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The Retinal Determination Gene *eyes absent* Is Regulated by the EGF Receptor Pathway Throughout Development in Drosophila

Claire L. Salzer, Yair Elias and Justin P. Kumar[1]

Department of Biology, Indiana University, Bloomington, Indiana 47405

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ABSTRACT

Members of the Eyes absent (Eya) protein family play important roles in tissue specification and patterning by serving as both transcriptional activators and protein tyrosine phosphatases. These activities are often carried out in the context of complexes containing members of the Six and/or Dach families of DNA binding proteins. *eyes absent*, the founding member of the Eya family is expressed dynamically within several embryonic, larval, and adult tissues of the fruit fly, *Drosophila melanogaster*. Loss-of-function mutations are known to result in disruptions of the embryonic head and central nervous system as well as the adult brain and visual system, including the compound eyes. In an effort to understand how *eya* is regulated during development, we have carried out a genetic screen designed to identify genes that lie upstream of *eya* and govern its expression. We have identified a large number of putative regulators, including members of several signaling pathways. Of particular interest is the identification of both *yan/anterior open* and *pointed*, two members of the EGF Receptor (EGFR) signaling cascade. The EGFR pathway is known to regulate the activity of Eya through phosphorylation via MAPK. Our findings suggest that this pathway is also used to influence *eya* transcriptional levels. Together these mechanisms provide a route for greater precision in regulating a factor that is critical for the formation of a wide range of diverse tissues.

IN Drosophila, an evolutionarily conserved regulatory network executes early decisions within the retina. This network includes a dozen known nuclear proteins that serve as DNA-binding proteins, transcriptional co-activators, phosphatases and kinases (Kumar 2009). Much effort into understanding the genetic, molecular, and biochemical mechanisms that underlie the function of this network has revealed that it does not function as a simple linear cascade with a unidirectional flow of information. Rather, the network is characterized by a meshwork of interactions that include numerous feedback loops and closed auto regulatory circuits (Kumar 2009). Additionally, several signaling transduction pathways function reiteratively within the network (Chen *et al.* 1999; Baonza and Freeman 2001; Kurata *et al.* 2000; Hsiao *et al.* 2001; Kumar and Moses 2001b,c; Baonza and Freeman 2002; Voas and Rebay 2004). Complicating our understanding of this network is that all of the interactions described to date do not necessarily occur uniformly throughout the eye. Instead, the functioning of the network seems to be influenced by spatial and temporal considerations (Salzer and Kumar 2009).

The *eyes absent* (*eya*) gene plays a central role within the retinal determination network. It encodes a transcriptional co-activator that also serves as a protein tyrosine phosphatase (Li *et al.* 2003; Rayapureddi *et al.* 2003; Silver *et al.* 2003; Tootle *et al.* 2003). Like the other members of the network, *eya* is expressed and functions within multiple tissues during development (Leiserson *et al.* 1998; Bonini *et al.* 1993, 1998; Bai and Montell 2002; Fabrizio *et al.* 2003). Null mutants die during embryogenesis while mutations within an eye specific enhancer lead to viable animals completely lacking the compound eye (Bonini *et al.* 1993, 1998; Leiserson *et al.* 1998; Bui *et al.* 2000a,b; Zimmerman *et al.* 2000). In contrast, forced expression of *eya* in several nonretinal tissues is sufficient to induce ectopic eye formation (Bonini *et al.* 1997).

Eya and its mammalian homologs influence development through two distinct biochemical mechanisms. First, they serve as transcriptional activators within a complex that often includes members of the Six and Dach families of homeobox DNA-binding proteins (Chen *et al.* 1997a; Pignoni *et al.* 1997; Xu *et al.* 1997; Ohto *et al.* 1999; Ikeda *et al.* 2002; Silver *et al.* 2003). As Six proteins appear to be lacking in strong intrinsic activation properties, Eya proteins are critical to promoting the expression of Six-Eya targets (Pignoni *et al.* 1997; Jemc and Rebay 2007a). Second, Eya proteins have been shown to possess tyrosine phosphatase activity (Rayapureddi *et al.* 2003; Tootle *et al.* 2003;

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[1]*Corresponding author:* Myers Hall 202, 915 E. 3rd St., Bloomington, IN 47401. E-mail: jkumar@indiana.edu

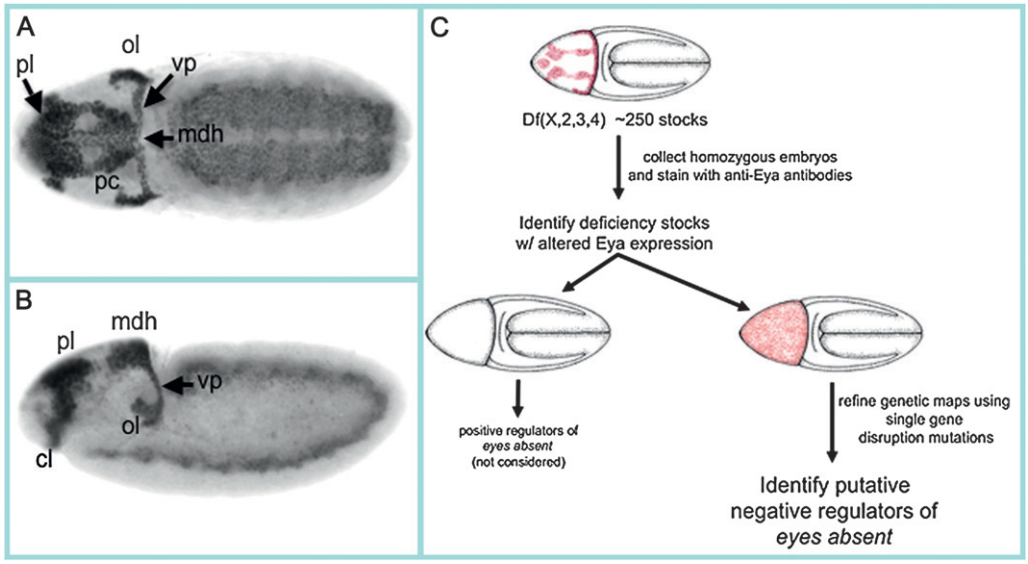

FIGURE 1.—Distribution of Eya within the developing embryonic head of Drosophila (A and B) Dorsal and lateral views respectively of wild type stage 9 embryos stained with an antibody that recognizes Eya. (C) A schematic drawing describing the genetic screen the yielded potential upstream transcriptional regulators of *eya* expression. ol = optic lobes, mdh = mid dorsal head, pl = procephalic head, pc = protocerebrum, cl = clypeolabrum. Anterior is to the left.

REBAY *et al.* 2005). This activity appears to be required for Eya to serve as a transcriptional activator, as mutations that reduce the phosphatase activity of Eya proteins reduce the ability of the Six-Eya complex to interact with DNA (LI *et al.* 2003; MUTSUDDI *et al.* 2005; JEMC and REBAY 2007b). More recently, Eya phosphatase activity has been shown to be required for appropriate embryonic CNS axonogenesis as well as photoreceptor axon guidance in Drosophila (XIONG *et al.* 2009). These recent findings, taken with work previously completed in mammalian cell culture, suggest that Eya had distinct developmental responsibilities in both the cytoplasm and the nucleus (FAN *et al.* 2000; EMBRY *et al.* 2004; XIONG *et al.* 2009).

The wide-ranging expression patterns of *eya* and the ability of Eya protein to function in both nuclear and cytoplasmic compartments suggests that its regulation may be complicated and occur at many levels. Indeed, Eya activity is modulated post-translationally via phosphorylation by EGFR/MAPK signaling (HSIAO *et al.* 2001) while its subcellular localization is regulated via interactions with select G-α subunits in mammalian cell culture (FAN *et al.* 2000; EMBRY *et al.* 2004). We set out to identify genes that lie genetically upstream of *eya* and regulate its expression. We conducted a screen for mutants that alter the distribution pattern of Eya protein in the developing embryonic head. From this effort we isolated a number of putative upstream transcriptional regulators including representatives from several signaling pathways. In particular, we demonstrate that the EGF Receptor signaling pathway regulates the expression of *eya* through the Ets transcription factors *pointed* (*pnt*) and *yan/anterior open*. We also describe the putative regulatory relationship between this signaling pathway and two other retinal determination genes, *sine oculis* (*so*) and *dachshund* (*dac*).

MATERIALS AND METHODS

Fly stocks and genetic screen: The Bloomington Drosophila Deficiency Kit was used to initially interrogate the genome for regions containing positive and negative regulators of *eya* expression. We collected stage 9 embryos homozygous for each chromosomal deletion within the kit and stained them with an antibody that recognizes the Eya protein. These deletions provide >95% coverage of the *Drosophila* genome. The embryos were assayed for changes in the *eya* expression pattern. As a secondary screen we repeated this analysis with single gene disruption mutations the lie within the subset of deficiencies that altered *eya* expression. Eya protein distribution was altered in the following mutant alleles: *yan*^{1}, *argos*^{W11}, *bib*^{1}, *cact*^{1}, *CG1455*^{d08265}, *CG3353*^{UY1730}, *da*^{1}, *dac*^{1}, *dl*^{4}, *dpp*^{H48}, *dve*^{k065515}, *Egfr*^{k05115}, *emc*^{1}, *exu*^{1}, *lab*^{14}, *l(1)G0344*^{G0344}, *l(1)G01290*^{G0129}, *l(1)G0145*^{G0145}, *l(2)k09221*^{k09221}, *l(2)k07433*^{e00176}, *l(2)k02107d*^{k02107a}, *l(2)k05713*^{k05713}, *l(2)k0711b*^{k0711b}, *l(2)k07237*^{k07237}, *l(2)k13704*^{k13704}, *l(3)k65ACf*^{D54}, *Mad*$^{1-2}$, *Me65d*, *mts*$^{XE-2258}$, *neur*^{11}, *Oce*^{WC1}, *osa*^{2}, *phl*^{G0475}, *scw*^{5}, *sec13*^{01031}, *pnt*^{D88}, *ptc*^{7}, *put*^{135}, *so*^{3}, *Sos*^{k05224}, *spi1*, *srw*^{1}, *stc*^{05441}, *tkv*^{7}, *tld*^{B4}, *tok*^{G3}, *tsg*^{4}, *twi*^{1}, *zen*^{2}. A second mutant allele of each gene was also shown to have altered eya expression patterns (a listing of these mutant alleles is available upon request).

The following GAL4 lines were used in this study in forced expression assays: *ey-GAL4, dpp-GAL4, act5C-GAL4, GMR-GAL4.* The following UAS lines were used in this study: *UAS-yan, UAS-aos, UAS-cact, UAS-da, UAS-dac, UAS-dl, UAS-dpp, UAS-dve, UAS-Egfr, UAS-emc, UAS-exu, UAS-ey, UAS-eya, UAS-eyg,, UAS-lab, UAS-Mad, UAS-mts, UAS-optix, UAS-osa, UAS-ptc, UAS-pnt P1, UAS-Pnt P2, UAS-put, UAS-Ras UAS-scw, UAS-so, UAS-spi, UAS-tkv, UAS-tld, UAS-toe, UAS-toy, UAS-tsg, UAS-twi, UAS-zen.* The following stocks were used to generate mutant retinal mosaic clones: *yweyflp; FRT42D Ubi-GFP/FRT42D aop*^{1} and *yweflp; FRT82B Ubi-GFP/FRT82B pnt*^{D88}. A *pnt-lacZ* line was used to monitor *pnt* transcription in embryos and eye discs. All experiments were conducted at 25°.

Reagents and microscopy: The following reagents were used in this study: mouse α-Dac (1:5), mouse α-Eya (1:5), guinea pig α-So (1:500, gift of Ilaria Rebay), rat α-Elav (1:100), mouse α-β Galactosidase, donkey α-mouse FITC (1:100), goat α-mouse Biotin (1:100), Streptavidin HRP (1:100), donkey α-rat FITC (1:100), goat α-guinea pig FITC (1:100), donkey

TABLE 1

Putative regulators of *eyes absent*

Class I	Class II	Class III	Class IV
osa (osa)[a]	★ *cactus (cact)*[a]	^ *argos (aos)*[a]	^ *anterior open (aop)*[a]
+ *tolkin (tok)*[a]	★ *dorsal (dl)*[a]	+ *decapentaplegic (dpp)*[a]	*big brain (bib)*
CG11455	*escargot (esg)*	*dachshund (dac)*[a]	*defective proventriculus (dve)*[a]
l(2)k07433	*extra macrochaetae (emc)*[a]	*daughterless (da)*[a]	*exuperantia (exu)*[a]
l(3)65ACf	*labial (lab)*[a]	^ *EGF Receptor (EGFR)*[a]	*neuralized (neu)*
l(3)j5E7	*microtubule star (mts)*	^ *pointed (pnt)*[a]	*Ocellarless (Oce)*
	Moire (Me)	*shrew (srw)*	*pole hole (phl)*
	+ *Mothers against dpp (Mad)*[a]	^ *spitz (spi)*[a]	*sine oculis (so)*[a]
	patched (ptc)[a]	+ *thick veins (tkv)*[a]	*CG31195*
	+ *punt (put)*[a]	+ *twisted gastrulation (tsg)*[a]	*l(1)19Cb*
	+ *screw (scw)*[a]	*zerknult (zen)*[a]	*l(1)G0120*
	Sec13	*l(1)G0344*	*l(1)G0145*
	shuttle craft (stc)	*l(2)44Ea*	*l(2)ry50*
	^ *Son of sevenless (Sos)*	*l(2)46Ca[37]*	
	+ *tolloid (tld)*[a]	*l(2)k09221*	
	★ *twist (twi)*[a]	*l(2)k02107a*	
	CG3353		
	l(2)k05713		
	l(2)k07118b		
	l(2)k07237		
	l(2)k13704		
56 genes identified in total			

+ indicates TGFb pathway member
★ indicates Dorsal/Toll pathway member
^ indicates EGFR pathway member
[a] indicates gene selected for overexpression assay

α-rat Cy5 (1:100), donkey α-mouse Cy5 (1:100), phalloidin-TRITC (1:1000; Molecular Probes) and DAB Detection Kit (Pierce). All primary antibodies (with the exception of α-So) are from the Developmental Studies Hybridoma Bank and all secondary antibodies are from the Jackson Laboratories. Embryos, imaginal discs and adult eyes were prepared for light, fluorescent and scanning electron microscopy as essentially described in Anderson *et al.* (2006).

