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RESEARCH ARTICLE

Eyeless/Pax6 initiates eye formation non-autonomously from the peripodial epithelium

Luke R. Baker[1], Bonnie M. Weasner[1], Athena Nagel[1], Sarah D. Neuman[2], Arash Bashirullah[2] and Justin P. Kumar[1,*,‡]

ABSTRACT

The transcription factor Pax6 is considered the master control gene for eye formation because (1) it is present within the genomes and retina/lens of all animals with a visual system; (2) severe retinal defects accompany its loss; (3) Pax6 genes have the ability to substitute for one another across the animal kingdom; and (4) Pax6 genes are capable of inducing ectopic eye/lens in flies and mammals. Many roles of Pax6 were first elucidated in *Drosophila* through studies of the gene *eyeless* (*ey*), which controls both growth of the entire eye-antennal imaginal disc and fate specification of the eye. We show that Ey also plays a surprising role within cells of the peripodial epithelium to control pattern formation. It regulates the expression of *decapentaplegic* (*dpp*), which is required for initiation of the morphogenetic furrow in the eye itself. Loss of Ey within the peripodial epithelium leads to the loss of *dpp* expression within the eye, failure of the furrow to initiate, and abrogation of retinal development. These findings reveal an unexpected mechanism for how Pax6 controls eye development in *Drosophila*.

KEY WORDS: Pax6, Eyeless, Twin of Eyeless, Eye, Retina, Peripodial epithelium, *Drosophila*, Morphogenetic furrow, Dorsal-ventral patterning

INTRODUCTION

The compound eyes of the fruit fly *Drosophila melanogaster* are derived from a pair of epithelial sacs called eye-antennal discs (Ferris, 1950; Haynie and Bryant, 1986). The precursor cells that give rise to the eye initiate their development during mid-embryogenesis by invaginating from the surface ectoderm and fusing with several other cell populations to form the eye-antennal disc (Cohen, 1993; Green et al., 1993). By late embryogenesis, these discs can be recognized by the expression of the two fly Pax6 genes: *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Czerny et al., 1999; Quiring et al., 1994). Ey and Toy sit atop the retinal determination (RD) network and together they activate the entire gene regulatory network (GRN) that controls the growth, specification, patterning and physiology of the retina (Kumar, 2010). Pax6 and core downstream members of the RD network have been conserved across evolutionary history and play a central role in eye formation in all animals with a visual system, including *Drosophila*, *Xenopus*, zebrafish, mouse and human (Wawersik and Maas, 2000).

[1]Department of Biology, Indiana University, Bloomington, IN 47405, USA. [2]Department of Pharmaceutical Sciences, University of Wisconsin, Madison, WI 53705, USA.

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‡Author for correspondence (jkumar@indiana.edu)

J.P.K., 0000-0001-9991-7932

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Mutations in members of the RD network lead to the drastic reduction or absence of compound eyes whereas overexpression of individual members in non-ocular tissues leads to the formation of ectopic eyes (Kumar, 2010). *ey* is the founding member of the RD network and the loss/gain-of-function phenotypes that characterize the network are based on the behavior of *ey* itself (Halder et al., 1995; Hoge, 1915). In the decades immediately following the isolation of mutations in *ey*, there was significant interest in understanding its role in eye development. Some studies examined eye development in *ey* mutants (Chen, 1929; Hinton, 1942; Richards and Furrow, 1922), others looked at genetic and environmental factors that influenced the severity and penetrance of the retinal phenotype (Baron, 1935; Sang and Burnet, 1963) and others attempted to determine if *ey* is part of the same genetic pathway as other genes that affect eye development (Steinberg, 1944). More recent studies have shown that Ey is homologous to vertebrate Pax6 (Quiring et al., 1994) and that forced expression of *ey* in non-ocular tissues can induce ectopic eyes (Halder et al., 1995), thus sparking another wave of interest in this transcription factor. Since then, several studies have identified genetic and molecular targets of Ey (Halder et al., 1998; Michaut et al., 2003; Niimi et al., 1999; Ostrin et al., 2006; Punzo et al., 2002) and others have compared the developmental, molecular and biochemical properties of Ey and Toy to each other (Czerny et al., 1999; Punzo et al., 2001; Punzo et al., 2004; Weasner et al., 2009; Zhu et al., 2017).

These studies have provided invaluable insights into the molecular and biochemical properties of Ey. Yet, beyond the reports that eye development is compromised and the identification of a few molecular targets of Ey, surprisingly little is known about the cellular and developmental consequences of losing *ey*. In fact, much of what is known about the RD network was revealed from studies that focused on other members of the network, such as *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*) (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994). Eya and Dac are targets of Ey (Halder et al., 1998; Niimi et al., 1999; Ostrin et al., 2006; Pappu et al., 2005; Punzo et al., 2002) and themselves regulate other members of the RD network as well as genes responsible for growth, the initiation/progression of the morphogenetic furrow, and cell fate specification (Jemc and Rebay, 2007; Kumar, 2010). In mutants of these genes, retinal progenitor cells undergo significant cell death and those remaining fail to adopt an eye fate and instead undergo homeotic transformations into head epidermis (Salzer and Kumar, 2009; Wang and Sun, 2012; Weasner and Kumar, 2013; Weasner et al., 2016).

It is widely assumed that the loss of any individual RD network gene has the same developmental consequence but whether this hypothesis is correct for *ey* mutants has never been formally tested. This is due, in part, to the fact that *ey* null mutants are difficult to examine in post-embryonic tissues because of the lack of mitotic recombination and appropriate FRT sites on the fourth chromosome

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where *ey* resides. Another reason is that the viable 'eye-specific' alleles (ey^1, ey^2 and ey^4) are extremely variable in their adult phenotypes and range from lacking eyes to having eyes that appear completely wild type (Baron, 1935; Chen, 1929; Guthrie, 1925; Hinton, 1942; Hoge, 1915; Morgan, 1929; Richards and Furrow, 1922; Sang and Burnet, 1963; Spofford, 1956; Steinberg, 1944). For these reasons, clear molecular and developmental mechanisms for how eye development continues to proceed in the absence of *ey* have been elusive. Furthermore, the collapse of the RD network, which has been proposed as a putative cause for the loss of the eye, is not a universally accepted explanation for the observed reductions in eye formation.

We have re-examined eye development in *ey* mutants and address the above observations. First, we demonstrate that the small/no-eyed phenotypes that characterize *ey* mutants are not caused by a collapse of the RD network or a transformation into non-ocular tissue. Instead, we find that the primary defect is the failure to properly express *decapentaplegic* (*dpp*) along the posterior margin and this, in turn, leads to a failure of the morphogenetic furrow to properly initiate and progress across the disc. Second, we show that Toy continues to be expressed in the retinal field and is responsible for the continuation of eye development in the absence of *ey*. Third, we demonstrate that the variation in the number of ommatidia in *ey* mutant eyes is the result of weaker and inconsistent activation of the downstream Six-Eya-Dac (SED) core by Toy. And lastly, we demonstrate that Ey contributes to eye development, in part, by controlling *dpp* expression and the initiation of the morphogenetic furrow. This activity is required in cells of the peripodial epithelium (PE). These findings indicate that Pax6 regulates eye development through a completely novel and unforeseen mechanism.

