosophila* eye, although structurally distinct from the vertebrate eye, shows striking parallels at the molecular level. Many genes that function in eye formation in the fly have homologs that are expressed during vertebrate eye development (Quiring et al., 1994; Zuker, 1994; Oliver et al., 1995). Among these is the *Drosophila eyes absent* (*eya*) gene which encodes a nuclear protein that, in the fly, functions prior to the first notable differentiation event – morphogenetic furrow formation – in eye progenitor cell development (Bonini et al., 1993; Leiserson et al., 1994). *eya* is the founding member of a class of vertebrate *Eya* genes, with homologs showing expression in the eye and other tissues (Abdelhak et al., 1997; Duncan et al., 1997; Xu et al., 1997; Zimmerman et al., 1997). Human mutations at the *Eya1* locus have been identified that result in defects in organ formation (Abdelhak et al., 1997).

eya functions at a similar time and place as the fly *eyeless* gene. *eyeless* is a counterpart of the human *ANIRIDIA* and mouse *Sey* (*Small eye*) genes, which are *Pax-6* family members containing a paired-box and homeobox and likely function as transcription factors (Quiring et al., 1994). Mutated *eyeless* results in loss of the fly eye (Quiring et al., 1994); similarly, mutation of the human or mouse counterparts leads to eye malformation and reduction of the eye in the extreme, and cataracts in mild forms (Hogan et al., 1986; Hill et al., 1991; Ton et al., 1991; Glaser et al., 1992; Jordan et al., 1992; see Hanson and van Heyningen, 1995). Expression of the *eyeless* cDNA in the fly using tissue-specific elements can direct the formation of ectopic eyes in the antennae, legs and wings (Halder et al., 1995a). The mouse *Sey* cDNA, when introduced into the fly, can similarly direct ectopic *Drosophila* eye development, suggesting potential conservation of fundamental molecular features by which these homologs act in eye formation. *Sey* mouse mutants have reduced expression of the *Eya1* and *Eya2* genes in eye progenitor tissue (Xu et al., 1997), suggesting that *Eya* genes might be mediators of *Pax-6* function for eye formation in vertebrates.

Since the vertebrate *Eya* homologs show expression in eye tissue, it is tantalizing to speculate that the vertebrate and fly genes are functional homologs. Here, we address the level of functional conservation between fly *eya* and a vertebrate *Eya* homolog. We then used the fly to define the relationship between *eya* and *eyeless* that was suggested by vertebrate work: that the *eya* gene may be a conserved mediator of *eyeless* function in eye formation. We found that not only is *eya* essential for *eyeless* function, but that the *eya* gene itself can serve as a master control gene for eye formation. These data indicate striking functional conservation of the genetic pathway of eye formation between flies and vertebrates, and suggest a model of combinatorial gene functions for eye formation.

MATERIALS AND METHODS

Drosophila strains and P-element-mediated transformation

Fly strains were grown on standard molasses, yeast and cornmeal medium at 25°C. *UAS-eya* transgenics were made by subcloning the full-length *Drosophila eya* typeI cDNA into the pUAST vector (Brand and Perrimon, 1993). *UAS-Eya2* mouse transgenes were made by subcloning a predicted full length mouse *Eya2* subclone (Zimmerman et al., 1997) into the pUAST vector. Flies were transformed using standard transgenic techniques (Rubin and Spradling, 1982). *eyeless-*

GAL4 was made by subcloning a fragment which reports *eyeless* staining in eye progenitor cells (Quiring et al., 1994), into the GAL4 vector (Brand and Perrimon, 1993), and transforming the construct into flies. Other GAL4 lines were obtained from *Drosophila* Stock Centers; *eya* mutant alleles are as previously described (Bonini et al., 1993); *eyeless*^{2} mutant strain was provided by courtesy of Dr Walter Gehring. GAL4 lines used included T59, T155 and *dpp-GAL4*, all of which express in the eye portion of the eye-antennal disc. *dpp-GAL4* is expressed in the imaginal discs in an expression pattern similar to that of *dpp* (Staehling-Hampton et al., 1994; see also Shen and Mardon, 1997). *eyeless-lacZ* lines are as described by Quiring et al. (1994); *UAS-eyeless* lines are as described by Halder et al. (1995a).

Immunohistology

Tissue preparations were fixed with 2% paraformaldehyde in TBS, permeabilized with 0.5% Triton X-100, and stained in primary antibody overnight. Primary antibodies were anti-Eya (Bonini et al., 1993) and anti-Glass (Ellis et al., 1993). After rinsing in TBS, tissue was stained with secondary antibodies conjugated to fluorescein or cyanine-3 (Jackson ImmunoResearch Laboratory), rinsed in TBS, then mounted in PDA-glycerol, as previously described (Bonini et al., 1993). Staining with β-galactosidase for *eyeless-lacZ* expression pattern was performed as described by Quiring et al. (1994). For viewing flies by scanning electron microscopy, flies were critical point dried and scanned at 5 kV. For sections of eye tissue, flies were fixed in glutaraldehyde, embedded in epon and thick sectioned (Bonini et al., 1993).