RESULTS

A screen for embryonic regulators of *eya* identifies four classes of regulators: The expression of the *eyes absent (eya)* gene, like all members of the eye specification network, is not restricted to the developing retinal epithelium but extends to several non-retinal tissues. Within the developing embryonic head (at stage 9), Eya protein is distributed within the optic lobes (ol), visual primordium (vp), mid-dorsal head (mdh), protocerebrum (pc), procephalic lobes (pl), and clypeolabrum (cl) (Bonini *et al.* 1998) (Figure 1, A and B). In an effort to better understand how this dynamic expression pattern is achieved, we executed a genetic screen designed to identify putative transcriptional regulators of *eya* (Figure 1C).

Initially, we systematically screened 235 deficiency stocks by collecting and staining stage 9 embryos with an anti-Eya antibody and looking for changes in Eya distribution within the embryonic head (supporting information, Table S1). Throughout the screen we eliminated candidates that demonstrated early embryonic lethality or had gross defects in head morphology. Deficiencies that exhibited visibly altered *eya* expression, 57 in total, where selected for further analysis via smaller deficiencies and single gene disruptions within the cytologically mapped breakpoints. In this second phase of the screen we tested 173 smaller deficiencies (100 were positive for changes in *eya*) and 316 single gene disruption stocks (53 were positive for altered *eya* expression) to refine our genetic maps (Table S2). As members of well-known signaling pathways emerged, we were able to select additional members of each pathway to identify genes that may have been missed in our initial screen (3 were positive for changes in *eya* pattern). We reasoned that these genes may have been passed over (during the screening of deficiency stocks) because of early lethality or catastrophic developmental defects caused by the loss of large amounts of genetic material. In all, we were able to identify a total of 56 putative regulators of *eya* in the embryonic head, many of which have phenotypes (change in *eya* expression) that very closely resemble those seen within the larger deficiencies (Table 1). This approach allowed us to rapidly scan the genome and revealed loci of interest for more detailed phenotypic analysis.

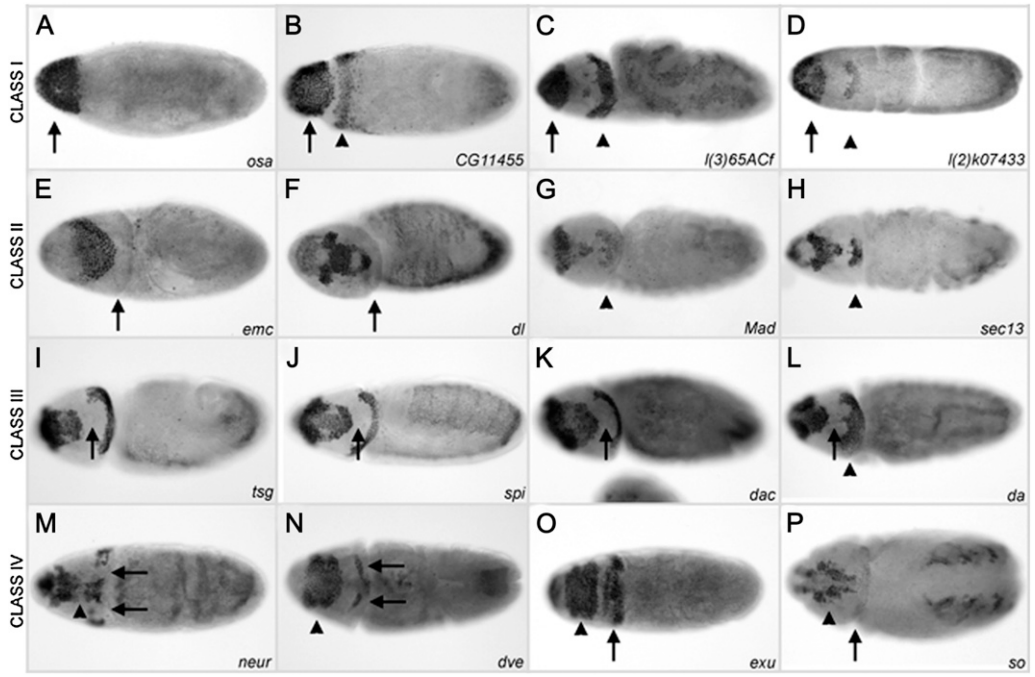

FIGURE 2.—Regulation of *eya* within the developing head of stage 9 embryos (A–P) Dorsal views of mutant stage 9 embryos stained with an antibody that recognizes Eya. Genotypes are listed in the bottom right corner of each panel. Arrows and arrowheads denote changes in the Eya expression pattern (see RESULTS for description). The mutant classifications on the left are assigned based on the primary effects on Eya expression. Anterior is to the left.

Alleles identified in this screen can be placed into four broad categories, which we arbitrarily refer to as Class I–IV mutants (Table 1, Figure 2). It should be noted that while each group is characterized by a primary effect on Eya protein distribution, in some cases secondary phenotypes that may be common to mutants in multiple group are also present. Class I mutants are characterized by the distribution of Eya protein throughout the anterior-most portion of the head (Figure 2, A–D, arrows). Interestingly, in these mutants the entire head does not express *eya;* rather the ectopic expression is restricted to the anterior third to half of the head. In several mutants such as *CG11455, l(3)65ACf* and *l(2)k07433* this pattern is accompanied by an expansion of *eya* expression (up to 10 cell diameters) within the vp (Figure 2, B–D, arrowhead). This is in contrast to the visual primordium of wild type embryos in which *eya* expression spans a width of only 2-3 cells (Figure 1, A and B). Additional defects such as a reduction of *eya* expression in the pc and ol can be seen in a subset of Class I mutants (Figure 2, C and D).

Mutants within Class II are grouped together based on the strong reduction in *eya* within the vp and/or ol (Figure 2, E and H, arrowheads). In some mutants, such as *extra macrochaetae (emc)* and *sec13*, Eya is completely lost in both regions (Figure 2, E and F, arrows). However in other mutants, such as *Mothers against dpp (Mad)* and *dorsal (dl),* the loss of Eya protein is mainly confined to the ol and a portion of the vp (Figure 2, F and G, arrowheads). These two mutants differ from *emc* and *sec13* in that Eya is still present in the mdh. There are additional defects in the more anterior expression domains; however these changes are variable among the mutants within this class.

Class III mutants share a reduction in *eya* within the pc and, to a lesser degree, diminished expression within the ol (Figure 2, I–L). For some of the members of this class, occasional embryos exhibiting Class II Eya patterns were observed. We attribute this phenotypic overlap between Class II and III to genes that fall in the same signaling pathways and may have different relationships with *eya* in different regions of the head (Table 1). Regardless, the majority of embryos for each Class III genotype exhibited Class III alterations to normal Eya protein distribution. Embryos mutant for *twisted gastrulation (tsg), spitz (spi), dachshund (dac),* and *daughterless (da)* exhibit similar changes in Eya distribution. In each mutant, *eya* is completely lost in regions just anterior to the vp and within the ol (Figure 2, I–L, arrows). In contrast, there is an increase in the number of rows of Eya positive cells within the vp. This is especially notable in *da* mutants (Figure 2L, arrowhead). The broadening of *eya* expression in *da* mutants is similar to that seen in class I mutants. However, *da* remains in its present grouping due to the relatively normal Eya expression in the anterior-most portions of the head (compare Figure 2L to 2, B–D).

Class IV represents mutants that we were not able to place in any of the three previously described classes. Changes in *eya* expression in members of this group vary from nearly global loss to selective reduction of *eya* within a limited number of cells. We have chosen four representatives from this group for discussion here (Figure 2, M–P); we will address another Class IV gene, *aop,* in more detail later (see below). The first example, *neuralized (neur),* is member of the Notch signaling pathway. In these mutants we observe a loss of Eya protein in regions of the vp that connect the ol to the

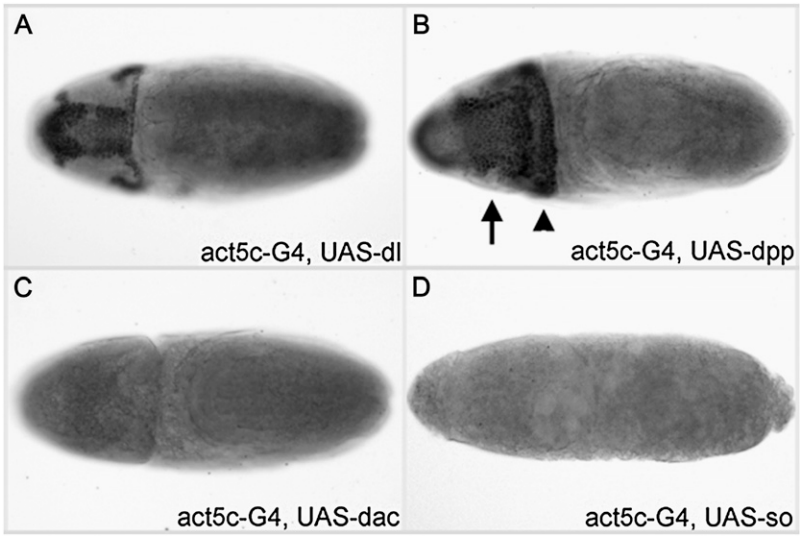

FIGURE 3.—Change in Eya distribution in response to the expression of putative regulators (A–D) Dorsal views of stage 9 embryos in which the *Act5C-GAL4* driver expresses individual regulators throughout the embryo. Genotypes are listed in the bottom right corner of each panel. Anterior is to the left.

mdh (Figure 2M, arrows). Additionally, *neur* mutants display a narrowing of the wild type *eya* expression pattern within the pc (Figure 2M, arrowhead). This is contrasted in *defective proventriculus (dve)* mutants where *eya* expression is lost in the mdh, the posterior portion of the pc, and the ol leaving only two narrow strips of the visual primordium to express *eya* (Figure 2N arrows). In addition, *dve* mutants show a slight broadening of *eya* across the anterior part of the head including the anterior portion of the pc (Figure 2N, arrowhead). Similarly, *exuperantia (exu)* mutants have a wider patch of *eya* expressing cells in the anterior portion of the head and thickening of the vp (Figure 2O, arrowhead, arrow). The vp thickening is a common characteristic of Class I mutants, however *exu* mutants do not display the same increase in Eya protein distribution in the anterior-most regions of the head that we see in all Class I members (compare Figure 2O to 2B,C). Finally, *sine oculis (so)* mutants exhibit some characteristics of *neur* and *dve* mutants: *eya* expression is lost in the ol and in subsets of cells of the pc. Levels of Eya protein are also dramatically reduced in the vp, which is a common feature of Class II mutants (Figure 2P, arrow & arrowhead).

Interestingly, two RD genes, *sine oculis (so) and dachshund (dac),* emerged from our screen as embryonic *eya* regulators (Figure 2, K and P). With respect to each other, *so* and *eya* are thought to reside at the same level within the RD hierarchy. In the developing retina they have both been shown to be directly activated by the transcription factor, *ey* (HALDER *et al.* 1998; NIIMI *et al.* 1999; OSTRIN *et al.* 2006) and So and Eya protein products form a biochemical complex whose function is thought to be crucial for promoting eye formation through the activation of downstream target genes (PIGNONI *et al.* 1997). One of these targets is *dac,* a gene that occupies the lowest known position within the cascade. Additionally, Dac and Eya themselves have been shown to form a biochemical complex *in vitro* though more recent work suggests that formation of this complex might not be necessary for eye development (CHEN *et al.* 1997a; TAVSANLI *et al.* 2004). In the eye disc, the So-Eya-Dac subcircuit is flexible and the regulatory relationships change in a position dependent manner (SALZER and KUMAR 2009). In most regions of the eye imaginal disc So and Eya participate in their canonical positive regulatory role with respect to *dac.* However in the very posterior regions of the tissue this relationship changes and So represses *dac* through partnership with a co-repressor. And at the margin of the eye disc there is feedback stability among all three members of this RD subcircuit (SALZER and KUMAR 2009). Interestingly, based on our data in the embryo, it appears that a feedback loop exists between *dac* and *eya* in this developmental context as well as in the margin of the eye disc. It should be noted that Eya and Dac protein distribution in stage 9 embryos is largely non-overlapping (KUMAR and MOSES 2001b) suggesting that part of the change we see in *eya* expression in *dac* mutants may be non-autonomous or may occur indirectly. At earlier stages in embryonic development (~5hr AEL), *so* and *dac* expression overlap in the ol, thus it is possible that at this stage *dac* positively regulates *so* and *so*, in turn, regulates *eya* (KUMAR and MOSES 2001b).