RESULTS

Variation in *ey* mutants results from disruptions to the stoichiometry of Pax-Six-Eya-Dac

The ey^2 allele, which has been used in nearly all studies of *ey*, contains a transposable element within the first intron (Fig. 1H). This insertion disrupts an eye-specific enhancer as the mutants are viable and *ey* expression, as assayed by RNA *in situ* hybridization, is eliminated from the eye field (Quiring et al., 1994). A 212 bp fragment surrounding the insertion drives reporter expression within the developing eye (Hauck et al., 1999). But, despite its name, very few flies from the ey^2 mutant strain (obtained from the Bloomington *Drosophila* Stock Center and outcrossed for 12 generations) lack eyes (Fig. 1A-D). The fly shown in Fig. 1B is the rare exception and

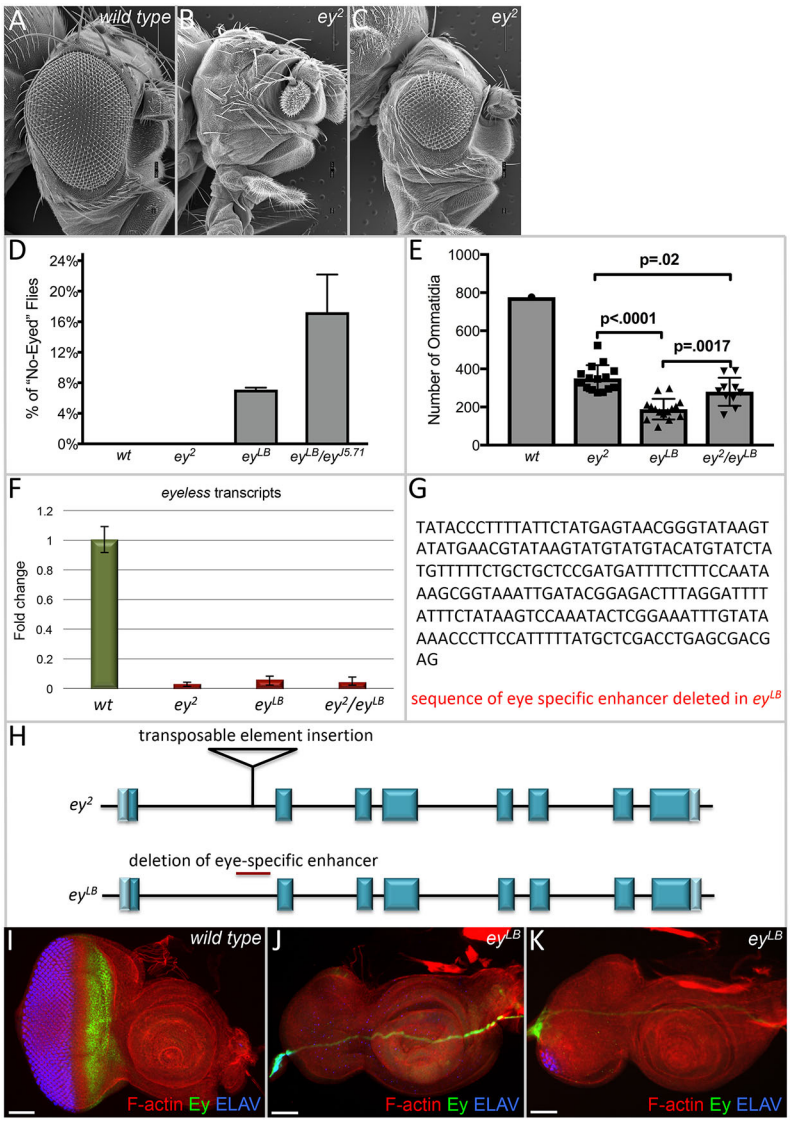

Fig. 1. *eyeless* loss-of-function mutant flies have variable defects in eye development. (A) Wild-type adult head. (B) ey^2 mutant head lacking compound eyes. (C) ey^2 mutant with a small eye. (D) Percentage of flies that lack compound eyes. Error bars indicate s.d. An unpaired *t*-test between ey^{LB} and $ey^{LB}/ey^{J5.71}$ yields a *P*-value of 0.0998 (not statistically significant). Sample numbers: wild type=100, ey^2=94, ey^{LB}=257, $ey^{LB}/ey^{J5.71}$=489. (E) Average number of ommatidia in wild-type and *ey* mutant flies. The right eyes of female flies were examined. Sample numbers: wild type=3, ey^2=30, ey^{LB}=30. An unpaired *t*-test was used in all pairwise comparisons. (F) *ey* transcripts in wild-type and *ey* mutant larvae expressed relative to wild type. Error bars represent s.e.m. (G) Sequence of enhancer element that has been deleted in ey^{LB}. (H) Schematic of the ey^2 and ey^{LB} mutants. Blue boxes, coding exons. (I-K) Wild-type (I) and ey^{LB} (J,K) third larval instar eye-antennal discs. The green stain within the Bolwig's nerve in J and K is due to a 3PXP3-dsRED transgene that is associated with the CRISPR plasmid that was used to create the ey^{LB} allele. Anterior is to the right. wt, wild type. Scale bars: 50 µm.

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is seen in <1% of the population. In fact, the average number of ommatidia is only slightly less than half that of wild type (Fig. 1E). The continued presence of substantial retinal tissue (Fig. 1C,E) raises the question of whether *ey* transcript levels are truly extinguished in the eye disc. Because RNA *in situ* hybridizations within imaginal discs are, in general, neither quantitative nor particularly sensitive, we used qRT-PCR to measure *ey* transcript levels in late third larval instar eye-antennal discs. We find that *ey* transcript levels in the ey^{2} mutant are about 10% of wild-type levels (Fig. 1F).

We used the CRISPR/Cas9 system to delete the regulatory region identified by Hauck et al. (1999) from a wild-type strain thereby creating the ey^{LB} allele (Fig. 1G,H). The elimination of this enhancer does increase the percentage of flies that lack compound eyes to 7% and causes the average number of ommatidia in the remaining flies to drop to 197 (Fig. 1D,E). Placing ey^{2} over ey^{LB} yields an intermediate number of ommatidia of roughly 250 (Fig. 1E). Consistent with these results is the observation that *ey* transcript levels do not drop any further in ey^{LB} compared with ey^{2} (Fig. 1F). Likewise, *ey* transcript levels in ey^{2}/ey^{LB} trans-heterozygotes are also not significantly different than either allele. Although *ey* continues to be transcribed in ey^{2} and ey^{LB} mutants, Ey protein levels cannot be detected by immunohistochemistry (Fig. 1I-K). At first, we thought that it is unlikely that this small amount is of *ey* transcript and protein, on its own, would be sufficient to support the formation of hundreds of ommatidia in nearly 93% of ey^{LB} mutant flies. However, we do see that placing ey^{LB} over a deficiency that removes the *ey* locus ($ey^{LB}/ey^{J5.71}$) increases the number of no-eyed flies to approximately 18% (Fig. 1D). This last result does imply that the remaining amount of Ey is contributing slightly to the size of the eye. Thus, we cannot completely rule out the possibility that the remaining Ey levels are contributing to the continued presence of eyes in *ey* mutants.