RESULTS

A vertebrate *Eya* homolog is able to functionally replace the fly gene

To test potential functional homology between the vertebrate and fly *eya* genes, we asked whether a vertebrate *Eya* homolog was capable of replacing the fly *eya* gene in the eye developmental pathway. To do this, we required functional replacement of *eya* activity in the *eya*^{2} mutant background. The *eya*^{2} mutant is a viable, eye-specific null for *eya* gene activity in eye progenitor cells anterior to the furrow (Bonini et al., 1993). These mutant flies are completely eyeless due to complete loss of eye progenitor cells by cell death (Fig. 1A). Thus, any survival and development of ommatidia to the adult eye in this mutant background would indicate functional replacement of fly *eya* gene activity.

To express the vertebrate gene, the GAL4-UAS system of tissue-specific targetting was used (Brand and Perrimon, 1993). UAS-*Eya2* transgenic animals were made using a mouse *Eya2* cDNA that is predicted to encode a full-length protein (Zimmerman et al., 1997). The protein encoded by this homolog shows an overall identity of 49% with the predicted fly protein sequence. To express the gene sufficiently early in eye progenitor cell development, we constructed an *eyeless*-GAL4 transgenic line that drives GAL4 expression in eye progenitor cells prior to furrow formation.

Remarkably, the mouse *Eya2* gene restored eye formation to *eya*^{2} mutant flies (Fig. 1A,B). Sections through the eyes indicated a normal pattern of photoreceptor neurons (Fig. 1C). These data indicate that the mouse *Eya2* gene can functionally replace the fly gene in eye formation. These data demonstrate that molecular features of the eye developmental pathway directly dependent on *eya* gene activity are conserved between flies and vertebrates.

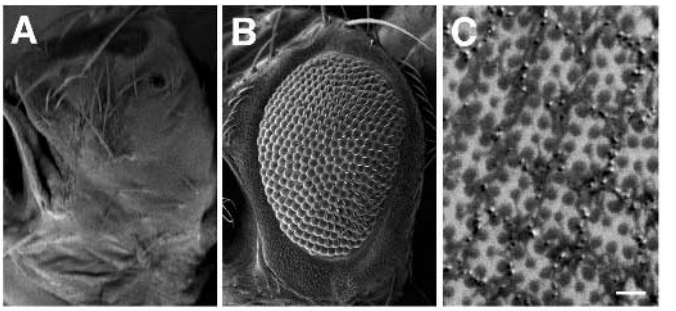

Fig 1. The mouse *Eya2* gene functionally complements the fly *eya*^{2} mutant. (A) Scanning electron micrograph of the head of an *eya*^{2} mutant fly. This allele of *eya* is null for the function of *eya* in eye progenitor cells prior to furrow formation, and results in complete lack of eyes (Bonini et al., 1993). (B) Scanning electron micrograph of an eye generated by the mouse *Eya2* gene in the *eya*^{2} mutant background, genotype *eya*^{2} UAS-*Eya2 eyeless*-GAL4. The eye shows a pattern of ommatidial units with bristle cells similar to the normal fly compound eye. (C) Tangential section of a fly eye generated by the mouse *Eya2* gene, genotype *eya*^{2} UAS-*Eya2 eyeless*-GAL4 fly. Photoreceptor cells have developed in a pattern typical of the compound eye (compare to Fig. 3C). In this section, it is possible to see all photoreceptor cells: to the right, ommatidia with R1-R6, plus R7 are present; to the left, ommatidia with R1-R6, plus R8 are seen. The ommatidia are surrounded by a pigment lattice, and the equator can be seen running through the eye. Bar, 50 μm for A,B; 3.5 μm for C.

eya function is essential for *eyeless* activity

Given this striking level of conservation of *eya* function, we were interested to determine the relationship between *eyeless* gene activity and *eya* gene activity suggested by expression studies in vertebrates. To determine whether *eya* gene expression occurred upon eye formation directed by the *eyeless* gene, we generated ectopic eyes with *eyeless*, and stained the tissue for ectopic expression of Eya protein. Flies bearing a UAS-*eyeless* insert (Halder et al., 1995a) were crossed to a *dpp*-GAL4 insert line, which expresses GAL4 in the imaginal disc expression pattern of *dpp* (Blackman et al., 1991). These animals generated ectopic eyes on the legs, wings and antennal region of the head (Fig. 2A,C; Table 1).

Immunostaining of third-instar larval imaginal discs confirmed that Eya was indeed ectopically expressed in regions where *eyeless* directed ectopic eye formation: the antennal portion of the eye-antennal disc, leg and wing discs (Fig. 2F,I,

Table 1. *eya* function is essential for ectopic eye formation by the *eyeless* gene

Structure with ectopic eye formation	UAS-*eyeless*/ *dpp*-GAL4	*eya*^{2}; UAS-*eyeless*/ *dpp*-GAL4*
Antennae	100%	0%
Legs	100%†	0%†
Wing	100%	0%
Haltere	24%	0%

50 animals scored for each data point; ectopic eye development is scored by the presence of ommatidia with pigment.

*Animals are completely eyeless in addition to lacking ectopic eye formation.

†Legs truncated and abnormally shaped (see Fig. 2C-E).