Expression of putative regulators is sufficient to alter Eya protein distribution: Using the UAS/GAL4 system we forcibly expressed a subset of the genes that are listed in Table 1 (marked with an asterisk) throughout the embryo in an attempt to determine if these genes, on their own, are sufficient, to influence *eya* expression. The genes were chosen based on the availability of extant UAS-driven transgenes. Several genes, such as *dl*, proved necessary for normal *eya* expression based on mutant analysis, but were incapable of altering Eya protein distribution in this forced

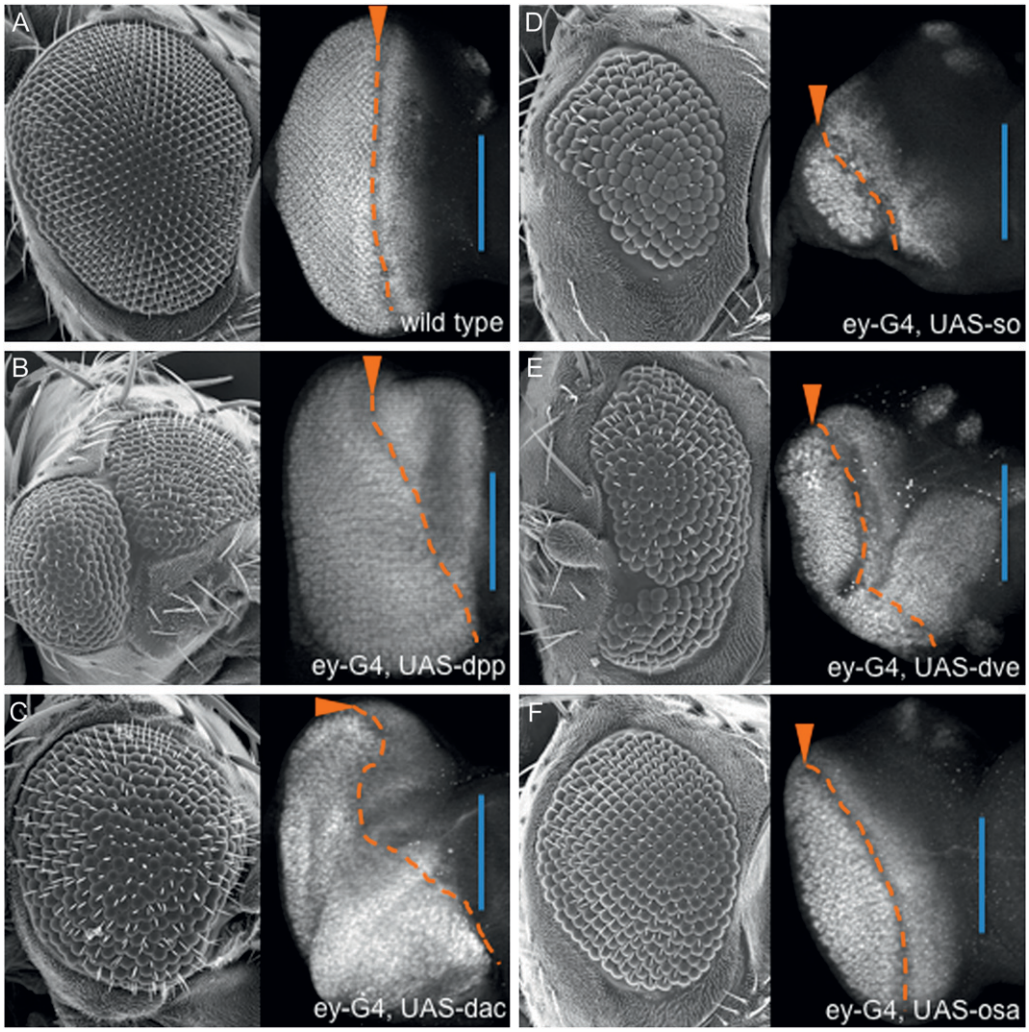

FIGURE 4.—Overexpression of putative *eya* regulators in cells ahead of the morphogenetic furrow. (A) Scanning electron micrograph of a wild type eye and immunofluorescence image of a wild type eye disc stained with an antibody that recognizes Eya protein. (B–F) Adult eye and corresponding imaginal disc stained with Eya antibody, genotype is indicated at lower right. In all panels, anterior is to the right. Blue bar indicates the anterior-most edge of the retinal field. Orange arrowhead and dashed line indicate the morphogenetic furrow.

expression assay (Table 1; Figure 2F, 3A). There are a few possible explanations for this result; it may be that these putative regulators require co-factors that have not been provided in the assay. Alternatively, they may not have been expressed at high enough levels to see a change in Eya distribution. Another possibility could be that there may be other factors at play within a signaling pathway that limit the function of the overexpressed gene and its encoded protein. And finally, it is possible that the connection between the putative regulator and *eya* is so indirect that overexpression of the regulator is incapable of having a direct effect on the transcription of *eya*.

On the other hand, many genes are, in fact, capable of altering *eya* expression. For instance, down regulation of the TGFβ signaling pathway leads to a strong loss of *eya* expression in the pc while increased levels has the opposite effect: Eya protein distribution is expanded within the pc and vp of animals overexpressing *dpp* transgenes (Figure 2I, 3B, arrows and arrowheads). Expression of a subset of genes such as *dac* is sufficient to completely abolish or drastically reduce *eya* expression from the embryo (Figure 3C). There are also occasional instances in which we cannot determine the effect that global overexpression of the regulators has on Eya protein distribution because forced expression leads to early embryonic lethality. This was the case when we expressed *so* throughout the developing embryo. In this case the embryo failed to even undergo germband extension (Figure 3D).

Effects of forced expression on Eya protein distribution in the developing eye: We were interested in determining if putative *eya* regulators could also function to govern *eya* expression in the retina. Normally *eya* is expressed in a broad stripe ahead of the morphogenetic furrow (approximately 20 cell diameters wide), in all developing photoreceptor and cone cells, all undifferentiated cells behind the morphogenetic furrow, and within the developing ocelli (Figure 4A, furrow marked with arrowhead and dashed line; (BONINI *et al.* 1993). An enhancer of the *ey* gene directs expression ahead of the furrow to a broader swathe of cells when compared to the known *eya* enhancer (data not shown). Using the *ey*-GAL4 driver, we expressed a subset of genes from Table 1 in all cells ahead of the furrow and then used an antibody to assay the effect on Eya protein distribution.

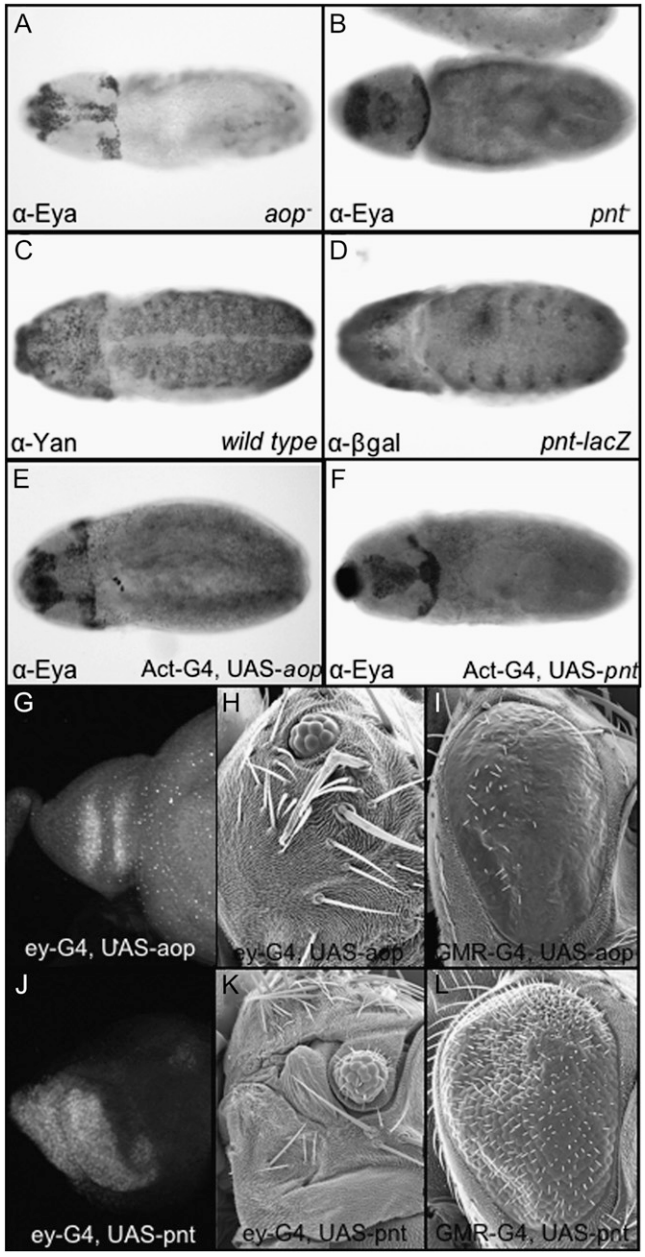

Figure 5.—EGFR regulation of *eya* in the embryo and eye. (A–D) Eya distribution in embryos that are either mutant for overexpressing activated transgenes of *aop* or *pnt*. Anterior is to the left. (E and H) Eya expression in eye discs overexpressing either *aop* or *pnt* transgenes. (F, G, I, and J) Scanning electron micrographs of adult eyes overexpressing *aop* or *pnt* via *ey*-GAL4 or GMR-GAL4. Anterior is to the right in E–J. Genotype is indicated at the bottom of each panel.

We first focused on genes known to function during retinal determination. Forced expression of *decapentaplegic (dpp)*, a member of the TGFβ superfamily, activates *eya* expression in all cells ahead of the furrow and appears to initiate ectopic eye formation at the anterior margin of the eye field. Often the end result is the production of two compound eyes being generated from a single retinal field (Figure 4B). This result is consistent with previous work that demonstrated that *dpp* functions within the retinal determination network to promote eye specification and furrow initiation (Pignoni and Zipursky 1997; Shen and Mardon 1997; Hazelett *et al.* 1998). It is also consistent with the effects that we see in the embryo in which activation of TGFβ signaling in the embryonic head leads to ectopic *eya* expression in the pc and the vp (Figure 3B). Similarly, expression of *dac* ahead of the furrow in the developing eye is also capable of inducing *eya* expression (Figure 4C). This in contrast to what we observe in the embryo where over-expression of *dac* results in the strong repression of *eya* (Figure 3C).

Forced expression of *so* did not have the same effect on *eya* expression. Instead of activating *eya* transcription and/or generating a larger eye field, the *ey-GAL4, UAS-so* eyes are smaller with little to no effect on the pattern of Eya protein distribution ahead of the furrow (Figure 4D). We then looked at how the forced expression of two genes from Table 1 [*dve* and *osa*] influence *eya* expression in the retina. These genes were chosen since they are not previously known to function in eye specification. Expression of *dve* in the eye disc leads to increases in both cell proliferation and *eya* expression (Figure 4E). In contrast, expression of *dve* within the embryonic head results in the downregulation of *eya* (data not shown). This represents another example of the same gene having distinctly opposite regulatory effects on its target depending on the stage of development. Expression of *osa*, which is known to be involved in furrow initiation and retinal differentiation does alter the structure of the eye but does not have a direct effect on *eya* expression (Figure 4F).

Since Eya is normally present in all cells behind the furrow, we also expressed each putative regulator under the control of *GMR-GAL4*, which drives expression in these cells. In cases where *GMR-GAL4/UAS-putative regulator* adults demonstrated aberrant retinal morphologies we dissected and stained eye discs and looked for changes in Eya expression. While several putative regulators caused eye phenotypes ranging from mildly rough to severely glazed (Figure S1), with the exception of *yan/anterior open* (see below), we observed no significant changes in Eya protein distribution in third instar eye discs (data not shown).

The EGF Receptor pathway regulates *eya* expression: In our screen we identified members of the TGFβ, Notch, and EGFR pathways, a result that connects these signaling cascades in the regulation of *eya* expression within the embryonic head (Figure 2, Table 1). Previous work has also implicated these pathways in regulating both *eya* and several other members of the retinal determination network (Chen *et al.* 1999; Curtiss and Mlodzik 2000; Kurata *et al.* 2000; Hsiao *et al.* 2001; Kumar and Moses 2001a; Firth and Baker 2009). Because the EGF Receptor pathway regulates Eya activity in the retina by phosphorylation via the downstream cytoplasmic effector protein MAPK (Hsiao *et al.*

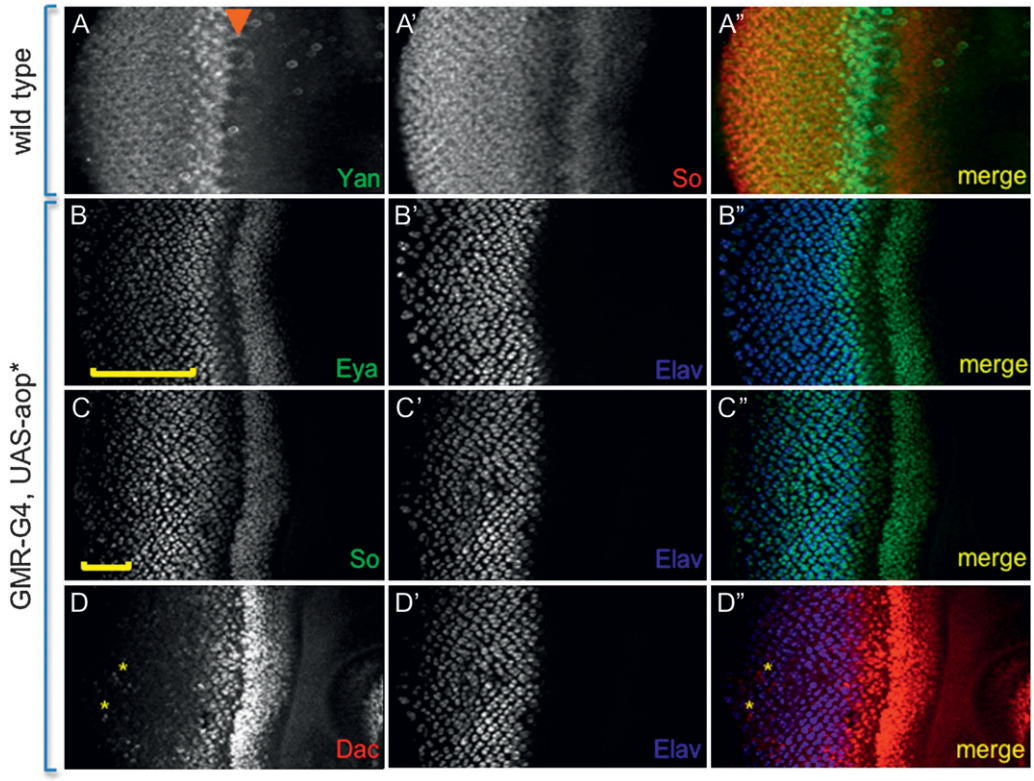

FIGURE 6.—*aop/yan* overexpression in the retina alters Eya, So and Dac distribution. (A–A") Immunoflourescent image of wild-type eye imaginal disc stained with antibodies that recognize Yan and So proteins. Orange arrowhead denotes morphogenetic furrow. (B–D") Immunofluorescence images of eye imaginal discs that express an activated *aop* transgene under the control of GMR-GAL4 and are stained with antibodies that recognize Eya, So, Dac, and Elav. Detected proteins are indicated at the bottom right corner of each panel. Yellow brackets denote portion of eye disc that displays reduced expression of the marker. Yellow asterisks neighbor cells that ectopically express Dac. Anterior is to the right.

2001; FIRTH and BAKER 2009), we chose to take a closer look at *yan/anterior open (aop)* and *pointed (pnt)*, two Ets transcription factors that lie downstream of the EGF Receptor (O'NEILL *et al.* 1994; REBAY and RUBIN 1995).