We also examined the contribution that Toy, a second Pax6 protein in *Drosophila* (Czerny et al., 1999), makes to eye development in the absence of Ey. Toy continues to be expressed in ey^{LB} mutant retinas (Fig. 2A-C) and is a good candidate for bypassing and/or substituting for *ey*. Such a proposition is supported by studies showing that Toy and Ey both bind and activate the downstream *so* gene (Niimi et al., 1999; Punzo et al., 2002). Toy has also been reported to partially restore eye development to ey^{2} mutants as well as induce ectopic eyes in the absence of *ey* (Punzo et al., 2002). However, other studies directly contradict these findings and suggest that Toy may not bind the *so* enhancer (Hens et al., 2011) and that it neither rescues the ey^{2} mutant nor is capable of inducing ectopic eyes in the absence of Ey protein (Czerny et al., 1999). To resolve this conflict, we obtained two *UAS-toy* transgenic lines from the lab of the late Walter Gehring (who initially reported the ability of Toy to rescue eye development in *ey* mutants) and generated three *UAS-toy* lines of our own (two were reported in Weasner et al., 2009; one line was generated in this report). We used an *ey-GAL4* driver to express the two *UAS-toy* lines that were obtained from the Gehring group in the eye field (these lines are the strongest in terms of inducing ectopic eyes) and do indeed see a partial rescue of the ey^{LB} mutant. We observe a reduction in the percentage of no-eyed flies and a substantial shift in the number of flies with small eyes (defined as 1-350 ommatidia) towards those having medium-sized eyes (defined as 351-600 ommatidia) (Fig. 2D-H). We used two different *ey-GAL4* lines to drive each *UAS-toy* line; all four *ey-GAL4/UAS-toy* combinations showed

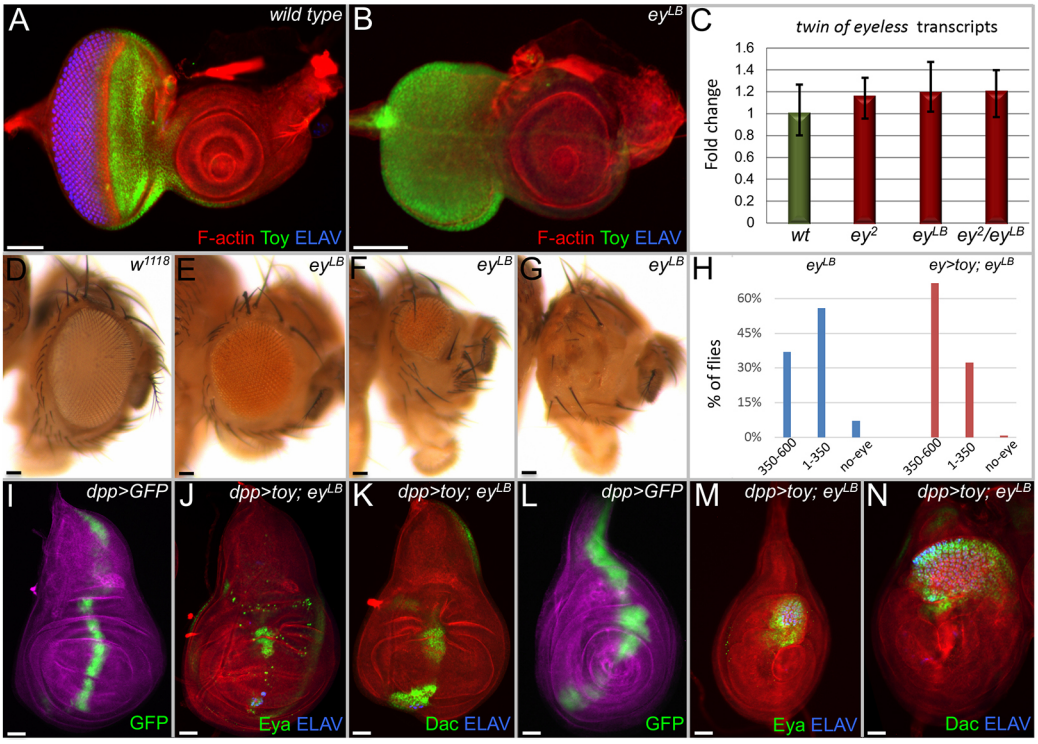

Fig. 2. Toy partially substitutes for Eyeless during eye development. (A,B) Third larval instar wild-type (A) and ey^{LB} (B) eye-antennal discs. (C) qRT-PCR analysis of *toy* transcript levels in wild-type and *ey* mutant retinas expressed relative to wild type. *n*=3. Error bars were determined by REST analysis (see Materials and Methods). (D-G) Examples of the medium-sized eye (E), small eye (F) and no-eyed (G) phenotypes observed in ey^{LB} mutants compared with w^{1118} (D). (H) Eye sizes in the ey^{LB} mutant alone (*n*=168) and in ey^{LB} mutants in which *toy* has been overexpressed using *ey-GAL4* (*n*=232). (I-N) Wing (I-K) and leg (L-N) discs in which either *GFP* (I,L) or *toy* (J,K,M,N) is being driven along the A/P axis by *dpp-GAL4*. wt, wild type. Scale bars: 50 µm.

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partial rescue of the ey^{LB} mutant phenotype. The graph in Fig. 2H represents the combination of all four experiments. If overexpression of *toy* rescues the *ey* mutant phenotype then double mutants of *ey* and *toy* would be expected to enhance the percentage of no-eyed flies. However, when expression of both genes is simultaneously reduced, the entire eye-antennal disc fails to form and the animals die as headless pharate adults (Zhu et al., 2017); thus, that prediction cannot be directly tested.

We also used a *dpp-GAL4* line, which drives expression along the anterior-posterior (A/P) axis of all imaginal discs (Staehling-Hampton et al., 1994), to direct expression of all five *UAS-toy* lines to all imaginal discs. These lines are all able to generate ectopic eyes in wild-type wings and leg discs. In ey^{LB} mutants, each *UAS-toy* line is able to activate downstream targets of Ey such as *eya* and *dac* as well as induce ectopic eye formation (Fig. 2I-N). Although Toy activates *ey* expression in wild-type leg and wing discs (Fig. S1A-F, green arrows), *ey* is not activated when we overexpress *toy* in the ey^{LB} background (Fig. S1G-L). This is because the deletion of the enhancer in the ey^{LB} mutant removes the Toy binding sites that were identified by Czerny and colleagues (Czerny et al., 1999). Thus, the activation of downstream target genes by Toy is independent of Ey. We conclude that Toy is partially redundant to Ey and that this functional redundancy is an underlying explanation for why eye development continues to be supported in *ey* mutants.