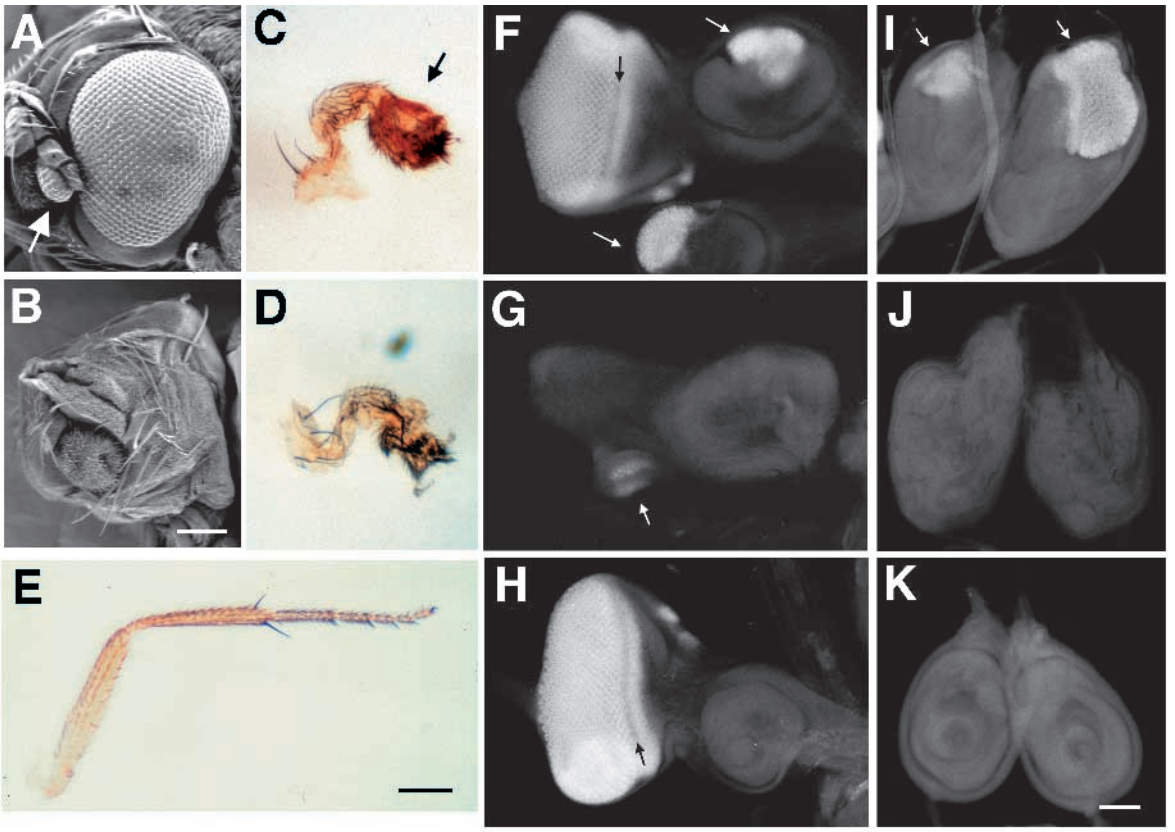

Fig 2. *eya* gene function is essential for ectopic eye formation by the *eyeless* gene. (A) Ectopic eye formation in the antennal lobe (arrow) of a UAS-*eyeless* × *dpp*-GAL4 adult fly. (B) Lack of normal and ectopic eye formation in an *eya*^{2} mutant animal bearing UAS-*eyeless* and *dpp*-GAL4 insertions. (C) First leg of a UAS-*eyeless* × *dpp*-GAL4 fly. The leg is stunted in length, and eye tissue is present in the distal portion (arrow). (D) First leg of a UAS-*eyeless* × *dpp*-GAL4 fly in the *eya*^{2} mutant background. No eye tissue is present, however the leg is gnarled and stunted in length. (E) First leg of a normal fly, for comparison. (F,I) Expression of Eya in animals of genotype UAS-*eyeless* × *dpp*-GAL4. Strong ectopic expression of Eya occurs in the antennal lobe (F, white arrows), and leg discs (I, arrow). Compare to normal pattern of Eya expression in H and K. (G, J) Expression of Eya in animals of genotype UAS-*eyeless* × *dpp*-GAL4, in the *eya*^{2} mutant background. In the *eya*^{2} mutant, no Eya expression is detectable in the eye portion of the disc except for the ocellar progenitors (arrow; the ocelli form normally in the *eya*^{2} mutant). No ectopic Eya protein is detectable in the antennal region of the disc (G) or the leg discs (J); the leg discs appear morphologically abnormal. (H,K) Expression of Eya in the (H) eye-antennal and (K) leg discs of a normal animal. Eya expression is limited to the eye portion of the eye-antennal disc, and is present both anterior and posterior to the furrow (arrow). Expression also occurs in the ocellar region of the eye disc far anterior to the furrow. Eya is not expressed in the leg discs. Anterior is to the right for F-H. Bar, 100 μm for A,B; 200 μm for C-E; 50 μm for F-K.

data not shown). Of these imaginal tissues, Eya is normally expressed only in eye and ocellar progenitor cells of the eye-antennal disc, and the peripodial membrane of the wing disc; Eya is not normally expressed in cells of the antennal, leg or wing disc proper (Fig. 2H,K, data not shown; Bonini et al., 1993). Activation of *eya* gene expression by *eyeless* suggested that *eya* may indeed be required for formation of eyes by *eyeless*, similar to the requirement for *eya* function during normal compound eye development.