Homozygous mutant *aop* and *pnt* embryos each exhibit striking changes to the *eya* expression pattern in the embryo. In *aop* mutants we see changes in Eya protein in the dorsally shifted ol and a narrowing of the expression domain throughout the mdh and pc (Figure 5A; ROGGE *et al.* 1995). In *pnt* mutants we see similar changes to Eya protein distribution in the ol, complete loss in the mdh and normal expression in the anterior-most portion of the head including the pc and pl (Figure 5B). While these loss-of-function effects are not identical, they are more similar than one might expect from two genes that are reportedly direct antagonists of one another. In a similar effort, Anderson et al conducted a screen in the embryo to identify transcriptional regulators of *dac*. Since neither *aop/yan* nor *pnt* were identified in that effort, we are confident that these mutants represent strong candidates for regulation of *eya* (ANDERSON *et al.* 2006). When we screened late stage embryos (post germband retraction) we saw very similar effects on *eya* expression presumably due to the breakdown in EGFR signaling. The developmental and patterning defects that we observe are ones that one would expect from loss of EGFR (Figure S2).

We used an antibody that recognizes the Aop/Yan protein to determine the *aop/yan* expression pattern in stage 9 embryos. We observe uniform expression across the head of embryos with particularly prominent expression within the mdh (Figure 5C). A *pnt-lacZ* stock was used to also determine the *pnt* expression pattern at this stage. *pnt* expression appears more restricted in the head, predominantly seen in two patches flanking the pc (Figure 5D).

Using an Ac5C-GAL4 driver we overexpressed *aop* and *pnt* throughout the developing embryo and assayed for changes in Eya protein distribution. When *aop* is expressed throughout the embryo we observe a broadening of the *eya* expression pattern throughout the visual primordium and mdh: an effect that is opposite to *aop* loss-of-function (Figure 5E). Interestingly, *pnt* overexpression results in an Eya protein distribution pattern that, aside from the striking increase in Eya at the anterior-most portion of the head, resembles the *aop* mutant (Figure 5F). This is consistent with an antagonistic relationship between these two genes. However, from this assay it appears that the relationship may be unidirectional since *aop* overexpression does not mimic *pnt* loss of function. This may be due to functional redundancy within the Ets family of transcription factors at sites which *aop/yan* regulates *eya* or a limitation in the ability of Yan protein itself to fully out-compete Pnt protein for regulatory binding sites.

Normally, *aop* is expressed at a high level posterior to the morphogenetic furrow where it serves to repress premature neuronal fate specification (Figure 6A; REBAY and RUBIN 1995). Since we identified *aop* in our screen and since both Aop and Eya proteins have partially overlapping distribution patterns posterior to the morphogenetic furrow, we were interested in de-

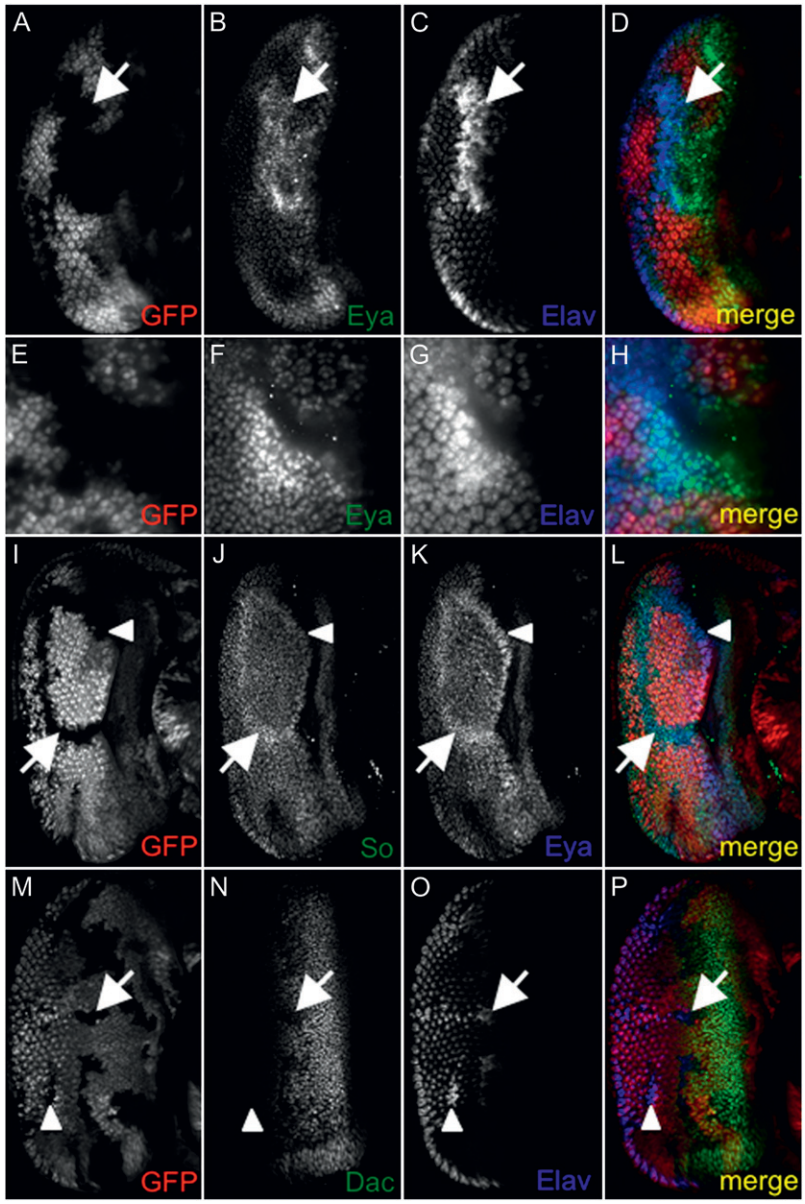

FIGURE 7.—Aop/Yan regulates the RD genes *eya*, *so*, and *dac*. (A–P) Immunofluorescence images of aop^{1} mutant clones in the eye imaginal disc. (A–H) Clones are marked by the absence of GFP and stained with antibodies that recognize Eya and Elav proteins. (I–L) Clones are marked by the absence of GFP and tissue is stained with antibodies that recognize RD genes Eya and So. (M–P) Clones are identified by the absence of GFP and the disc is stained with antibodies that recognize Dac and Elav. Arrows and arrowheads denote changes in expression attributable to the loss of *aop/yan*. Please refer to the text for specific arrow and arrowhead notations. Detected proteins are indicated at the bottom right corner of each panel. Anterior is to the right.

termining if EGF Receptor signaling through *aop* also regulates *eya* expression in the eye. We carried out *ey*-GAL4 and *GMR*-GAL4 driven overexpression experiments in the eye with activated *aop* and *pnt* responder lines. Expression of *aop* leads to a dramatic reduction in the size of the eye field (Figure 5, G and H). While Eya protein is still distributed within the anterior remnants of the eye field, the pattern is different than that seen in discs that express other genes such as *so, osa* or *dve* (compare 5G to 4D–F). Specifically, *eya* expression is not seen fully behind the small furrow suggesting that, in this example, the very small eye field may be due to a direct loss of *eya*. With surprising similarity, overexpression of a UAS-*pnt* transgene caused a dramatic reduction in the size of the adult eye (Figure 5K compare to 5H). However, unlike *aop*, Eya protein is still present ahead of and behind the distorted furrow within the reduced eye field (Figure 5J). Overexpression of either *aop* or *pnt* in developing photoreceptors via GMR-GAL4 resulted in animals with rough eyes (Figure 5, I and L). The rough eyes are likely to be due, in part, to alterations in Eya and So protein levels (Figure 6).

Yan/Aop regulates the expression of *so* and *eya* but not *dac*: Forced expression of *aop* in all developing photoreceptors via the *GMR-GAL4* driver resulted in severe retinal defects. We assayed for changes in Eya protein distribution in response to activated *aop* overexpression and observed a marked, uniform depletion of Eya behind the furrow that began about 10 cell diameters behind the furrow (Figure 6B, yellow bracket). Since *so, eya,* and *dac* have a complex, position dependent regulatory relationship within the developing eye and since *so* and *dac* were also identified in our screen for regulators of *eya* we assayed for So and Dac protein distribution in *aop* over-expressing retinas. As with Eya we observed downregulation of So, however

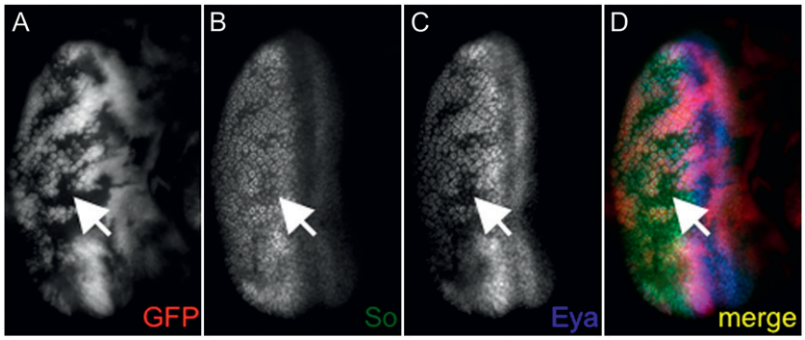

Figure 8.—So and Eya protein levels are reduced in *pnt* clones. (A–D) Immunofluorescence images of pnt^1 mutant clones in the eye imaginal disc. Clones are marked by the absence of GFP and stained with antibodies that recognize So and Eya proteins. Arrows point to pnt mutant clones. Detected proteins are indicated at the bottom right corner of each panel. Anterior is to the right.

this effect was delayed in developmental time and occurred in much more posterior regions of the eye disc (Figure 6C yellow bracket). Dac, on the other hand showed no significant reduction within its normal expression domain. Instead, we observed rather patchy ectopic *dac* expression in the posterior-most region of the eye disc (Figure 6D, yellow asterisks). The upregulation of *dac* in response to the loss of *so* in the most posterior sections of the retina is consistent with our previous findings that normally So can function to repress *dac* in this region of the developing eye (Salzer and Kumar 2009).

Next we generated aop^1 loss-of-function mosaic clones in the developing retina. In agreement with previous reports, removal of *aop* leads to a disorganization of the retina and an increase in photoreceptor cells (O'Neill *et al.* 1994). We extended these observations by demonstrating that Eya protein levels are elevated in aop^1 clones behind the furrow (Figure 7, A–H). This finding suggests that in addition to the modification of Eya activity by MAPK phosphorylation (Hsiao *et al.* 2001), the EGFR pathway also regulates the expression levels of the *eya* transcriptional unit. In addition it appears that So protein levels are also elevated in *aop* clones (Figure 7, I–L) suggesting that either EGFR signaling via Aop regulates both *so* and *eya* or there is a serial link between these factors. It should be noted that we also observe an increase in ELAV protein within cells that are mutant for *aop/yan*. Since both *eya* and *so* are required for the maintenance of photoreceptor fate (Pignoni *et al.* 1997) it is possible that these factors regulate a number of neuron/photoreceptor specific genes including *elav*. As the down-regulation of *aop/yan* results in an increase in Eya and So protein levels it is possible that this in turn leads to an increase in ELAV protein levels. Downstream of both factors lies *dac*, which is regulated by the So-Eya composite transcription factor (Pappu *et al.* 2005). Interestingly, in *aop* clones, which have elevated levels of both *so* and *eya*, we do not see the expected elevation of Dac protein levels. Instead we observe a complete loss or severe downregulation of *dac* expression within *aop* clones (Figure 7, M–P arrowhead). As distinct regulatory relationships exist among the So-Eya-Dac subcircuit within different spatial regions of the eye field (Salzer and Kumar 2009) and since *dac* genetically interacts with the EGFR signaling cascade (Mardon *et al.* 1994), the loss of *dac* expression in *aop* clones is likely the result of the combined activities of the EGFR pathway and the So-Eya complex.

The removal of *pnt* in the eye should result in the loss of both *eya* and *so* expression. And that is indeed what we observe. In clones that are lacking *pnt* activity, the levels of both So and Eya proteins are reduced but not eliminated (Figure 8). This is consistent with reports in which the loss of *pnt* results in the reduction but not completely loss of photoreceptor development (O'Neill *et al.* 1994). This result suggests that the loss of photoreceptor neurons in *pnt* mutants may in fact be due to the loss of *eya* and *so* expression.

DISCUSSION

In this report we describe a genetic screen that identified factors that direct the expression of the retinal determination gene *eyes absent* to the developing embryonic head and eye imaginal disc. We identified putative regulators by the loss or expansion of Eya protein distribution within the embryonic head of stage 9 loss-of-function mutants. Our findings indicate multiple signaling cascades including Notch, Hedgehog, TGFβ, and the EGFR regulate *eya* expression. These results are consistent with previous studies identifying Hedgehog, Ras, and TGFβ as regulators of *eya* function in eye development (Chen *et al.* 1999; Curtiss and Mlodzik 2000; Hsaio *et al.* 2001; Pappu *et al.* 2003; Firth and Baker 2009). We did not recover mutations in any of known Wingless pathway members. This was slightly unexpected as Wnt signaling and *eya* are known to reciprocally regulate each other (Hazelett *et al.* 1998). This result could imply, however, that *eya* is regulated differently in diverse tissues.

A screen similar to the one described here successfully identified the TGFβ pathway as an important upstream regulator of another retinal determination gene, *dachshund* (Anderson *et al.* 2006). Of interest is the observation that the loss of TGFβ signaling has differential effects on *eya* and *dac* expression. In TGFβ mutant embryos ectopic *dac* expression was observed in cells of the visual primordium. However, *eya* expression remains unaffected in this tissue and is instead lost in

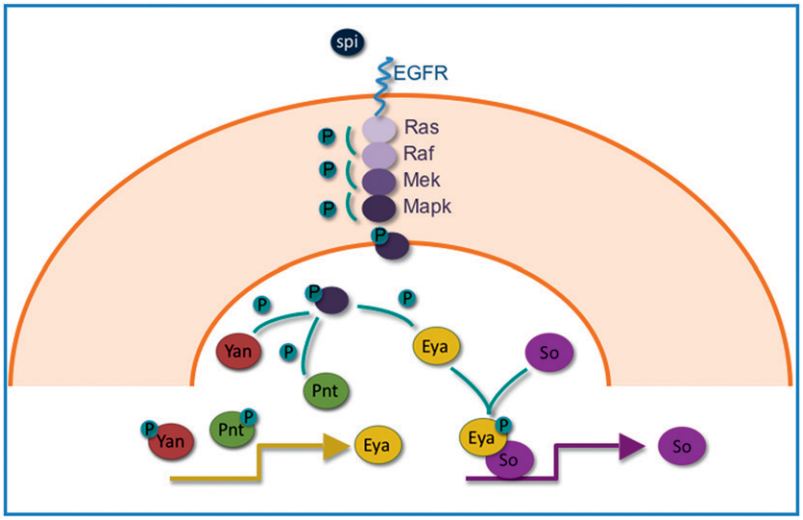

FIGURE 9.—Model for EGFR mediated regulation of RD cofactors *so* and *eya*. Note that EGFR regulates Eya activity via phosphorylation by MAPK and also through transcriptional regulation at the genetic level. It remains unclear if the transcriptional regulation of eya by Yan and Pnt is direct or through additional intermediates. Teal molecules with "P" represent phosphate groups.

the subsets of cells that give rise to the protocerebrum. These differential effects are interesting as *eya* and *dac* interact genetically within the retinal determination network. Therefore it seems that these regulatory relationships vary among different tissues. It also appears that the number of distinct signaling pathways that regulate *eya* expression outnumbers that of *dac* (this report; (ANDERSON *et al.* 2006). This is unsurprising as the expression pattern of *eya*, when compared to *dac*, is considerably more dynamic, at least within the embryonic head.