In ey^{LB} mutants, Toy is expressed at levels that are similar to those observed in control flies (Fig. 2C). Even in discs that completely lack photoreceptor development, Toy protein is found at robust levels (Fig. 2A,B). As increasing the levels of Toy can only partially rescue the ey^{LB} mutant, the amount of Toy protein (no matter how high) cannot solely account for why eye development persists nor can it be the only explanation for why the size of the ey^{LB} retina is variable. An earlier study from our group hints that the difference in the relative strengths of Ey and Toy as transcriptional activators might provide for a more comprehensive explanation. Yeast-based transcription assays were used to demonstrate that the activation domain within Ey is significantly stronger than that of Toy and swapping the activation domains makes Toy function more like Ey in both ectopic eye and transcription activation assays (Weasner et al., 2009). This, we believe, is the reason why Ey can induce ectopic eyes within a wider number of tissues and at higher frequencies compared with Toy (Weasner et al., 2009). If this model is correct, then expression of *ey* should suppress the small/no-eyed phenotype of ey^{LB} mutants better than *toy*. We first generated a stock in which the 212 bp enhancer that is deleted in the ey^{LB} mutant is directly fused to and drives expression of an *ey* cDNA. This enhancer drives expression along the margins and within a broad swatch of cells within the posterior region of the eye disc (Fig. S2A-C). This matches the expression pattern reported by Hauck et al. (1999). Expression of *ey* is restored ahead of the morphogenetic furrow (Fig. S2D-F, arrow) and eye development is partially rescued in animals carrying two copies of the rescue cassette (Fig. S2G, arrow). We then fused the 212 bp enhancer to GAL4 and used this ey^{212}-*GAL4* construct to drive expression of *UAS-ey* and *UAS-toy* responders. Both responder lines were inserted into the same genomic landing site so that we could compare the ability of each to rescue the ey^{LB} mutant. Expression of *ey* rescues the small/no-eyed phenotype of ey^{LB} significantly better than *toy* (Fig. S2H, arrow). These findings suggest that Toy expression might be sufficient to allow for transcription of downstream targets but not at the levels that are needed to fully maintain eye development in *ey* mutant retinas.

We recorded a developmental delay of approximately 24 h in ey^{LB} mutants (Fig. S3). This delay takes place during the third larval instar. In normal development, about five ommatidial rows are present at 84 h after egg laying (AEL) (Spratford and Kumar, 2013). However, in ey^{2} and ey^{LB} mutants there are no photoreceptor clusters at this stage. By 108 h AEL we observe some discs with photoreceptors and some discs without ommatidia. The percentage of discs with and without ommatidia matches the percentages of adults with and without eyes. The images of all discs in this manuscript (unless otherwise stated) are from animals that were dissected at 108 h.

The expectation is that the expression of direct Ey/Toy targets such as *so* and *eya* should be reduced (but not absent) in ey^{LB} mutants. This is the case as qPCR reveals that the expression of both genes is reduced by approximately 40% (Fig. 3A,B). In fact, both genes continue to be transcribed in discs that completely lack photoreceptor neurons albeit in fewer cells (Fig. 3C-F). The reduction in transcript levels appears to be specific to *so* and *eya* as the expression levels of the remaining RD genes is largely unaffected (Fig. S4A-H). The lone exception is *dac*, transcript levels of which are lower in the ey^{LB} mutant than in control eye-antennal discs (Fig. S4A). This is consistent with *dac* being a direct target of Ey, So and Eya (Pappu et al., 2005). If the weaker activation of *so* and *eya* by Toy is the underlying reason for the variability of the *ey* mutant phenotype, then eliminating one copy of either of these two factors should increase the percentage of *ey* mutant flies that have either small eyes or lack them altogether. Although heterozygotes of each gene alone (ey^{LB}/+, so^{1}/+ or eya^{2}/+) are completely wild type in appearance, combining either so^{1}/+ or eya^{2}/+ with ey^{LB}/+ (so^{1}/+; ey^{LB}/+ and eya^{2}/+; ey^{LB}/+) results in 100% of the double heterozygotes having smaller, disorganized eyes (Fig. 3G). We also removed one copy of the remaining 11 RD genes through the use of genomic deficiencies. Deficiencies, instead of loss-of-function mutants, were used because the molecular lesions of some alleles are not known and it is not clear if some mutants are null or hypomorphic alleles. Of these, we observed high numbers of flies with small and disorganized eyes only when one copy each of *dac* and *ey* [*Df(2L)Exel7066*/+; ey^{LB}/+] are simultaneously removed (Table S1, top set). The genetic interaction between *ey* and *dac* makes sense considering that Ey, So and Eya together regulate the activity of several eye-specific enhancers within the *dac* gene and as expression of *dac* is reduced slightly in ey^{LB} mutants (Fig. S4A) (Pappu et al., 2005). In contrast, removing one copy of each of the other ten RD genes individually, had minimal effects on the structure of the compound eyes of ey^{LB}/+ heterozygotes. These data suggest that the stoichiometry amongst the Pax-Six-Eya-Dac (PSED) core members of the RD network is crucial for regulating the overall size of the compound eye. Mutations that disrupt that stoichiometric relationship lead to smaller eyes that are variable in size. We can force the RD network to collapse if we remove one copy of most RD genes in an ey^{LB} homozygote background (Fig. 3H, Table S1, bottom set). In these genetic backgrounds, the percentage of no-eyed flies rises substantially. The most dramatic results are obtained when one copy of *so* or *eya* is removed either individually (so^{1}/+; ey^{LB}/ey^{LB} or eya^{2}/+; ey^{LB}/ey^{LB}) or simultaneously in an ey^{LB} background (so^{1}/+, eya^{2}/+; ey^{LB}/ey^{LB}). In these cases, the percentage of no-eyed flies swells to values between 40% and 80% compared with 7% for the ey^{LB} strain alone (Fig. 3H).

Ey controls the size of the eye through correct positioning of the dorsal-ventral midline

Our finding that all members of the RD network are transcribed in *ey* mutants (Fig. 2C, Fig. 3A,B, Fig. S4A-H) differs from an earlier