To test whether *eya* gene activity was essential for *eyeless*-driven ectopic eye formation, we attempted to induce ectopic eyes with UAS-*eyeless* crossed to *dpp*-GAL4, but now in the *eya*^{2} mutant background. As noted, the *eya*^{2} mutant is completely eyeless and null for the early eye function of the *eya* gene. If eye formation by *eyeless* were dependent upon *eya* gene activity, then ectopic eye formation should fail in the *eya*^{2} mutant background. Eye formation indeed failed in all tissues of the fly where ectopic eyes had previously developed: the legs, wings and antennal segments of the head (Table 1, Fig. 2B,D). In the imaginal tissues, as anticipated, no ectopic Eya protein expression was detectable in the antennal, leg or wing discs of animals bearing UAS-*eyeless* in *trans* to *dpp*-GAL4 in the *eya*^{2} mutant background (Fig. 2G,J). These experiments also demonstrated that UAS-*eyeless* was not able to restore normal eye formation to the *eya*^{2} mutant (Fig. 2B), indicating that *eyeless* gene activity cannot replace or substitute for the function of *eya* in eye development. Taken together, these date clearly indicate that *eya* gene function is essential for *eyeless* to form eyes; the *eya* gene thus appears to be an essential biological target of *eyeless* gene activity in eye formation.

The *eya* gene directs ectopic eye formation

Given the high conservation demonstrated above of the *eya* pathway at the functional level between flies and vertebrates, and given the essential role of *eya* for *eyeless* function that may well extend between flies and vertebrates, we were interested

to determine what were potential effects of the *eya* gene itself for eye formation. Could *eya*, like *eyeless*, mediate eye formation? Previously, we had expressed the *eya* gene in the fly with a heat shock promoter (Bonini et al., 1993); whereas such expression could restore the eyes to *eya* mutants that lacked eye formation, no other consistent effects were observed in the rescued animals. Nevertheless, we attempted to express *eya* at higher levels using the GAL4-UAS system (Brand and Perrimon, 1993), to determine whether it was possible to induce dominant phenotypes that would yield clues to the function of the gene.

A *UAS-eya* construct was made with the *eya* cDNA. To determine whether the construct was functional, we attempted rescue of the eya^2 mutant phenotype using various GAL4 lines that express in the eye progenitor field prior to furrow formation. A number of GAL4 lines were tested, including *eyeless*-GAL4, *dpp*-GAL4, T59, and T155. These GAL4 insertions, when crossed to a UAS-*eya* insert, could restore to the eya^2 mutant eyes up to three quarters of normal size. Given that rescue was partial, we attempted to increase eye size by increasing gene dosage of the *UAS-eya* and GAL4 insertions. In doing this, we found that *UAS-eya* lines in *trans* to GAL4 insertion lines were lethal in two doses of the transgenes; for those lines that were lethal at the late pupal stage (*dpp*-GAL4, T59), the homozygous animals could be observed by dissection of the pupae. This analysis showed that these lines displayed not only rescue of the eye, but also ectopic eye formation in other regions of the animals where GAL4 was expressed with these constructs. The chromosomes bearing the *UAS-eya* and GAL4 inserts were then crossed out of the *eya* mutant background and into a normal background for additional analysis. We focused on expression of *UAS-eya* driven by *dpp*-GAL4, since this combination led to late pupal lethals that could be readily observed; in these animals, GAL4 expression occurs in the imaginal disc expression pattern of *dpp*, in the eye and antennal portions of the eye-antennal disc, the leg and wing discs, among other tissues (Blackman et al., 1991).

In a wild-type background, UAS-*eya dpp*-GAL4 in single copy generated rare examples of ectopic eyes, which resembled normal eyes, on the antennal segment (10% of the animals, Fig. 3A,B). Tangential sections of the ectopic eyes formed indicated that photoreceptor cells developed in a pattern similar to that of the normal compound eye (Fig. 3C). With two copies of UAS-*eya dpp*-GAL4, ectopic eye formation was induced in the antennal region of the head in almost all animals (96% ectopic eye formation on antennae); 80% also showed ommatidial formation on the legs, and occasionally on the wings. Glass, a photoreceptor-specific gene, was used as a marker to detect development of retinal tissue in the larval imaginal discs. Glass expression is normally restricted to the eye portion of the eye-antennal disc and does not occur in the antennal portion or other imaginal discs (Ellis et al., 1993). Ectopic expression of Glass was seen in the antennal and leg imaginal discs; in these tissues, rosettes of developing photoreceptor clusters similar to the normal pattern were seen (Fig. 3D,E). These data indicate that *eya* has the capacity to function as a master regulatory gene for eye formation.