We were particularly interested in finding that mutations in *spitz*, *argos*, *anterior open/yan* and *pointed*, all members of the EGFR signaling pathway, altered the transcriptional pattern of *eya*. Previous work has demonstrated that the EGFR pathway post-translationally regulates Eya activity in the developing eye through phosphorylation via Ras/MAPK at two sites within the transactivation domain (Figure 9; HSIAO *et al.* 2001). Experiments in both flies and in insect cell culture indicate that phosphorylation augments, but is not absolutely essential, for either the transcriptional activation potential of Eya or for the induction of ectopic eyes in forced expression assays (HSIAO *et al.* 2001; SILVER *et al.* 2003).

Our findings suggest that the EGFR pathway is also required to regulate *eya* transcription (Figure 9). This is consistent with findings that *eya* expression is lost in *mago*$^{-}$ clones, which reduce Ras signaling (FIRTH and BAKER 2009). Indeed, loss of *aop/yan* behind the morphogenetic furrow results in the higher levels of Eya and its facultative partner So. Both proteins are required for photoreceptor cell fate specification and maintenance (PIGNONI *et al.* 1997; SALZER and KUMAR 2009). Elevated levels of Eya and So proteins in *yan* mutant clones are consistent with roles for Yan in suppressing photoreceptor cell fate during normal development (O'NEILL *et al.* 1994; REBAY and RUBIN 1995). We see that, in *yan* clones, Eya protein levels are activated to significantly higher levels than that of So. One possible explanation for these results is that EGFR signaling may in fact regulate *eya* expression but not that of *so*. As EGFR signaling also regulates Eya activity, in a *yan* clone there may be a feedback loop that ultimately results in lowered levels of Eya phosphorylation. Reduced levels of the Eya phospho-protein, while still able to stimulate *so* transcription, may do so at a less efficient rate thereby leading to lower levels of ectopic So protein (Figure 9).

Unexpectedly, we find that *dac*, a putative downstream target of the So-Eya complex, is not up regulated in *yan* clones. Rather, *dac* expression is down-regulated when *yan* is removed. As So-Eya is thought to positively regulate *dac* expression this result is somewhat puzzling. The result does suggest that *dac* is regulated not only by the Eya-So complex but also by other mechanisms, possible through EGFR signaling and an intermediate repressor. As our prior work has recently shown, the So-Eya-Dac subcircuit is under complex regulatory control (SALZER and KUMAR 2009). The work presented here suggests that still greater complexity exists in the form of differential regulation by signal transduction cascades both at transcriptional and post-translational levels.

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LITERATURE CITED

ANDERSON, J., C. L. SALZER and J. P. KUMAR, 2006 Regulation of the retinal determination gene dachshund in the embryonic head and developing eye of Drosophila. Dev Biol **297:** 536–549.

Bai, J., and D. Montell, 2002 Eyes absent, a key repressor of polar cell fate during Drosophila oogenesis. Development **129:** 5377–5388.

Baonza, A., and M. Freeman, 2001 Notch signalling and the initiation of neural development in the Drosophila eye. Development **128:** 3889–3898.

Baonza, A., and M. Freeman, 2002 Control of Drosophila eye specification by Wingless signalling. Development **129:** 5313–5322.

Bonini, N. M., W. M. Leiserson and S. Benzer, 1993 The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell **72:** 379–395.

Bonini, N. M., Q. T. Bui, G. L. Gray-Board and J. M. Warrick, 1997 The Drosophila eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. Development **124:** 4819–4826.

Bonini, N. M., W. M. Leiserson and S. Benzer, 1998 Multiple roles of the eyes absent gene in Drosophila. Dev. Biol. **196:** 42–57.

Bui, Q. T., J. E. Zimmerman, H. Liu and N. M. Bonini, 2000a Molecular analysis of Drosophila eyes absent mutants reveals features of the conserved Eya domain. Genetics **155:** 709–720.

Bui, Q. T., J. E. Zimmerman, H. Liu, G. L. Gray-Board and N. M. Bonini, 2000b Functional analysis of an eye enhancer of the Drosophila eyes absent gene: differential regulation by eye specification genes. Dev. Biol. **221:** 355–364.

Chen, R., G. Halder, Z. Zhang and G. Mardon, 1999 Signaling by the TGF-b homolog decapentaplegic functions reiteratively within the network of genes controlling retinal cell fate determination in Drosophila. Development **126:** 935–943.

Chen, Z., D. L. Kaplan, K. Yang, J. Kumar, K. A. Marx *et al.*, 1997 Two-photon-induced fluorescence from the phycoerythrin protein. Appl Opt **36:** 1655–1659.

Curtiss, J., and M. Mlodzik, 2000 Morphogenetic furrow initiation and progression during eye development in Drosophila: the roles of decapentaplegic, hedgehog and eyes absent. Development **127:** 1325–1336.

Embry, A. C., J. L. Glick, M. E. Linder and P. J. Casey, 2004 Reciprocal signaling between the transcriptional co-factor Eya2 and specific members of the Galphai family. Mol. Pharmacol. **66:** 1325–1331.

Fabrizio, J. J., M. Boyle and S. DiNardo, 2003 A somatic role for eyes absent (eya) and sine oculis (so) in Drosophila spermatocyte development. Dev. Biol. **258:** 117–128.

Fan, X., L. F. Brass, M. Poncz, F. Spitz, P. Maire *et al.*, 2000 The alpha subunits of Gz and G_i interact with the eyes absent transcription cofactor Eya2, preventing its interaction with the six class of homeodomain-containing proteins. J. Biol. Chem. **275:** 32129–32134.

Firth, L. C., and N. E. Baker, 2009 Retinal determination genes as targets and possible effectors of extracellular signals. Dev Biol **327:** 366–375.

Halder, G., P. Callaerts, S. Flister, U. Walldorf, U. Kloter *et al.*, 1998 Eyeless initiates the expression of both sine oculis and eyes absent during drosophila compound eye development. Development **125:** 2181–2191.

Hazelett, D. J., M. Bourouis, U. Walldorf and J. E. Treisman, 1998 decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. Development **125:** 3741–3751.

Hsiao, F. C., A. Williams, E. L. Davies and I. Rebay, 2001 Eyes Absent mediates cross-talk between retinal determination genes and the receptor tyrosine kinase signaling pathway. Developmental Cell **1:** 51–61.

Ikeda, K., Y. Watanabe, H. Ohto and K. Kawakami, 2002 Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. Mol. Cell. Biol. **22:** 6759–6766.

Jemc, J., and I. Rebay, 2007a Identification of transcriptional targets of the dual-function transcription factor/phosphatase eyes absent. Dev. Biol. **310:** 416–429.

Jemc, J., and I. Rebay, 2007b The eyes absent family of phosphotyrosine phosphatases: properties and roles in developmental regulation of transcription. Annu. Rev. Biochem. **76:** 513–538.

Kumar, J. P., 2009 The molecular circuitry governing retinal determination. Biochim. Biophys. Acta **1789:** 306–314.

Kumar, J. P., and K. Moses, 2001a EGF receptor and Notch signaling act upstream of Eyeless/Pax6 to control eye specification. Cell **104:** 687–697.

Kumar, J. P., and K. Moses, 2001b Expression of evolutionarily conserved eye specification genes during Drosophila embryogenesis. Dev Genes Evol **211:** 406–414.

Kumar, J. P., and K. Moses, 2001c EGF receptor and Notch signaling act upstream of Eyeless/Pax6 to control eye specification. Cell **104:** 687–697.

Kurata, S., M. J. Go, S. Artavanis-Tsakonas and W. J. Gehring, 2000 Notch signaling and the determination of appendage identity. Proc Natl Acad Sci U S A **97:** 2117–2122.

Leiserson, W. M., S. Benzer and N. M. Bonini, 1998 Dual functions of the Drosophila eyes absent gene in the eye and embryo. Mech. Dev. **73:** 193–202.

Li, X., K. A. Oghi, J. Zhang, A. Krones, K. T. Bush *et al.*, 2003 Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature **426:** 247–254.

Mardon, G., N. M. Solomon and G. M. Rubin, 1994 dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. Development **120:** 3473–3486.

Mutsuddi, M., B. Chaffee, J. Cassidy, S. J. Silver, T. L. Tootle *et al.*, 2005 Using Drosophila to decipher how mutations associated with human branchio-oto-renal syndrome and optical defects compromise the protein tyrosine phosphatase and transcriptional functions of eyes absent. Genetics **170:** 687–695.

Niimi, T., M. Seimiya, U. Kloter, S. Flister and W. J. Gehring, 1999 Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in Drosophila. Development **126:** 2253–2260.

Ohto, H., S. Kamada, K. Tago, S. I. Tominaga, H. Ozaki *et al.*, 1999 Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. Mol. Cell. Biol. **19:** 6815–6824.

O'Neill, E. M., I. Rebay, R. Tjian and G. M. Rubin, 1994 The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell **78:** 137–147.

Ostrin, E. J., Y. Li, K. Hoffman, J. Liu, K. Wang *et al.*, 2006 Genome-wide identification of direct targets of the Drosophila retinal determination protein Eyeless. Genome Res **16:** 466–476.

Pappu, K. S., R. Chen, B. W. Middlebrooks, C. Woo, U. Heberlein *et al.*, 2003 Mechanism of hedgehog signaling during Drosophila eye development. Development **130:** 3053–3062.

Pappu, K. S., E. J. Ostrin, B. W. Middlebrooks, B. T. Sili, R. Chen *et al.*, 2005 Dual regulation and redundant function of two eye-specific enhancers of the Drosophila retinal determination gene dachshund. Development **132:** 2895–2905.

Pignoni, F., B. Hu, H. Z. Kenton, J. Xiao, P. A. Garrity *et al.*, 1997 The eye-specification proteins So and Eya form a complex and regulate multiple steps in Drosophila eye development. Cell **91:** 881–891.

Pignoni, F., and S. L. Zipursky, 1997 Induction of Drosophila eye development by Decapentaplegic. Development **124:** 271–278.

Rayapureddi, J. P., C. Kattamuri, B. D. Steinmetz, B. J. Frankfort, E. J. Ostrin *et al.*, 2003 Eyes absent represents a class of protein tyrosine phosphatases. Nature **426:** 295–298.

Rebay, I., and G. M. Rubin, 1995 Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell **81:** 857–866.

Rebay, I., S. J. Silver and T. L. Tootle, 2005 New vision from Eyes absent: transcription factors as enzymes. Trends Genet. **21:** 163–171.

Rogge, R., P. J. Green, J. Urano, S. Horn-Saban, M. Mlodzik *et al.*, 1995 The role of yan in mediating the choice between cell division and differentiation. Development **121:** 3947–3958.

Salzer, C. L., and J. P. Kumar, 2009 Position dependent responses to discontinuities in the retinal determination network. Dev Biol **326:** 121–130.

Shen, W., and G. Mardon, 1997 Ectopic eye development in Drosophila induced by directed dachshund expression. Development **124:** 45–52.

Silver, S. J., E. L. Davies, L. Doyon and I. Rebay, 2003 Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. Mol Cell Biol **23:** 5989–5999.

Tavsanli, B. C., E. J. Ostrin, H. K. Burgess, B. W. Middlebrooks, T. A. Pham *et al.*, 2004 Structure-function analysis of the Drosophila retinal determination protein Dachshund. Dev Biol **272:** 231–247.

Tootle, T. L., S. J. Silver, E. L. Davies, V. Newman, R. R. Latek *et al.*, 2003 The transcription factor Eyes absent is a protein tyrosine phosphatase. Nature **426:** 299–302.

Voas, M. G., and I. Rebay, 2004 Signal integration during development: insights from the Drosophila eye. Dev. Dyn. **229:** 162–175.

Xiong, W., N. M. Dabbouseh and I. Rebay, 2009 Interactions with the abelson tyrosine kinase reveal compartmentalization of eyes absent function between nucleus and cytoplasm. Dev. Cell **16:** 271–279.

Xu, P. X., I. Woo, H. Her, D. R. Beier and R. L. Maas, 1997 Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. Development **124:** 219–231.

Zimmerman, J. E., Q. T. Bui, H. Liu and N. M. Bonini, 2000 Molecular genetic analysis of Drosophila eyes absent mutants reveals an eye enhancer element. Genetics **154:** 237–246.