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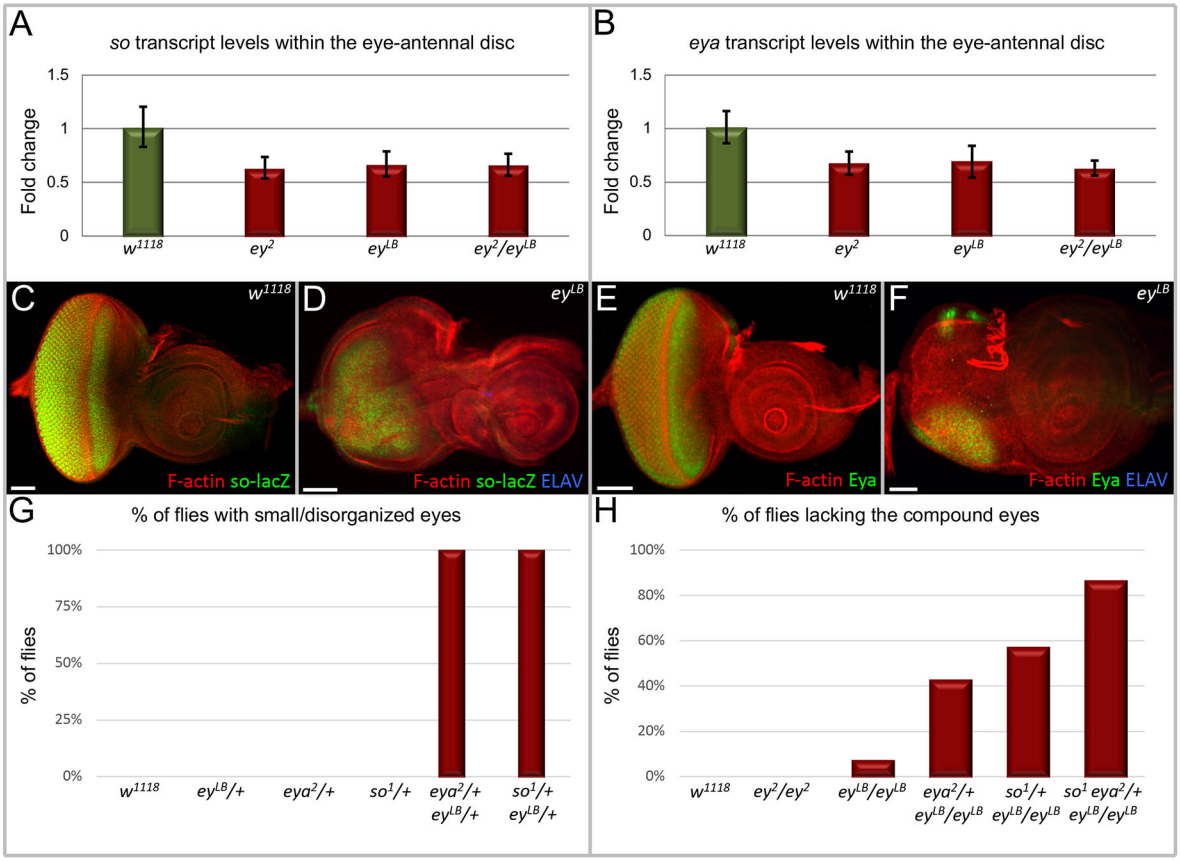

Fig. 3. The stoichiometry between core RD network members is essential for supporting eye development. (A,B) *so* (A) and *eya* (B) transcript levels in control and *ey* mutant eye-antennal discs expressed relative to wild type. *n*=3. Error bars were determined by REST analysis (see Materials and Methods). (C-F) Control and ey^{LB} eye-antennal imaginal discs. Anterior is to the right. Scale bars: 50 µm. (G) Simultaneous removal of one copy of *ey* and either *so* or *eya* results in adults with small, disorganized compound eyes. 30 animals were analyzed for each genotype. (H) Removal of one copy of *so*, *eya* or both together in an ey^{LB} mutant synergistically increases the percentage of flies that lack the compound eyes. Sample sizes: w^{1118}=30, ey^{2}/ey^{2}=94, ey^{LB}/ey^{LB}=257, $eya^{2}/+$; ey^{LB}/ey^{LB}=146, $so^{1}/+$; ey^{LB}/ey^{LB}=51, so^{1} $eya^{2}/+$; ey^{LB}/ey^{LB}=36.

report, which suggested that the RD network might collapse when Ey is lost (Halder et al., 1998). We looked specifically at 108 h ey^{LB} discs in which photoreceptor development is completely abrogated (no-eyed flies) and still observe continued expression of the RD genes (Fig. 2B, Fig. 3D,F, Fig. S5A-H). In contrast, in so^{1} and eya^{2} mutants, the expression of *so* and *eya* are both lost and *dac* levels are drastically reduced (Salzer and Kumar, 2009; Weasner and Kumar, 2013; Weasner et al., 2016). As a consequence, there is significant cell death and non-ocular genes are de-repressed in both *so* and *eya* mutants resulting in a homeotic transformation of the eye into head epidermal tissue (Salzer and Kumar, 2009; Wang and Sun, 2012; Weasner and Kumar, 2013; Weasner et al., 2016). Because *so* and *eya* continue to be expressed in the *ey* mutant retinas, we suspected that the reduction/loss of the eye is not due to a fate transformation. Consistent with this hypothesis, we continue to see antennal and head epidermal genes being expressed within their normal expression domains and not expressed ectopically within the eye (Fig. S6A-N).

If the RD network remains largely intact and if the eye field does not undergo a fate change, then what is the primary defect in this mutant that leads to smaller retinas? To address this question, we examined a number of important cellular and developmental processes that affect retinal size, such as cell proliferation, apoptosis, compartment boundary formation, and the initiation of the morphogenetic furrow. We have previously shown that cell proliferation is not affected in *ey* RNAi-expressing clones (Zhu et al., 2017); therefore, it is unlikely that reduction of tissue growth is the primary reason for the smaller ey^{LB} retinas. We then turned our attention to the possibility that cell death may account for the smaller size for the ey^{LB} retina. We examined control and ey^{LB} mutant discs for the presence of Dcp-1, a marker for apoptotic cells. Control discs almost completely lack any dying cells (Fig. S7A). However, in ey^{LB} mutants we do see increased numbers of apoptotic cells. In mutant retinas that are small to moderate in size we observe scattered Dcp-1-positive cells both ahead and behind the morphogenetic furrow (Fig. S7B). The amount of cell death increases dramatically in mutant retinas that completely lack photoreceptors (Fig. S7C) and approaches that observed in so^{1} and eya^{2} mutants (Bonini et al., 1993; Cheyette et al., 1994; Weasner and Kumar, 2013). In order to determine whether this amount of cell death is primarily responsible for the smaller retina in ey^{LB} mutants, we expressed DIAP1 and P35, two inhibitors of cell death (Hay et al., 1995; Hay et al., 1994). Unexpectedly, the percentage of flies lacking eyes increased (Fig. S7D, arrows). We interpret this to mean that we have rescued an embryonic and/or larval lethality that is associated with the ey^{LB} allele. Similar results were obtained when cell death was blocked in ey^{D} mutants; in this case, embryonic lethality was rescued but there was no restoration of eye development (Kronhamn et al., 2002). In our experiments we do see a slight increase in the percentage of flies that have medium- to

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large-sized eyes (Fig. S7D). This suggests that cell death does contribute to the smaller eye size of ey^{LB} mutants, but it is unlikely to be the primary reason for the small eyes or absence of eyes in *ey* flies.