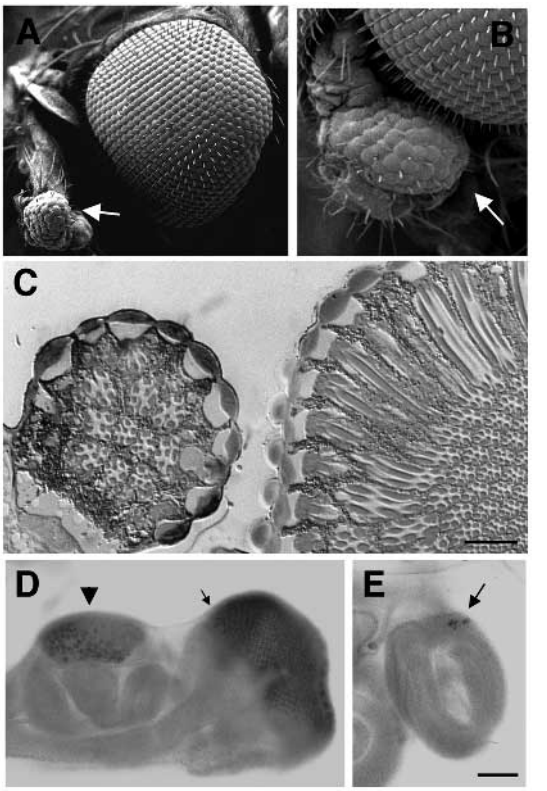

Fig 3. The *eya* gene directs ectopic eye formation. (A,B) Scanning electron micrographs of ectopic eyes (arrows) formed on the antennal segment of flies heterozygous for UAS-*eya dpp*-GAL4. The regular ommatidial array and bristle formation is similar to that of the normal eye. (C) Tangential eye section through the normal eye (right) and the ectopic eye (left) formed on the antennal segment of a fly heterozygous for UAS-*eya dpp*-GAL4. (D,E) Ectopic expression of the photoreceptor-specific protein Glass in antennal (D, arrowhead) and leg (E, arrow) imaginal discs of larval animals of genotype UAS-*eya dpp*-GAL4. Normally in the imaginal discs, Glass protein occurs only in the eye portion of the eye-antennal disc (Ellis et al., 1993), posterior to the furrow (arrow in D). Reverse images of fluorescent-stained tissue; anterior to the left. Bar 100 μm for A; 50 μm for B; 10 μm for C; 50 μm for D,E.

Requirement for *eyeless* in ectopic eyes produced by *eya*

These observations raised questions regarding the relationship between the *eya* and *eyeless* gene functions during eye formation. Since *eya* was essential for ectopic eye formation by *eyeless* (see Table 1; Fig. 2), was *eyeless* gene function essential for ectopic eye formation by *eya*? To address this, we first asked whether *eyeless* gene expression was induced during ectopic eye formation directed by the *eya* gene. Normally, *eyeless* expression is restricted to the eye portion of the eye-antennal imaginal disc (Fig. 4A; Quiring et al., 1994). In UAS-*eya dpp*-GAL4 animals, expression of *eyeless* occurred ectopically in the antennal region of the eye-antennal disc, in the region where *eya* directed ectopic eye formation (Fig. 4B). Although *eya* also directed ectopic eye formation in the leg discs, ectopic *eyeless* expression was not detectable in that tissue upon *eya* expression (Fig. 4C); *eyeless* was capable of autoregulation in leg discs when *eyeless* itself was ectopically expressed (Fig. 4D). This suggested that *eyeless* might be required for *eya* activity to form eyes in some tissues, but dispensible in others. We also determined that *eyeless* remained expressed in the eye progenitor cells of the eya^2 mutant (Fig.

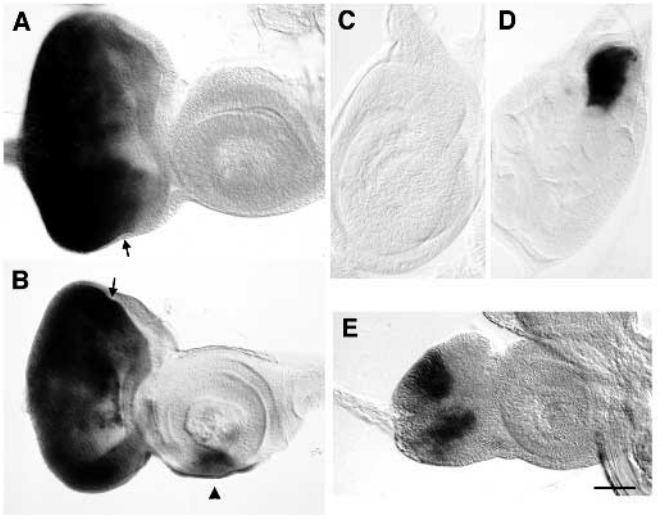

Fig 4. Ectopic eye formation by *eya* turns on *eyeless* gene expression. (A) Expression of *eyeless* in eye progenitor cells of a normal eye-antennal imaginal disc. *eyeless* expression is detected with a β-galactosidase reporter construct (Quiring et al., 1994). Arrow indicates position of the morphogenetic furrow. (B,C) Ectopic expression of *eyeless* directed by the *eya* gene. In animals of genotype UAS-*eya dpp*-GAL4, ectopic *eyeless* expression occurred in the antennal region of the eye-antennal imaginal disc (B, arrowhead; small arrow indicates the position of the morphogenetic furrow). In the leg discs (C), however, ectopic *eyeless* is not detectable upon *eya* expression. (D) When *eyeless* itself is ectopically expressed, *eyeless* expression is detectable in leg discs (D) as well as in the antennal portion of the eye-antennal disc (not shown), consistent with autoregulation of the *eyeless* gene (Glardon et al., 1997). Animals of genotype *eyeless-lacZ*; UAS-*eyeless* in *trans* to *dpp*-GAL4. (E) *eyeless* expression is present in the eye progenitor field of eya^2 mutant discs. The eye portion of the disc is reduced in size due to loss of the eye progenitor cells by programmed cell death, no morphogenetic furrow is present (Bonini et al., 1993). Anterior to the right. Bar 50 μm.