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The Retinal Determination Gene *eyes absent* Is Regulated by the EGF Receptor Pathway Throughout Development in Drosophila

Claire L Salzer, Yair Elias and Justin P. Kumar

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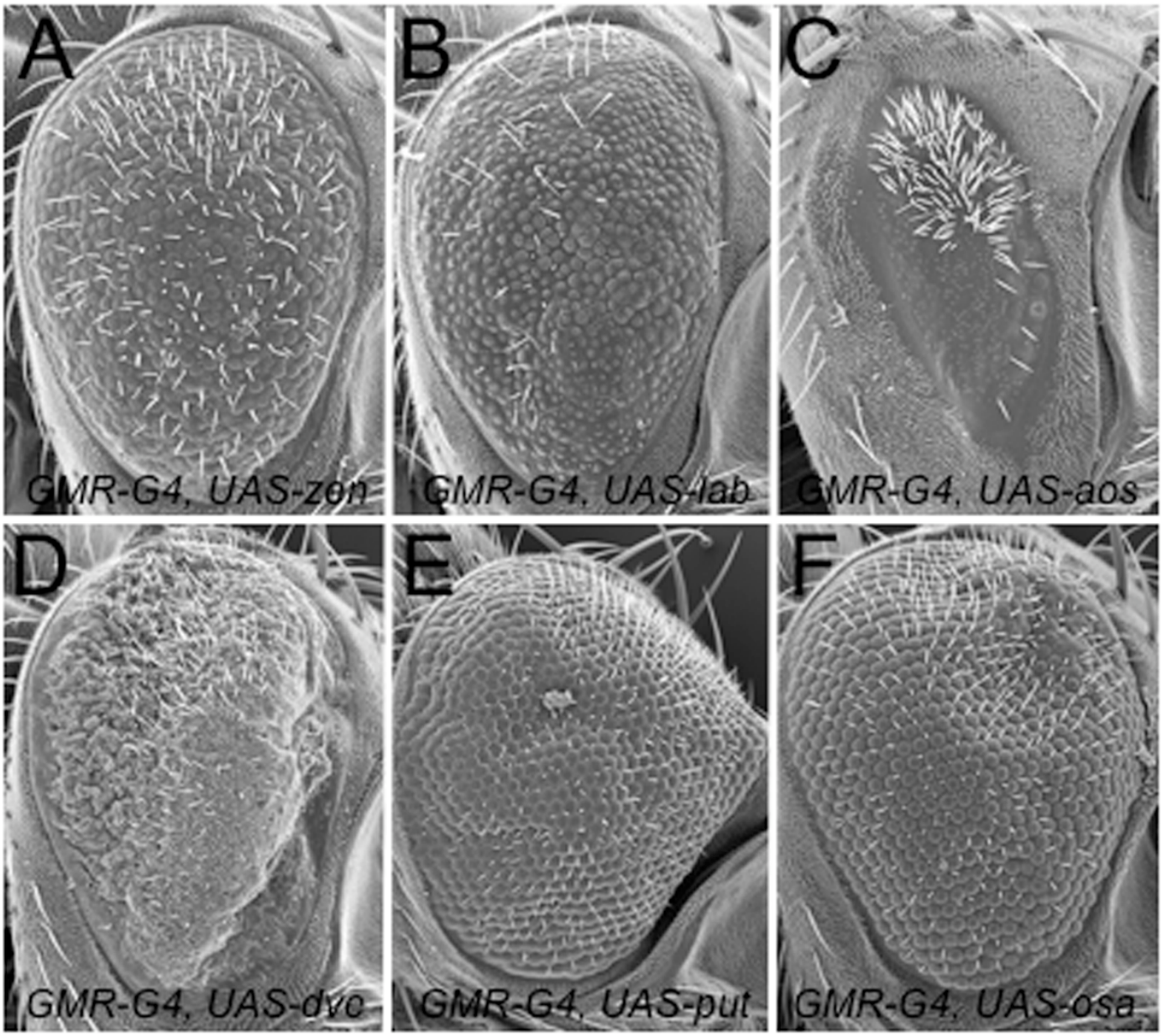

FIGURE S1.—Over-expression of putative *eya* regulators via GMR-GAL4. (A-F) Scanning electron micrographs of adult eyes that are expressing putative regulators of *eya*. Genotypes are listed in the bottom of each panel. Anterior is to the right.

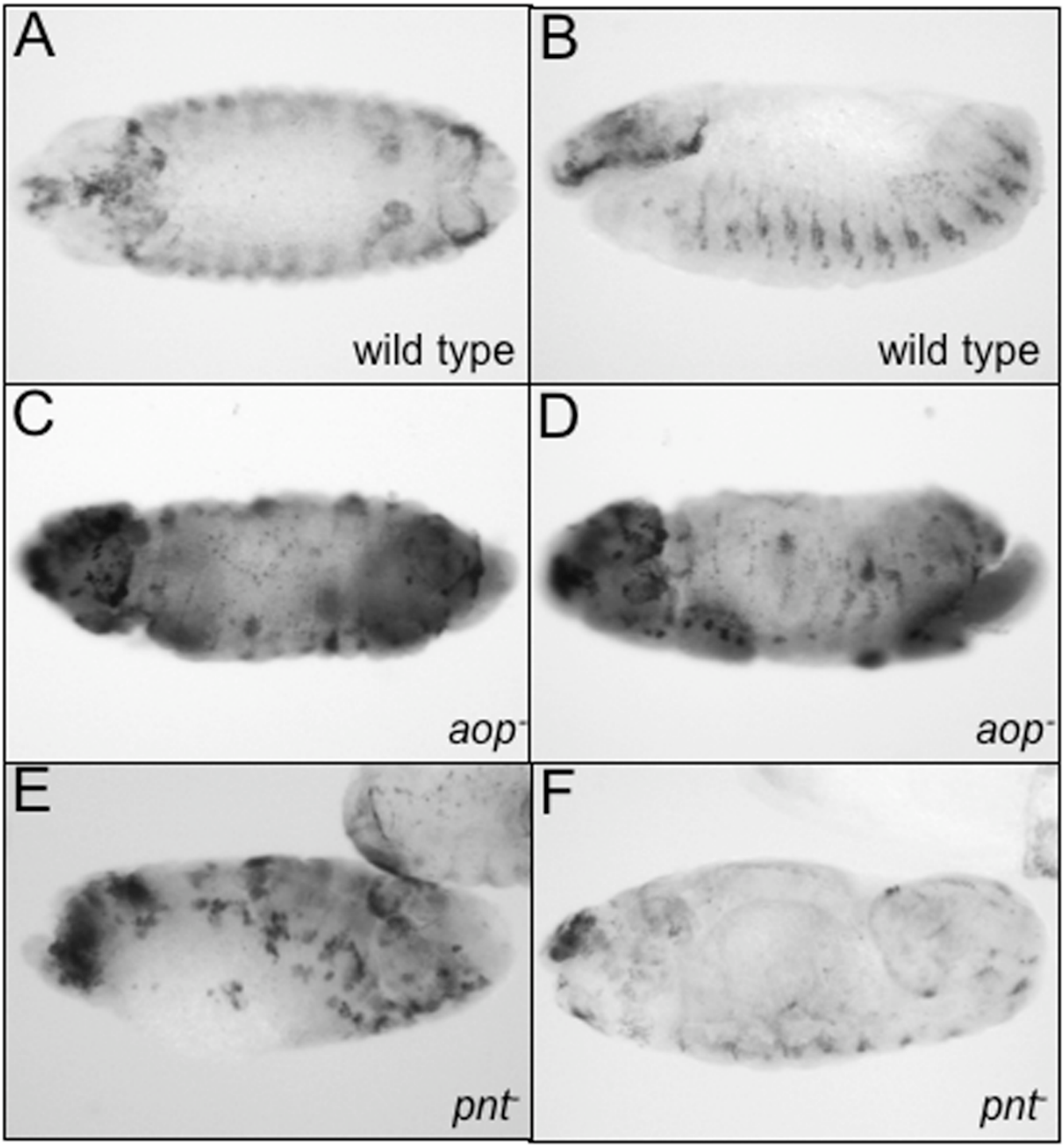

FIGURE S2.—Eya expression in wild type, *aop*, and pnt mutant embryos that are post-germband retraction. (A, C, E) Dorsal views of wild type and mutant embryos stained with Eya antibody. (B, D, F) Lateral views of wild type and mutant embryos

TABLE S1

List of Deficiencies Used in the Genetic Screen

Arm	BL Df Kit#	Genotype	Mutant?
X	125	Df(1)4b18, y[1] cv[1] v[1] nonA[4b18] f[1] car[1]/FM0	N
X	727	Df(1)g, f[1] B[1]/In(1)AM	N
X	729	Df(1)N-8/FM7c	Y
X	935	Df(1)JC19/FM7c	N
X	936	Df(1)64c18, g[1] sd[1]/Dp(1;2;Y)w[+]/C(1)DX, y[1] w[1] f[1]	N
X	939	Df(1)dm75e19/FM7c	N
X	940	Df(1)A113/C(1)DX, y[1] w[1] f[1]; Dp(1;2)w[+]64b/+	N
X	944	Df(1)JC70/FM7c, sn[+]	N
X	945	Df(1)C149/FM6	N
X	946	Df(1)N73/FM6	N
X	948	Df(1)ct-J4, In(1)dl-49, f[1]/C(1)DX, y[1] w[1] f[1]; Dp(1;3)sn[13a1]/+	N
X	949	Df(1)C128/FM6	N
X	950	Df(1)RA2/FM7c	N
X	951	Df(1)KA14/FM7c	N
X	952	Df(1)C52, flw[C52]/FM6	N
X	954	Df(1)v-L15/FM6	N
X	957	Df(1)KA7/C(1)DX, y[1] w[1] f[1]; Dp(1;2)v[+]65b/+	N
X	959	Df(1)HA85/FM7c	N
X	962	Df(1)N105/FM6	Y
X	964	Df(1)JA26/FM7c	Y
X	966	Df(1)N12, ras[1] v[1]/FM6	N
X	967	Df(1)C246/FM6	Y
X	970	Df(1)N19/FM6	N
X	971	Df(1)JA27/FM7c	N
X	972	Df(1)HF396/FM7c	Y
X	977	Df(1)DCB1-35b/FM6/Dp(1;Y)y[+]mal[106], mal[106]	N
X	998	Df(1)RK2/FM7a	N
X	1039	Df(1)RK4/FM7k/Dp(1;Y)y[+], y[1]	Y
X	1329	Df(1)BA1, w[*]/FM7a; Dp(1;2)E1, y[+]/+	N
X	1546	Df(1)sc-J4, sc[J4]/C(1)DX, y[1] f[1]; Dp(1;f)z9, y[+]	N
X	3196	Df(1)Sxl-bt, y[1]/Binsinscy	N
X	3217	Tp(1;2)r[+]75c, sl[3]/CyO; C(1)M4, y[2]	Y
X	3221	Df(1)ct4b1, y[1]/Binsn	N
X	3347	Df(1)sd72b/FM7a	N
X	3560	Df(1)v-N48, f[*]/Dp(1;Y)y[+]v[+]#3/C(1)DX, y[1] f[1]	N
X	3651	Df(1)lz-90b24, y[2] w[a]/FM7c	N
X	3714	Df(1)A209/FM7a	Y

X	4741	Df(1)B25, Sh[14]/FM6	N
X	4953	Df(1)BK10, r[*] f[1]/FM7c	N
X	5272	Df(1)r-D1, v[1] f[1]/C(1)DX, y[1] f[1]; Dp(1;4)r[+]m	Y
X	5281	Df(1)dx81, w[*]/Dp(1;Y)dx[+]1/C(1)M5	Y
X	6217	Df(1)RR79, w[1118]/FM7a	Y
X	7339	In(1)AC2[L]AB[R], y[1] w[1]/FM7c	Y
2L	90	Df(2L)C144, dpp[d-ho] ed[1]/In(2LR)Gla, wg[Gla-1] Bc[1] Egfr[E1]	N
2L	140	y[1] w[67c23]; Df(2L)Trf-C6R31/CyO	N
2L	167	Df(2L)TW161, cn[1] bw[1]/CyO	Y
2L	179	In(1)w[m4h], y[1]; Df(2L)TE29Aa-11, dp[*]/CyO	N
2L	420	Df(2L)TW137, cn[1] bw[1]/CyO, Dp(2;2)M(2)m[+]	N
2L	490	In(1)w[m4]; Df(2L)E110/CyO	N
2L	567	Df(2L)pr-A16, cn[1] bw[1]/CyO	Y
2L	693	Df(2L)sc19-8/SM6b; Dp(2;1)B19, y[1], ed[1] dp[o2] cl[1]	N
2L	781	Df(2L)cl-h3/SM6b	N
2L	1045	Df(2L)Mdh, cn[1]/Dp(2;2)Mdh3, cn[1]	Y
2L	1491	Df(2L)r10, cn[1]/CyO	Y
2L	1567	Df(2L)JS17, dpp[d-ho]/CyO, P{ry[+t7.2]=en1}wg[en11]	N
2L	2414	w[*]; Df(2L)spd[j2], wg[spd-j2]/CyO, P{ry[+t7.2]=ftz/lacB}E3	Y
2L	2583	Df(2L)cact-255rv64, cact[chif64]/CyO; ry[506]	Y
2L	2892	Df(2L)N22-14/CyO	Y
2L	3079	Df(2L)Prl, Prl[1] nub[Prl]/CyO	N
2L	3084	Df(2L)ast2/SM1	N
2L	3133	Df(2L)dp-79b, dp[DA] cn[1]/In(2LR)bw[V1], b[1] bw[V1]	Y
2L	3138	Df(2L)b87e25/CyO	Y
2L	3366	y[*]; Df(2L)J2/SM1	Y
2L	3588	Df(2L)TE35BC-24, b[1] pr[1] pk[1] cn[1] sp[1]/CyO	Y
2L	3638	Df(2L)net-PMF/SM6a	Y
2L	3813	Df(2L)sc19-4/In(2L)Cy[L]t[R] In(2R)Cy	Y
2L	4956	Df(2L)XE-3801/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	N
2L	4959	Df(2L)C'/CyO	N
2L	5330	Df(2L)ed1/CyO; P{ry[+t7.2]=ftz/lacC}1	N
2L	5420	w[*]; Df(2L)Dwee1-W05/CyO; P{ry[+t7.2]=ftz/lacC}1	N
2L	5869	Df(2L)FCK-20, dp[ov1] bw[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	Y
2L	6283	Df(2L)BSC4, w[+mC], net[1] cn[1]/SM5	N
2L	6299	Df(2L)BSC5, w[+mC]/SM6a	N
2L	6338	Df(2L)BSC6, dp[ov1] cn[1]/SM6a	N
2L	6374	w[1118]; Df(2L)BSC7/CyO	N
2L	6478	Df(2L)BSC17/SM6a	N
2L	6507	y[1] w[*]; Df(2L)drm-P2, P{w[+mC]=lacW}Pdsw[k10101]/SM6b	N
2L	6608	Df(2L)BSC16, net[1] cn[1]/SM6a	N