Instead, we propose that the loss of *ey* affects the placement of the dorsal/ventral (D/V) midline and that this mis-positioning prevents the eye from achieving its normal size. During normal development, the D/V midline forms at the point at which the optic stalk meets the posterior margin and, as a result, the eye is divided into two halves of approximately equal size. Each compartment expresses different domain-specific transcription factors (Sato and Tomlinson, 2007). The juxtaposition of the expression patterns induces JAK/STAT and Notch signaling at the midline and this, in turn, promotes growth of the retina (Chao et al., 2004; Dominguez et al., 2004; Gutierrez-Aviño et al., 2009). The homeobox-containing transcription factor Mirror (Mirr) is found within cells of the dorsal compartment, which constitutes about 50% of wild-type eye discs and adult eyes (Fig. 4A-D) (McNeill et al., 1997). However, *mirr* is expressed throughout the entire retina of ey^{LB} mutants that either lack or have drastically reduced numbers of ommatidia (Fig. 4E,F). Retinal sections through flies with smaller eyes reveal that the ommatidia are almost exclusively oriented with dorsal chirality (Fig. 4G,H). A smaller retina containing mainly dorsal ommatidia would be the expected result when the D/V axis shifts ventrally – the further the shift along the posterior margin, the smaller the eye and the higher percentage of remaining ommatidia that will be oriented with dorsal chirality. A recent study showed that the eye is initially composed of dorsal-fated tissue and that the ventral compartment initiates growth later in development (Won et al., 2015). The dorsal dominance that we observe makes sense in light of this result and the position of a ventrally shifted midline.

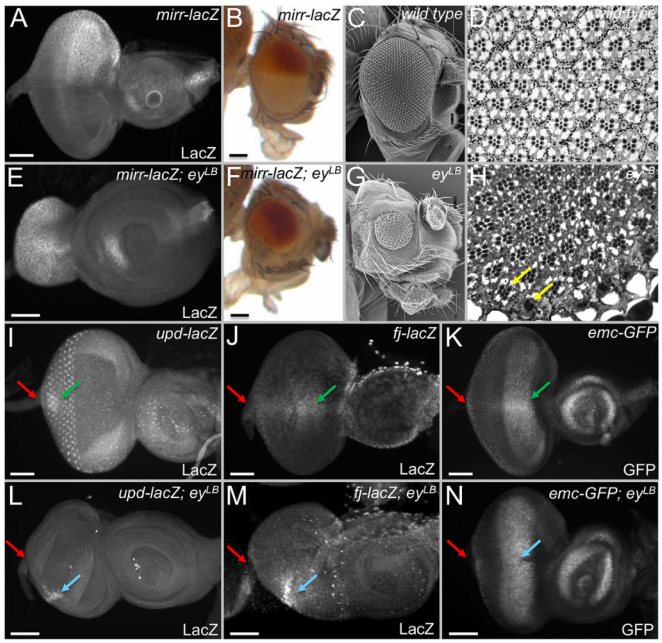

Fig. 4. Mis-positioning of the D/V midline is correlated with a smaller eye field in ey^{LB} mutants. (A) Control third larval instar eye-antennal disc showing the expression pattern of the dorsal selector gene *mirr*. (B) Control adult fly that contains *mirr-lacZ* transgene. (C) Control adult eye. (D) Retinal section of a control adult eye. (E) ey^{LB} third larval instar eye-antennal disc. (F) ey^{LB} adult with a small eye. (G) ey^{LB} adult with a small eye. (H) ey^{LB} retinal section. The yellow arrows mark the two unit eyes that have ventral chirality. (I-N) Control (I-K) or ey^{LB} (L-N) third larval instar eye-antennal discs. Red arrows point to the optic stalk, green arrows show the position of midline markers in control discs and blue arrows indicate shifted or lost midline expression. Anterior is to the right. Scale bars: 50 µm.

To confirm that the midline is actually shifted in ey^{LB} mutant discs, we examined the expression of *unpaired* (*upd*), *four-jointed* (*fj*) and *extramacrochaetae* (*emc*) (Fig. 4I-N) (Bach et al., 2003; Brodsky and Steller, 1996; Spratford and Kumar, 2015). *upd* expression marks the point at which the furrow initiates along the posterior margin (Fig. 4I, green arrow) whereas *fj* and *emc* are enriched along the midline (Fig. 4J,K, green arrows). In ey^{LB} mutant retinas that are drastically reduced in size, the position of the firing point is shifted ventrally (Fig. 4L, blue arrow). As a consequence of shifting the firing point, the expression of *fj* is also shifted ventrally (Fig. 4M, blue arrow). In contrast, both genes are expressed correctly in mutant retinas that are closer to the size of wild-type retinas (Fig. S8A,B). The expression pattern of *emc* remains largely intact with the exception that it is no longer enriched at the midline of small mutant discs that contain either no or very few ommatidia (Fig. 4N, Fig. S8C). The shift in the midline and the subsequent dorsal dominance is also seen in ey^{2} mutant discs, suggesting that this effect is not due to either the transposable insertion or the deletion of the eye-specific enhancer. Our conclusion is that the mis-positioning of both the firing point and the D/V midline reduces the size of the retina because of uneven starting compartment sizes.

Ey controls *dpp* expression and the initiation of the morphogenetic furrow

Although the mis-positioning of the D/V midline explains a smaller retina, we were still interested in understanding why some *ey* mutant retinas completely lack photoreceptor neurons. We turned our attention to the morphogenetic furrow because So, Eya and Dac are required for its initiation and progression across the eye field. Reductions in these factors prevent the furrow from either initiating at the margin or progressing across the eye field (Mardon et al., 1994; Pignoni et al., 1997). The point at which the furrow initiates at the posterior margin is marked by the expression of both *upd* (Fig. 4I) and *hedgehog* (*hh*; Fig. 5A, green arrow) (Dominguez and Hafen, 1997). At this stage, *hh* is also expressed within the posterior compartment of the antennal disc. In older discs, *hh* expression is lost from the posterior margin but is now expressed in photoreceptor neurons and the ocellar region (Fig. 5B) (Ma et al., 1993). In ey^{LB} mutant discs that lack photoreceptors, *hh* expression is absent from the posterior margin (Fig. 5C, red arrow) while still being maintained in the antenna and ocelli (Fig. 5C). As expected, *hh* expression is maintained in developing photoreceptors in any mutant retina that contains ommatidia (Fig. 5D).

The Decapentaplegic (Dpp) pathway, which itself is activated by the JAK/STAT and Hh cascades, is also involved in controlling initiation of the furrow (Chanut and Heberlein, 1997; Ekas et al., 2006; Heberlein et al., 1993; Tsai et al., 2007). In wild-type retinas, *dpp* is expressed along the posterior margins in nascent discs and, once the furrow has initiated, it is then expressed within the advancing furrow (Fig. 5E,F) (Blackman et al., 1991). It is also expressed in a ventral pie sector of the antennal disc. The Dpp pathway is integrated into the RD network at several levels (Chen et al., 1999); of particular note, this pathway is regulated by members of the RD network including Eya (Curtiss and Mlodzik, 2000; Hazelett et al., 1998). In developing *ey* mutant retinas that completely lack photoreceptors, *dpp* expression is lost from the posterior margin and consequently the furrow fails to initiate (Fig. 5G). In contrast, in any *ey* mutant retina that develops photoreceptors, *dpp* expression is maintained within the furrow