4E), suggesting that *eya* gene function is not essential for the normal expression pattern of *eyeless*.

To address a functional requirement for *eyeless*, we investigated whether there were detectable genetic interactions between the *eyeless* and *eya* genes. Such experiments are limited by the mutants of *eyeless* currently available – there are no null mutants for the eye function of *eyeless* which would allow us to remove *eyeless* gene activity completely (Quiring et al., 1994). Nevertheless, we attempted to induce ectopic eye formation with *eya* in a background of reduced *eyeless* gene activity, by using the $eyeless^2$ mutant allele. $eyeless^2$ mutant flies show a range of reduced eye phenotypes, with about 30% of the flies missing at least one eye completely. Directed expression of the *eya* gene in the $eyeless^2$ background, did not result in ectopic eye formation in the antennal segments of the head, or the legs (data not shown). These data indicated a dependence on *eyeless* gene activity in the ability of *eya* to direct eye development both in the head and in the legs. Thus, *eya* appears to function both downstream and upstream of *eyeless* gene activity in eye formation.

Potentiation between *eyeless* and *eya* in eye formation

This regulatory relationship between the two genes prompted us to ask whether we could detect additional interactions between the genes. To do this, we examined the ability of *eyeless* to direct eye formation when combined with additional doses of *eya* gene activity. Animals bearing UAS-*eya* in *trans* to *dpp*-GAL4 show limited dominant effects (see above and Table 2); in animals bearing UAS-*eyeless* in trans to *dpp*-GAL4, Eya protein is already highly expressed (see Fig. 2). Nevertheless, ectopic eye formation by *eyeless* was dramatically enhanced when additional *eya* gene activity was provided (Table 2, Fig. 5). The ectopic eyes were larger and formed with higher penetrance than with *eyeless* or *eya* alone, and eye formation now occurred on the genitalia, a condition never previously observed in individuals with either gene alone (Table 2 and Fig 5B,E). This effect did not appear additive (Table 2). Rather, these data suggest functional synergy between *eyeless* and *eya* gene activities in eye formation.

Table 2. Functional synergy between *eya* and *eyeless* in ectopic eye formation

Structures with ectopic eye formation	UAS-*eya*	UAS-*eyeless*	UAS-*eyeless* + UAS-*eya*
Antennae	10%	95%	100%
Proboscis	0%	13%	91%
Legs	0%	100%	100%
Wing	0%	100%	100%
Haltere	0%	33%	93%
Genitalia	0%	0%	55%

110 animals scored for antennal data point, 50-55 animals scored for other data points. Ectopic eye development is scored by the presence of ommatidia with pigment. Expression of the UAS constructs driven with *dpp*-GAL4.

DISCUSSION

Our data reveal an active role of the *eya* gene in eye formation, and suggest a model of gene regulatory interactions between *eyeless* and *eya* in eye formation in the fly that may extend to their mammalian counterparts.

Conservation of *eya* function between vertebrates and flies

We found that a vertebrate homolog of *eya*, the mouse *Eya2* gene, can functionally replace the fly gene in eye formation. These data suggest that the role of the *eya* gene in eye formation has been conserved through evolution, between flies and vertebrates, despite dramatic differences in eye structure between the two (see Zuker, 1994). Such functional homology has been shown for various *Pax-6* homologs of the *eyeless* gene (Halder et al., 1995a; Glardon et al., 1997): we have extended those studies to *eya* and its homologs, a second gene of the eye developmental pathway. The vertebrate *Eya* homologs identified to date are all expressed in the developing or adult eye, suggesting all homologs may function in aspects of vertebrate eye formation and maintenance (Duncan et al., 1997; Xu et al., 1997; Zimmerman et al., 1997). For the *Eya2* homolog, we demonstrate here a homologous role in the eye developmental pathway.