2L	6648	Df(2L)dpp[d14]/In(2LR)Gla, wg[Gla-1]	N
2L	6875	Df(2L)BSC28/SM6a, bw[k1]	Y
2L	6965	Df(2L)BSC31, net[1] cn[1]/CyO, b[81f2] rk[81f2]	N
2L	6999	Df(2L)BSC30/SM6a, bw[k1]	N
2L	7142	Df(2L)BSC32/SM6a, bw[k1]	N
2L	7143	Df(2L)BSC36/SM6a, bw[k1]	N
2L	7144	Df(2L)BSC37, dpp[EP2232]/CyO	N
2L	7147	Df(2L)BSC41, dp[ov1] cn[1]/CyO	Y
2R	190	Df(2R)en-A/CyO	N
2R	198	w[118]; Df(2R)H3C1/CyO	N
2R	201	w[118]; Df(2R)H3E1/CyO	Y
2R	282	Dp(1;Y)y[+]/y[1]; Df(2R)X58-12/SM5	Y
2R	442	Df(2R)CX1, wg[12] b[1] pr[1]/SM1	N
2R	543	Df(2R)017/SM1	N
2R	739	Df(2R)M41A4/SM1	N
2R	749	In(2R)bw[VDe2L]Cy[R]/In(2LR)Gla, wg[Gla-1]	Y
2R	754	Df(2R)vg-C/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	N
2R	757	y[1] w[*]/Dp(1;Y)y[+]; Df(2R)P34/CyO	Y
2R	1007	Df(2R)nap9/Dp(2;2)BG, In(2LR)Gla, wg[Gla-1]	N
2R	1145	Dp(1;Y)B[S]; Df(2R)en30/SM5	Y
2R	1547	Df(2R)PC4/CyO	N
2R	1682	Df(2R)or-BR6, cn[1] bw[1] sp[1]/In(2LR)lt[G16L]bw[V32gR], bw[V32g]	Y
2R	1702	Df(2R)X1, Mef2[X1]/CyO, Adh[nB]	N
2R	1743	w[1118]; Df(2R)B5, px[1] sp[1]/CyO, Adh[nB]	Y
2R	1888	Df(2R)ST1, Adh[n5] pr[1] cn[*]/CyO	Y
2R	2471	Df(2R)M60E/In(2LR)bw[V32g], bw[V32g]	N
2R	2604	Df(2R)Px2/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	N
2R	3368	Df(2R)cn9/CyO, amos[Roi-1] sp[*]	Y
2R	3467	Df(2R)AA21, c[1] px[1] sp[1]/SM1	Y
2R	3518	w[a] N[fa-g]; Df(2R)Jp1/CyO	Y
2R	3520	w[a] N[fa-g]; Df(2R)Jp8, w[+]/CyO	N
2R	3591	w[1]; Df(2R)Np5, In(2LR)w45-32n, cn[1]/CyO	N
2R	3909	w[*]; Df(2R)59AD/SM1	N
2R	4959	Df(2L)C'/CyO	N
2R	4960	Df(2R)CB21/CyO; ry[506]	N
2R	4961	Df(2R)Kr10, b[1] pr[1] Bl[1] c[1]/CyO	N
2R	4966	w[1]; Df(2R)w45-30n, cn[1]/CyO	N
2R	5246	Df(2R)Egfr5, b[1] pr[1] cn[1] sca[1]/CyO, P{ry[+t7.2]=sevRas1.V12}FK1	N
2R	5574	y[1] w[67c23]; Df(2R)k10408, P{w[+mC]=lacW}mthl3[k10408] veil[k10408]/CyO	Y
2R	5680	Df(2R)robl-c/CyO, y[+]	N
2R	5879	Df(2R)BSC3, w[+mC] unch[k15501] cn[1] bw[1] sp[1]/SM6a, bw[k1]	N

2R	6404	y[1]; Df(2R)P803-Delta15, cn[1]/SM1; sv[spa-pol]	N
2R	6455	Df(2R)BSC11/SM6a	N
2R	6516	Df(2R)BSC18/SM6a	N
2R	6609	Df(2R)BSC19, cn[1] bw[1]/SM6a	N
2R	6647	Df(2R)BSC22/SM6a	N
2R	6779	y[1] w[67c23]; Df(2R)14H10Y-53/SM6a	N
2R	6780	y[1] w[67c23]; Df(2R)14H10W-35/SM6a	N
2R	6866	Df(2R)BSC26/CyO	N
2R	6916	w[1118]; Df(2R)ED1, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED1/SM6a	Y
2R	6917	Df(2R)BSC29, cn[1] bw[1] sp[1]/CyO	N
2R	7145	Df(2R)BSC39, cn[1] bw[1]/SM6a, bw[k1]	N
2R	7146	Df(2R)BSC40/SM6a	N
2R	7273	Df(2R)vir130/CyO	N
2R	7414	Df(2R)BSC44/SM6a	N
2R	7441	Df(2R)BSC45, w[+mC]/SM6a	N
2R	7445	Df(2R)BSC49/SM6a	N
3L	439	Df(3L)Ar14-8, red[1]/TM2, p[p]	N
3L	463	w[1118]; Df(3L)GN34/TM3, ry[*] su(Hw)[2] Sb[1]	N
3L	997	Df(3L)AC1, rn[roe-1] p[p]/TM3, Sb[1]	N
3L	1420	Df(3L)pbl-X1/TM6B, Tb[1]	N
3L	1541	y[1] w[1] N[spl-1]; Df(3L)66C-G28/TM3, Sb[1]	N
3L	2052	Df(3L)rdgC-co2, th[1] st[1] in[1] kni[ri-1] p[p]/TM6C, cu[1] Sb[1] Tb[1] ca[1]	Y
3L	2400	Df(3L)R-G7, rho[ve-1]/TM6B, Tb[1]	y
3L	2577	Df(3L)emc-E12/TM6B, Tb[1] ca[1]	Y
3L	2608	Df(3L)W10, ru[1] h[1] Cap-H2[TH1] Sb[sbd-2]/TM6B, Tb[1]	N
3L	2611	Df(3L)vin5, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	N
3L	2612	Df(3L)vin7, h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	N
3L	2990	Df(3L)Cat, kni[ri-1] Sb[sbd-1] e[*]/TM3, Ser[1]	N
3L	2993	Df(3L)st-f13, Ki[1] rn[roe-1] p[p]/TM6B, Tb[1]	Y
3L	2998	Df(3L)81k19/TM6B, Tb[1]	Y
3L	3024	Df(3L)h-i22, h[i22] Ki[1] rn[roe-1] p[p]/TM3, Ser[1]	N
3L	3096	Df(3L)ZN47, ry[506]/TM3, Sb[1]	Y
3L	3124	Df(3L)fz-GF3b, P{w[+tAR] ry[+t7.2AR]=wA[R]}66E/TM6B, Tb[+]	N
3L	3126	Df(3L)fz-M21, st[1]/TM6	N
3L	3127	Df(3L)ri-79c/TM3, Sb[1]	N
3L	3617	Df(3L)kto2/TM6B, Tb[+]	N
3L	3640	Df(3L)brm11/TM6C, cu[1] Sb[1] ca[1]	N
3L	3649	Df(3L)HR119/TM6B, Tb[+] ca[1]	Y
3L	3650	Df(3L)M21, kni[ri-1] p[p]/In(3LR)T33[L]f19[R], h[1] th[1] st[1] cu[1] sr[1]	N
3L	3686	Df(3L)GN24/TM8, l(3)DTS4[1]	Y
3L	4366	In(3LR)C190[L]Ubx[42TR], Ubx[-]/sti[1]	N

3L	4393	w[*]; Df(3L)XDI98, e[1]/TM6B, Tb[1]	N
3L	4429	Df(3L)ME107, mwh[1] red[1] e[1]/TM1, red[*]	N
3L	4430	Df(3L)Pc-2q, ry[506]/TM2	N
3L	4500	Df(3L)Scf-R6, th[1] st[1] cu[1] sr[1] e[s] ca[1]/TM3, Sb[1]	N
3L	4506	Df(3L)Ten-m-AL29/TM3, ry[RK] Sb[1] Ser[1]	N
3L	5126	Df(3L)XS533/TM6B, Sb[1] Tb[1] ca[1]	N
3L	5411	Df(3L)Aprt-32/TM6	N
3L	5492	w[*]; Df(3L)eyg[C1]/TM3, P{ry[+t7.2]=ftz/lacC}SC1, ry[RK] Sb[1] Ser[1]	N
3L	5877	w[1]; Df(3L)ZP1/TM3, Sb[1] Ser[1]	N
3L	5878	Df(3L)ri-XT1, ru[1] st[1] e[1] ca[1]/TM3, P{w[+m*]=Ubx-lacZ.w[+]}TM3, Sb[1]	N
3L	5951	w[*]; Df(3L)HD1/TM3, Sb[1] Ser[1]	N
3L	6411	Df(3L)BSC8/TM3, Ser[1]	N
3L	6456	Df(3L)BSC10, rho[ve-1] e[1]/TM3, Ser[1]	N
3L	6457	Df(3L)BSC12, rho[ve-1] e[1]/TM3, P{w[+m*]=Ubx-lacZ.w[+]}TM3, Sb[1]	N
3L	6460	Df(3L)BSC13, rho[ve-1] e[1]/TM2, p[p]	N
3L	6471	Df(3L)BSC14, rho[ve-1] p[*] e[1]/TM3, Ser[1]	N
3L	6551	Df(3L)XG5/TM3, Sb[1] Ser[1]	N
3L	6646	Df(3L)BSC20, st[1] ca[1]/TM6B, Tb[1]	N
3L	6649	Df(3L)BSC21, st[1] sr[1] e[s] ca[1]/TM3, Sb[1] Ser[1]	N
3L	6754	w[*]; Df(3L)fz2/TM6B, Tb[1]	N
3L	6755	Df(3L)BSC23, rho[ve-1] e[1]/TM2, p[p]	N
3L	6867	Df(3L)BSC27/TM6B, Tb[1]	N
3L	6964	Df(3L)BSC33, rho[ve-1] e[1]/TM2	N
3L	7079	Df(3L)BSC35, rho[ve-1] e[1]/TM3, P{w[+m*]=Ubx-lacZ.w[+]}TM3, Sb[1]	N
3R	383	Df(3R)ea, kni[ri-1] p[p]/TM3, Ser[1]	N
3R	430	w[1118]; Df(3R)3450/TM6B, Tb[1]	N
3R	669	w[*]; Df(3R)Dr-rv1, ry[506]/TM3, ry[RK] Sb[1] Ser[1]	N
3R	756	Df(3R)sbd105, p[p] Ubx[bx-1] sr[1] e[s]/TM3, Ser[1]	N
3R	823	Df(3R)D605/TM3, Sb[1] Ser[1]	N
3R	1467	Dp(3;1)P115/+; Df(3R)P115, e[11]/TM1	N
3R	1518	Df(YS)bb[-]; Df(3R)ME15, mwh[1] red[1] e[4]/MKRS	N
3R	1534	Tp(3;Y)ry506-85C/MKRS	N
3R	1842	Df(3R)Antp17/TM3, Sb[1] Ser[1]	N
3R	1884	Df(3R)Scr, p[p] e[s]/TM3, Sb[1]	N
3R	1910	Df(3R)Tl-P, e[1] ca[1]/TM3, Ser[1]	N
3R	1920	C(1;Y)1, y[+]; Df(3R)sbd104/TM2, ry[*]	N
3R	1931	Df(3R)by10, red[1] e[1]/TM3, Sb[1] Ser[1]	N
3R	1962	Df(3R)p-XT103, ru[1] st[1] e[1] ca[1]/TM3, Sb[1]	N
3R	1968	Df(3R)p712, red[1] e[1]/TM3, Sb[1] Ser[1]	Y
3R	1990	Df(3R)Tpl10, Dp(3;3)Dfd[rv1], kni[ri-1] Dfd[rv1] p[p] Doa[10]/TM3, Sb[1]	N
3R	2363	Df(3R)crb87-5, st[1] e[1]/TM3, Ser[1]	N

3R	2393	Df(3R)WIN11, Ki[1] rn[roe-1] p[p]/TM3, Sb[1]	Y
3R	2425	Df(3R)e-N19/TM2	N
3R	2585	cn[1]; Df(3R)mbc-R1, ry[506]/TM3, ry[*] Sb[1] Ser[1]	Y
3R	2586	Df(3R)23D1, ry[506]/TM3, Sb[1] Ser[1]	N
3R	3003	Df(3R)T-32, (kni[ri-1]) cu[1] sr[1] e[s]/MRS	N
3R	3007	Df(3R)ry615/TM3, Sb[1] Ser[1]	N
3R	3011	Df(3R)Cha7, red[1]/TM6B, Tb[1]	N
3R	3012	Df(3R)Dl-BX12, ss[1] e[4] ro[1]/TM6B, Tb[1]	N
3R	3128	Df(3R)M-Kx1/TM3, Sb[1]	N
3R	3340	Df(3R)e-R1, Ki[1]/TM3, Sb[1] Ser[1]	Y
3R	3468	Df(3R)slo8/Dp(3;3)Su[8]	Y
3R	3546	Df(3R)B81, P{ry[+t7.2]=RP49}F2-80A e[1]/TM3, Sb[1]; Dp(3;1)67A	N
3R	3547	Df(3R)L127/TM6; Dp(3;1)B152	N
3R	4431	Df(3R)DG2/TM2, red[1]	N
3R	4432	Df(3R)crb-F89-4, st[1] e[1]/TM3, P{w[+m*]=Ubx-lacZ.w[+]}TM3, Sb[1]	N
3R	4787	Df(3R)3-4, ru[1] th[1] st[1]/TM3, Sb[1] Ser[1]	N
3R	4940	cn[1]; Df(3R)mbc-30/TM3, Sb[1]	Y
3R	4962	Df(3R)H-B79, e[*]/TM2	Y
3R	5601	Df(3R)Espl3/TM6C, cu[1] Sb[1] Tb[1] ca[1]	N
3R	5694	w[*]; Df(3R)e1025-14/TM6B, Tb[1]	N
3R	6676	y[1] w[*]; P{ry[+t7.2]=neoFRT}82B Mtl[Delta]/TM3, Sb[1]	N
3R	6756	Df(3R)BSC24, st[1] ca[1]/TM3, Ser[1]	N
3R	7080	Df(3R)BSC38, st[1] ca[1]/TM2, p[p]	N
3R	7412	Df(3R)BSC42, st[1] ca[1]/TM3, Sb[1]	N
3R	7413	Df(3R)BSC43, st[1] ca[1]/TM2, p[p]	N
3R	7443	Df(3R)BSC47, st[1] ca[1]/TM3, P{w[+m*]=Ubx-lacZ.w[+]}TM3, Sb[1]	Y
4-	759	Df(4)G/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	N
4-	1082	Df(4)M101-63a/In(4)ci[D], ci[D] pan[ciD]	N
4-	1197	Df(4)38/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	N
4-	1785	C(4)RM, ci[1] ey[R]/0	N
4-	7082	y[1] w[*]; Df(4)J2/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	N
4-	7083	w[*]; Df(4)C3/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	N
4-	7084	y[1] w[*]; Df(4)O2/In(4)ci[D], ci[D] pan[ciD] sv[spa-pol]	N
4-	8067	w[1118]; Df(4)ED6366, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}CG2316[ED6366]	N