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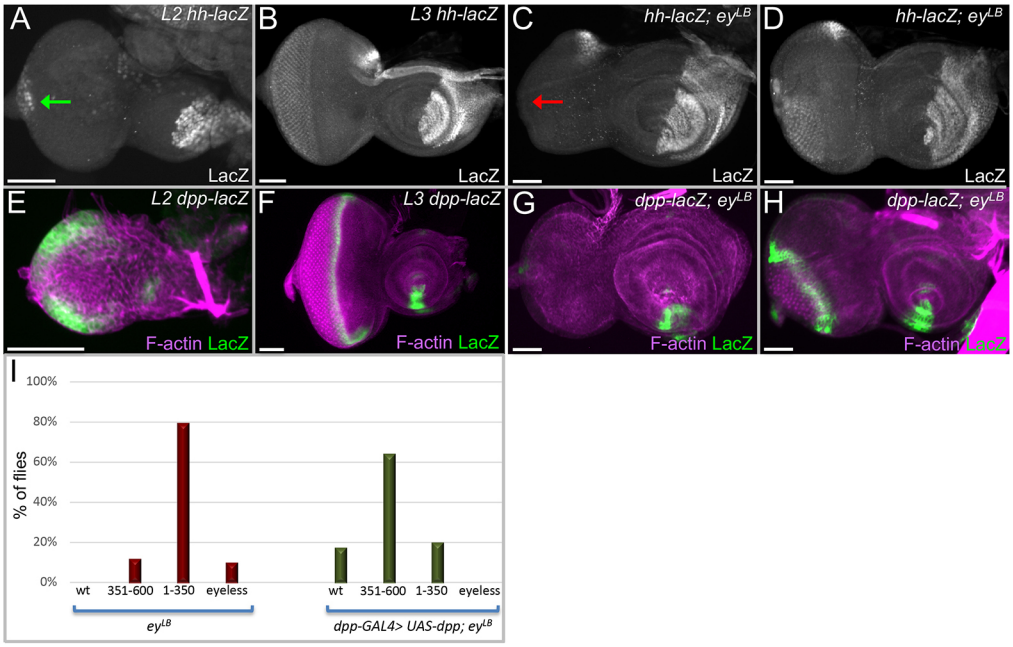

Fig.5. Loss of *dpp* expression is the underlying cause of the no-eyed phenotype of ey^{LB} mutants. (A-H) Control (A,B,E,F) and ey^{LB} mutant (C,D,G,H) eye-antennal imaginal discs. Anterior is to the right. Scale bars: 50 µm. (I) Distribution of eye sizes in ey^{LB} mutants (*n*=209) and ey^{LB} mutants in which *dpp* has been restored to the posterior margin (*n*=77). wt, wild type.

(Fig. 5H). We noticed that the expression of *dpp* within the furrow of ey^{LB} mutants is weaker than that seen in wild-type discs (Fig. 5H). Although not tested here, this weaker expression may prevent the furrow from traversing across the entire disc. This could then contribute to the generation of a small/medium-sized eye. If the loss of Dpp is the primary reason for the loss of eye development, then one would expect that restoring *dpp* expression to the posterior margin should result in the initiation of the morphogenetic furrow and the recovery of eye development to *ey* mutants. Indeed, that is precisely what we observe (Fig. 5I). With the addition of Dpp we see a complete absence of any no-eyed flies, a shift in the percentage of small-eyed flies (1-350 ommatidia) to ones that are medium in size (350-600 ommatidia), and a sudden emergence of flies with wild-type appearance (~20%). This finding, taken with the continued expression of downstream eye specification genes, suggests that the underlying cause for the no-eyed phenotype of ey^{LB} mutants is the failure of the morphogenetic furrow to initiate pattern formation. Interestingly, removal of Ey within the disc itself (*DE-GAL4, UAS-ey* RNAi) does not affect *dpp* expression or the progression of the furrow (Zhu et al., 2017).

Ey is required in the peripodial epithelium to control initiation of the morphogenetic furrow

Because several of our results contrast with current models, we sought to confirm these findings by removing *ey* using an independent method; namely, we expressed an RNAi line that targets *ey* in the eye disc using an *ey-GAL4* driver. This RNAi line is efficient at blocking *ey* expression (Fig. 6A,B) (Zhu et al., 2017) and the *ey-GAL4* line that we are using drives expression within the eye field including cells at the margin of the disc (Hauck et al., 1999). *ey-GAL4>UAS-ey* RNAi flies exhibit a wide range of eye sizes including small eyes and no eyes (Fig. 6D,E). An example of an eye field that completely lacks photoreceptor development is shown in Fig. 6A-C. The percentage of no-eyed flies and the range of eye sizes are similar to ey^{LB} mutants (Fig. 6F). In addition to the eye field, *ey* is expressed within the overlying PE and margin cells (Bessa and Casares, 2005; Lee et al., 2007). The PE is important for the development of the eye-antennal disc and the adult head (Gibson and Schubiger, 2000; Milner et al., 1983; Milner et al., 1984; Milner and Haynie, 1979). We first confirmed that Ey protein and the *ey-GAL4* driver are both present in the PE (Fig. 7A,B,D). We used a

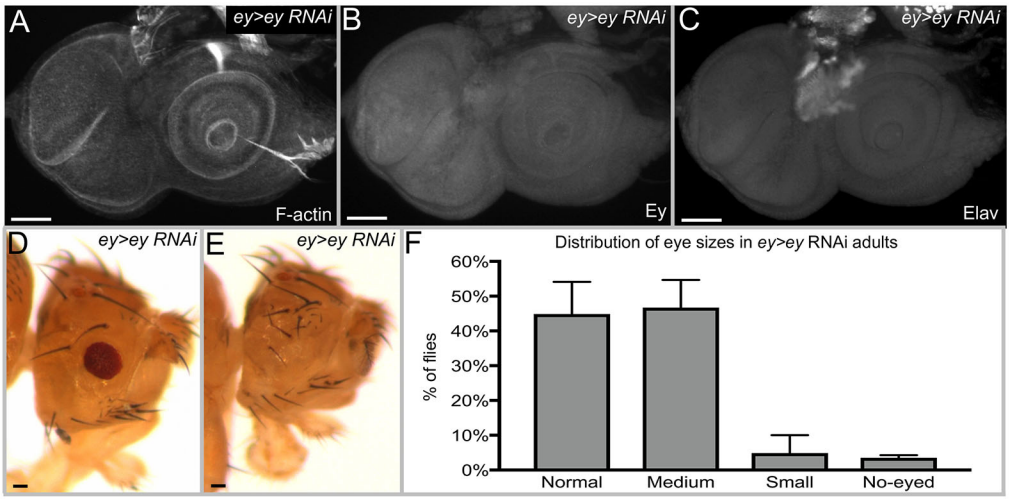

Fig. 6. Removal of *ey* using RNAi recapitulates the ey^{LB} mutant phenotype. (A-C) *ey-GAL4>UAS-ey* RNAi third larval instar eye-antennal discs. (D,E) *ey-GAL4>UAS-ey* RNAi adult heads showing examples of a fly with small eyes (D) and a fly lacking eyes (E). Anterior is to the right. Scale bars: 50 µm. (F) Distribution of eye sizes when *ey* is removed using RNAi (*n*=279). Error bars indicate s.d.