Relation of *eya* gene activity to *eyeless*

We have addressed and clarified the relationship of *eya* gene

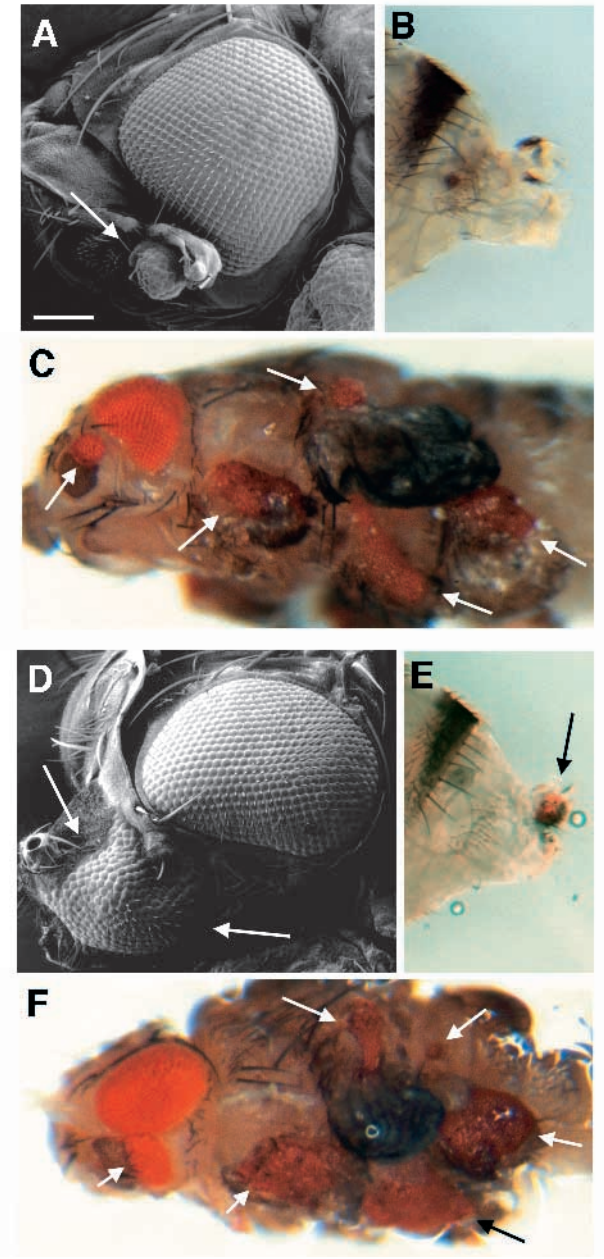

Fig 5. *eya* activity functionally enhances ectopic eye formation directed by the *eyeless* gene. (A-C) Ectopic eye formation on an animal of genotype UAS-*eyeless* in *trans* to *dpp*-GAL4. Ectopic eyes form on the antennal segment of the head (A, arrow), the wings and legs (C, arrows). No ectopic eye formation occurs on the genitalia (B). (D-F) Ectopic eye formation on an animal of genotype UAS-*eyeless* in *trans* to UAS-*eya dpp*-GAL4. Ectopic eyes which form on the antennal segment (D, arrows), wings and legs (F, arrows) are larger than without UAS-*eya*. Ectopic eye tissue is now also observed on the genitalia (E, arrow). Bar, 100 μm for A and D; 200 μm for B-C, E-F.

activity to that of the *eyeless* gene. Previous data indicate that, normally, *eyeless* expression precedes that of *eya* in eye progenitor cells. Whereas *eyeless* is expressed in the eye primordium from embryonic stages (Quiring et al., 1994), *eya* expression is initiated during the mid-larval stages (Bonini et al., 1993). We found that *eya* gene activity was essential for

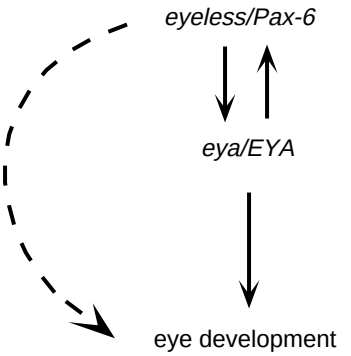

Fig 6. Model for gene interactions between *eyeless*/*Pax-6* and *eya/EYA* in eye development. *eya* is placed downstream of *eyeless* as data indicate that expression of *eyeless* (Quiring et al., 1994) occurs prior to expression of *eya* (Bonini et al., 1993) in normal eye development. However, a loop between *eya* and *eyeless* is proposed because results suggest that not only is *eya* activity essential for *eyeless* function, but also *eyeless* function is essential for *eya* function. Since *eya* and *eyeless* together are more effective in eye formation than either gene alone, *eya* and *eyeless* may function in at least partially distinct pathways (curved arrow to the left and the pathway through *eya*), both of which are critical for eye formation. We propose these same gene interactions may exist for the mammalian counterparts, given the conservation of function of *eyeless* with mouse *Sey* shown previously (Halder et al., 1995a), and functional conservation between mammalian *Eya2* and fly *eya* shown here.

eye formation by *eyeless*; these data, along with the observation that *eyeless* remains expressed in fly *eya* mutants, suggest that *eya* is downstream of *eyeless* gene function (Fig. 6). Consistent with this, *Eya* expression was induced upon ectopic *eyeless* expression; mammalian *Eya* expression is also affected by mutation of *Pax-6* in the mouse (Xu et al., 1997). *eya/Eya* thus appears to be essential for eye formation by *eyeless /Pax-6*. In humans reduction of *EYA* gene activity may be a critical consequence of mutation in the *ANIRIDIA* gene, leading to improper eye formation.

In the *eya* mutant background, we note that *eyeless* activity was not completely ineffective – although ectopic eye formation did not occur, leg development remained severely affected (see Fig. 2 D,J). This suggests that *eyeless* is activating gene functions, in addition to that of *eya*, that are interfering with normal leg development. These additional genes may be upstream of *eya* in the eye developmental pathway, or in a different branch of the eye developmental pathway (see below and Fig. 6). Furthermore, target genes of *eyeless* may have functions in addition to those associated with eye development. For example, the *dac* (*dachshund*) gene is a target of *eyeless* gene activity that functions both in eye and leg development (Shen and Mardon, 1997).