Arm	BL Df Kit#	Cytological Breakpoints	Gene Name
X	125	14B8;14C1	
X	727	12A3-10;12E9	
X	729	3C2-3;3E3-4	*Ocellarless* and *pole hole*
X	935	2F6;3C5	
X	936	2E1-2;3C2	
X	939	3C11-3E4	
X	940	3D6-E1;4F5	
X	944	4C15-16;5A1-2	
X	945	5A8-9;5C5-6	
X	946	5C2;5D5-6	
X	948	7A2-3;7C1, 6C	
X	949	7D1;7D5-6	
X	950	7D10;8A4-5	
X	951	7F1-2;8C6	
X	952	8E;9C-D	
X	954	9B1-2;10A1-2	
X	957	10A9;10F6-7	
X	959	10C1-2;11A1-2	
X	962	10F7;11D1	
X	964	11A1;11D-E	*twisted gastrulation*
X	966	11D1-2;11F1-2	
X	967	11D-E;12A1-2	
X	970	17A1;18A2	
X	971	18A5;18D	
X	972	18E1-2;20	*l(1)G0120*
X	977	19F1-2;20E-F	
X	998	12D2-E1;13A2-5	
X	1039	12F5-6;13A9-B1	*l(1)G0344*
X	1329	1A1;2A	
X	1546	1B2-14;3A3	
X	3196	6E2;7A6	
X	3217	14B13;15A9;35D-E	
X	3221	7B2-4;7C3-4	
X	3347	13F1;14B1	
X	3560	9F;10C3-5	
X	3651	8B5-6;8D8-9 or 8D1-2;8E1-2	
X	3714	20A;20F	*l(1)19Cb*
X	4741	15D3;16A4-6	
X	4953	16A2;16C7-10	
X	5272	14C5-6;15B1	

X	5281	5C3-10;6C3-12	
X	6217	16C;16F	
X	7339	9D5-E1;9E7-8 and 13B5-6;13E1-2	*l(1)G0145*
2L	90	22F4-23A1;23C2-4	
2L	140	28D2-28E1;28E5	
2L	167	38A6-B1;40A4-B1	*screw* and *Mad*
2L	179	28E4-7;29B2-C1	
2L	420	36C2-4;37B9-C1	
2L	490	25F3-26A1;26D3-11	
2L	567	37B2-12;38D2-5	*spitz*
2L	693	24C2-8;25C8-9	
2L	781	25D2-4;26B2-5	
2L	1045	30D-30F;31F	*big brain*
2L	1491	35D1;36A6-7	*escargot*
2L	1567	23C1-2;23E1-2	
2L	2414	27C1-2;28A	
2L	2583	35F-36A;36D	*cactus*, *dachshund*, and *dorsal*
2L	2892	29C1-2;30C8-9	*l(2)k07118b*
2L	3079	32F1-3;33F1-2	
2L	3084	21D1-2;22B2-3	
2L	3133	22A2-3;22D5-E1	*aop/yan*
2L	3138	34B12-C1;35B10-C1	*Son of sevenless*
2L	3366	31B;32A	*daughterless*
2L	3588	35B4-6;35F1-7	*shuttlecraft*
2L	3638	21A1;21B7-8	*CG11455*
2L	3813	25A5;25E5	
2L	4956	27E2;28D1	
2L	4959	40h35;40h38L	
2L	5330	24A2;24D4	
2L	5420	27C2-3;27C4-5	
2L	5869	32D1;32F1-3	
2L	6283	21B7-C1;21C2-3	
2L	6299	26B1-2;26D1-2	
2L	6338	26D3-E1;26F4-7	
2L	6374	26D10-E1;27C1	
2L	6478	30C3-5;30F1	
2L	6507	23F3-4;24A1-2	
2L	6608	21C3-4;21C6-8	
2L	6648	22E4-F2;22F3-23A1	
2L	6875	23C5-D1;23E2	*dpp*

2L	6965	23E5;23F4-5	
2L	6999	34A3;34B7-9	
2L	7142	32A1-2;32C5-D1	
2L	7143	32D1;32D4-E1	
2L	7144	22D2-3;22F1-2	
2L	7147	28A4-B1;28D3-9	*microtubule star*
2R	190	47D3;48B2	
2R	198	43F;44D3-8	
2R	201	44D1-4;44F12	*patched*
			defective proventriculus
2R	282	58D1-2;59A	and *l(2)ry50*
2R	442	49C1-4;50C23-D2	
2R	543	56F5;56F15	
2R	739	41A;41A	
2R	749	h42-h43;42A2-3	
2R	754	49A4-13;49E7-F1	
2R	757	55E2-4;56C1-11	
2R	1007	42A1-2;42E6-F1	
2R	1145	48A3-4;48C6-8	
2R	1547	55A;55F	
2R	1682	59D5-10;60B3-8	*twist and EGFR*
2R	1702	46C;47A1	
			l(2)k09221,
			l(2)46Ca[37], and
2R	1743	46A;46C	*l(2)k07237*
2R	1888	42B3-5;43E15-18	*sine oculis*
2R	2471	60E2-3;60E11-12	
2R	2604	60C5-6;60D9-10	
			l(2)k02107a and
2R	3368	42E;44C	*l(2)44Ea*
2R	3467	56F9-17;57D11-12	*exuperantia*
2R	3518	51D3-8;52F5-9	*l(2)k05713*
2R	3520	52F5-9;52F10-53A1	
2R	3591	44F10;45D9-E1	
2R	3909	59A1-3;59D1-4	
2R	4959	40h35;40h38L	
2R	4960	48E;49A	
2R	4961	60F1;60F5	
2R	4966	45A6-7;45E2-3	
2R	5246	57D2-8;58D1	
2R	5574	54B16;54B16	*l(2)k07433*
2R	5680	54B17-C4;54C1-4	

2R	5879	48E12-F4;49A11-B6	
2R	6404	53E;53F11	
2R	6455	50E6-F1;51E2-4	
2R	6516	50D1;50D2-7	
2R	6609	56F12-14;57A4	
2R	6647	56D7-E3;56F9-12	
2R	6779	54D1-2;54E5-7	
2R	6780	54E5-7;55B5-7	
2R	6866	56C4;56D6-10	
2R	6916	53E4;53F8	*l(2)k13704*
2R	6917	45D3-4;45F2-6	
2R	7145	48C5-D1;48D5-E1	
2R	7146	48E1-2;48E2-10	
2R	7273	59B;59D8-E1	
2R	7414	54B1-2;54B7-10	
2R	7441	54C8-D1;54E2-7	
2R	7445	53D9-E1;54B5-10	
3L	439	61C5-8;62A8	
3L	463	63E6-9;64A8-9	
3L	997	67A2;67D7-13 or 67A5;67D9-13	
3L	1420	65F3;66B10	
3L	1541	66B8-9;66C9-10	
3L	2052	77A1;77D1	
3L	2400	62B4-7;62D5-E5	
3L	2577	61A;61D3	*extra macrocheate*
3L	2608	75A6-7;75C1-2	
3L	2611	68A2-3;69A1-3	
3L	2612	68C8-11;69B4-5	
3L	2990	75B8;75F1	
3L	2993	72C1-D1;73A3-4	*argos*
3L	2998	73A3;74F	
3L	3024	66D10-11;66E1-2	
3L	3096	64C;65C	*Moire, l(3)65ACf*
3L	3124	70C1-2;70D4-5	
3L	3126	70D2-3;71E4-5	
3L	3127	77B-C;77F-78A	
3L	3617	76B1-2;76D5	
3L	3640	71F1-4;72D1-10	
3L	3649	63C2;63F7	
3L	3650	62F;63D	
3L	3686	63F6-7;64C13-15	*shrew*

3L	4366	70A1-2;70C3-4	
3L	4393	65A2;65E1	
3L	4429	77F3;78C8-9	
3L	4430	78C5-6;78E3-79A1	
3L	4500	66E1-6;66F1-6	
3L	4506	79C1-3;79E3-8	
3L	5126	76B4;77B	
3L	5411	62B1;62E3	
3L	5492	69A4-5;69D4-6	
3L	5877	66A17-20;66C1-5	
3L	5878	77E2-4;78A2-4	
3L	5951	79D3-E1;79F3-6	
3L	6411	74D3-75A1;75B2-5	
3L	6456	69D4-5;69F5-7	
3L	6457	69F6-70A1;70A1-2	
3L	6460	66B12-C1;66D2-4	
3L	6471	67E3-7;68A2-6	
3L	6551	71C2-3;72B1-C1	
3L	6646	76A7-B1;76B4-5	
3L	6649	79E5-F1;80A2-3	
3L	6754	75F10-11;76A1-5	
3L	6755	62E8;63B5-6	
3L	6867	65D4-5;65E4-6	
3L	6964	65E10-F1;65F2-6	
3L	7079	66F1-2;67B2-3	
3R	383	88E7-13;89A1	
3R	430	98E3;99A6-8	
3R	669	99A1-2;99B6-11	
3R	756	88F9-89A1;89B9-10	
3R	823	97E3;98A5	
3R	1467	89B7-8;89E7;20	
3R	1518	81F3-6;82F5-7	
3R	1534	87D1-2;88E5-6;Y	
3R	1842	84A5;84D9	
3R	1884	84A1-2;84B1-2	
3R	1910	97A;98A1-2	
3R	1920	1A;20F;20F, 89B5;89C	
3R	1931	85D8-12;85E7-F1	
3R	1962	85A2;85C1-2	
3R	1968	84D4-6;85B6	*neuralized*
3R	1990	83C1-2;84B1-2, 83D4-5;84A4-5;98F1-2	

3R	2363	95F7;96A17-18	
3R	2393	83E1-2;84A5	*labial* and *zerknullt*
3R	2425	93B;94	
3R	2585	95A5-7;95D6-11	*sec13*
3R	2586	94A3-4;94D1-4	
3R	3003	86E2-4;87C6-7	
3R	3007	87B11-13;87E8-11	
3R	3011	90F1-F4;91F5	
3R	3012	91F1-2;92D3-6	
3R	3128	86C1;87B1-5	
3R	3340	93B6-7;93D2	*CG3353*
3R	3468	96A2-7;96D2-4	*tolloid* and *tolkin*
3R	3546	99C8;100F5	
3R	3547	99B5-6;99E4-F1	
3R	4431	89E1-F4;91B1-B2	
3R	4432	95D7-D11;95F15	
3R	4787	82F3-4;82F10-11	
3R	4940	95A5-7;95C10-11	
3R	4962	92B3;92F13	*osa* and *CG31195*
3R	5601	96F1;97B1	
3R	5694	82F8-10;83A1-3	
3R	6676	82B	
3R	6756	85C4-9;85D12-14	
3R	7080	85F1-2;86C7-8	
3R	7412	98B1-2;98B3-5	
3R	7413	92F7-93A1;93B3-6	
3R	7443	83B7-C1;83C6-D1	*l(3)j5E7*
4-	759	102E2--7;102E--F2	
4-	1082	101F2-102A1;102A3	
4-	1197	102E02;102E10	
4-	1785	101F1;102F8	
4-	7082	?-102D4;102D5-?	
4-	7083	102D06;102F	
4-	7084	?+102D4-5;102F4-5+?	
4-	8067	102A1;102A6	

TABLE S2

List of Genes Recovered in Genetic Screen and Chromosomal

Deficiency breakpoints	Gene
3C2-3;3E3-4	*Ocellarless*
	pole hole
11A1;11D-E	*twisted gastrulation*
12F5-6;13A9-B1	*l(1)G0344*
18E1-2;20	*l(1)G0120*
20A;20F	*l(1)19Cb*
21A1;21B7-8	*CG11455*
22A2-3;22D5-E1	*anterior open/yan*
23C5-D1;23E2	*dpp*
28A4-B1;28D3-9	*microtubule star*
29C1-2;30C8-9	*l(2)k07118b*
30D-30F;31F	*big brain*
31B;32A	*daughterless*
34B12-C1;35B10-C1	*Son of sevenless*
35B4-6;35F1-7	*shuttlecraft*
35D1;36A6-7	*escargot*
	cactus
35F-36A;36D	*dachshund*
	dorsal
37B2-12;38D2-5	*spitz*
38A6-B1;40A4-B1	*screw*
	Mothers against Dpp
42B3-5;43E15-18	*sine oculis*
42E;44C	*l(2)k02107a*
	l(2)44Ea
44D1-4;44F12	*patched*
	l(2)k09221
46A;46C	*l(2)46Ca[37]*
	l(2)k07237
51D3-8;52F5-9	*l(2)k05713*
53E4;53F8	*l(2)k13704*
54B16;54B16	*l(2)k07433*
56F9-17;57D11-12	*exuperantia*
58D1-2;59A	*defective proventriculus*
	l(2)ry50
59D5-10;60B3-8	*twist*
	EGFR
61A;61D3	*extra macrocheate*

63F6-7;64C13-15	*shrew*
64C;65C	*Moire*
	l(3)65ACf
72C1-D1;73A3-4	*argos*
83B7-C1;83C6-D1	*l(3)j5E7*
83E1-2;84A5	*labial*
	zerknullt
84D4-6;85B6	*neuralized*
92B3;92F13	*osa*
	CG31195
93B6-7;93D2	*CG3353*
95A5-7;95D6-11	*sec13*
96A2-7;96D2-4	*tolloid*
	tolkin
9D5-E1;9E7-8 and 13B5-6;13E1-2	*l(1)G0145*
	punt
Specially selected candidates	*pointed*
	thick veins