eya as a master control gene for eye formation

We found that *eya* shares with *eyeless* the capacity to function as a ‘master control gene’ for eye formation. By this term, we refer to the fact that *eya* has the capacity to direct the appropriate genetic program of the many genes required for eye development (Halder et al., 1995a). Using loss-of-function *eyeless* mutants, we found evidence that *eyeless* activity is

required for *eya* to form eyes – similar to the requirement for *eya* gene activity in the proper *eyeless* function in eye formation. Thus, the activities of the *eya* and *eyeless* genes appear connected by a regulatory loop, with each functionally required by the other in eye formation (Fig. 6). One qualification of these conclusions is that in leg discs, we were unable to detect *eyeless* expression upon ectopic *eya* activity. Nevertheless, genetic studies in the *eyeless* mutant background indicated that eye formation by *eya* in legs appeared dependent on *eyeless* gene activity. Thus, we suggest that *eyeless* function is required for ectopic eye formation by the *eya* gene. In Fig. 6, we place *eya* downstream of, but connected back to, *eyeless* gene function. Genetically, there is little to argue which gene is first; however, the normal expression patterns of the genes indicate that *eyeless* expression temporally precedes that of *eya* during normal eye formation (Bonini et al., 1993; Quiring et al., 1994). In ectopic eye formation, the genes are each essential for the others' function, thus are interchangeable in placement.

Moreover, we found that *eya* and *eyeless* displayed functional synergy in eye formation – the same dosage of *eyeless* was potentiated when combined with additional *eya* gene function. This synergy was observed with a *dpp* enhancer construct, and whether other regulatory elements will mediate a similar level of synergy remains to be determined. However, that *eyeless* has functions in addition to activating *eya* activity is also suggested by the severely affected leg morphology observed upon *eyeless* expression in the *eya* mutant background (see above). These data suggest that *eyeless* and *eya* may function in at least partially distinct pathways for eye formation (Fig. 6). By such a model, expression of either gene alone, if expressed strongly enough, will eventually drive both pathways because they form part of a regulatory loop. However, when both genes are expressed strongly, both pathways will be strongly driven, leading to an enhancement of eye formation compared to that with either gene alone.

Taken together, these data suggest that early events of eye formation proceed not by a simple linear pathway, but rather by a combinatorial code of gene function. Thus, there may be no single 'master control gene' for eye formation, but a complex regulatory network of gene activities required to trigger the biological event of eye development. These initial events of eye formation include additional genes, such as *dac* and *sine oculis*. The position of *dac* in this regulatory pathway will be of interest, as *dac* has also been shown able to direct eye formation (Shen and Mardon, 1997). Given the increase in *eyeless* expression upon *dac*-directed eye formation (Shen and Mardon, 1997), *dac* likely also requires *eyeless* gene activity to form eyes. This suggests that, minimally, *dac* and *eya* are connected through common regulation of, and regulation by, *eyeless* gene activity.

The relationship between *eya*, *eyeless* and other genes central to eye formation conserved in both flies and vertebrates, such as *sine oculis*/*Six-3* (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Oliver et al., 1995), are also of great interest. Moreover, how these early events of eye determination subsequently merge with pattern formation events of furrow movement (Heberlein and Moses, 1994) and cell cycle regulation (Thomas and Zipursky, 1994), are key aspects of generating a patterned neural structure like an eye. Somehow, these different aspects of the eye developmental process must be triggered by the eye differentiation pathway and coordinately regulated, to achieve this exquisitely organized neural center.

The role of the *eya* gene

The *eya* gene has additional roles in development. In flies mutations in *eya* can be embryonic lethal (Nüsslein-Volhard et al., 1984; Bonini et al., 1993; Leiserson et al., 1994), or result in defects in gonad formation (Boyle et al., 1997), whereas humans mutant in *EYA1* show developmental defects of the branchial arches, ear and kidney (Abdelhak et al., 1997). Thus, the *eya* gene has roles in development of the animal in addition to a function in eye formation. It is thus of interest to determine whether expression of *eya* has consequences over and above ectopic eye formation. Expression in other tissues or at other times in development may lead to elucidation of additional roles of the gene, as well as insight into the specificity of expression for eye formation. Toward this end, it is rather surprising that genes like *eyeless*, *eya* and *dac*, with roles in the animal in addition to eye formation, should induce eye development when ectopically expressed. What leads to this specificity is of particular interest. With respect to the role of *eya* in eye formation, loss-of-function *eya* mutants show death of eye progenitor cells (Bonini et al., 1993).

Taken together, these data indicate that, although the function of *eya* in eye differentiation is coupled to both differentiation and survival, the most dramatic effects of the gene upon strong expression are in the differentiation pathway. Thus, we anticipate that the relationship of *eya* activity with cell death will be indirect, through an effect on the pathway of differentiation. What gene activites might be altered in *eya* mutants, such that the cells become directed down a death pathway, remain to be defined.

With respect to the eye developmental pathway, the biological activities of *eyeless* and *eya* in eye formation extend to their mammalian counterparts *Pax-6* and *Eya*. The mouse *Sey* gene has been shown to function in the fly (Halder et al., 1995a) and we have shown that a mouse homolog of *eya* has the ability to functionally replace the endogenous fly gene in eye development. These data indicate a remarkable level of conservation of gene function in eye formation between flies and mammals. These data lend support to the idea (Quiring et al., 1994; Halder et al., 1995a,b; see Zuker, 1994) that common genetic pathways may be used for the formation of eyes of widely divergent structure in organisms as evolutionarily distant as flies and man.

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