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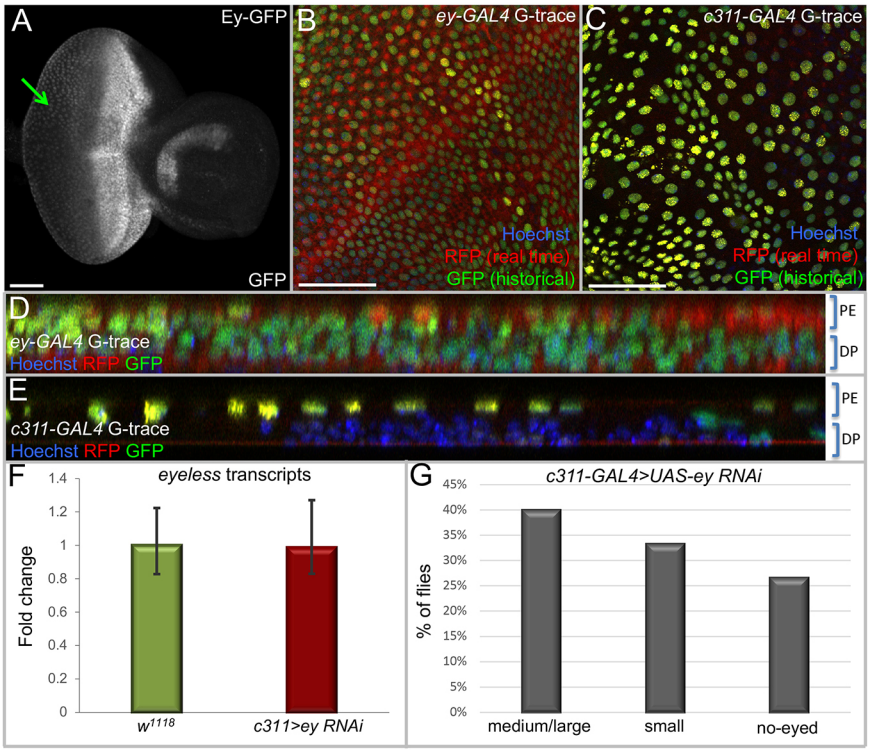

Fig. 7. Ey is required in the peripodial epithelium for proper development of the neural retina. (A) Third instar eye-antennal disc showing that Ey protein is present in cells of the peripodial epithelium (green arrow). (B,C) High magnification views of the peripodial epithelia of eye-antennal discs that carry either *ey-GAL4* (B) or *c311-GAL4* (C) drivers. Scale bars: 50 µm. (D,E) Orthogonal views of eye-antennal discs that carry either *ey-GAL4* (D) or *c311-GAL4* (E) drivers. Anterior is to the right. DP, disc proper; PE, peripodial epithelium. (F) *ey* transcripts in *c311-GAL4>UAS-ey* RNAi eye-antennal discs. Error bars were determined by REST analysis (see Materials and Methods). (G) Distribution of eye sizes when *ey* is removed from the peripodial epithelium (*n*=30).

BAC construct in which GFP has been fused to the *ey* coding region to demonstrate that Ey is present in the PE during normal development (Fig. 7A). We then used the G-trace method (Evans et al., 2009) to show that the *ey-GAL4* driver that we are using to express the *ey* RNAi construct is expressed in the PE during early larval stages (GFP marks historical expression) as well as within the third larval instar (RFP expression marks real-time expression) (Fig. 7B). This led us to realize that in both ey^{LB} mutants and *ey-GAL4>UAS-ey* RNAi knockdowns, *ey* expression is being removed from both the eye field proper and the PE. This led us to consider whether *ey* expression in the PE contributes to normal retinal development and whether the loss of *ey* in the PE is responsible for the reductions in retinal development.

Signaling via the Dpp pathway from the PE does influence development of the eye-antennal disc (Cho et al., 2000; Gibson et al., 2002; Stultz et al., 2006; Stultz et al., 2012; Stultz et al., 2005). Having demonstrated a genetic link between Ey and Dpp (Fig. 5E-I), we then investigated whether Ey regulates *dpp* expression in the PE and if this is important for the initiation of the morphogenetic furrow. To do this, we eliminated *ey* expression in the PE while maintaining it within the eye field itself. This was accomplished by expressing the *ey* RNAi line specifically within the PE by using the *c311-GAL4* driver (Fig. 7C,E) (Gibson et al., 2002; Manseau et al., 1997). We again used the G-trace method to determine whether the *c311-GAL4* driver is expressed exclusively in the PE. We find that cells of the PE, but not of the disc proper, expresses the *c311-GAL4* driver at multiple stages of development – a disc dissected during the late third larval instar shows both historical and real-time expression of the driver (Fig. 7C,E). We also examined expression of a *c311-GAL4, UAS-lacZ* reporter at 48, 60, 72, 84, 96 and 108 h AEL (Fig. S9A-F). This developmental expression profile analysis corroborated the expression pattern obtained using the G-trace method.

The expression of *ey* within the eye field continues to be maintained whereas it is removed from the PE (Fig. 7F,G and Fig. 8A). Surprisingly, we were able to recapitulate the loss of eye development (and its variance) that is seen in ey^{LB} mutants by just removing *ey* from the PE (Fig. 7F, Fig. 8B). In retinas that completely lack photoreceptor neurons, *dpp* expression is lost along the posterior margin, which is consistent with its loss in ey^{LB} mutants (Fig. 8B,C). This loss occurs despite the continued presence of Ey protein within the eye field (Fig. 8A). Restoration of *dpp* expression to the PE in ey^{LB} mutant discs (*c311-GAL4>UAS-dpp; ey^{LB}*) is sufficient to rescue 80% of flies back to normal size (Fig. 8D,E). We tried to restore *dpp* expression to discs in which *ey* expression is removed specifically in the PE (*c311-GAL4, UAS-ey RNAi, UAS-dpp*); however, a synthetic lethality between the two UAS constructs exists (likely as a result of the insertion sites) therefore we could not conduct this experiment. Nonetheless, our data supports a model in which Ey activates *dpp* expression in the squamous cells of the PE or the cuboidal cells of the disc margin, which are themselves derived from the PE (McClure and Schubiger, 2005), to initiate the morphogenetic furrow. Dpp is most likely required in the peripodial/margin cells as expression of Dpp in these two tissues completely rescues eye development in 80% of flies. Once the furrow has initiated, other members of the RD network function within the eye field itself to promote furrow progression. The loss of the eye in ey^{LB} mutants is actually caused by the loss of *dpp* expression at the disc margin. This results in failure of the morphogenetic furrow to initiate and pattern the eye field. These findings indicate that Ey controls eye development via a mechanism and from a location that are both completely unexpected.

Toy is also distributed broadly within the PE (Fig. S10A, red arrows). We sought to remove it just within the PE via expression of a *UAS-toy* RNAi line with the *c311-GAL4* driver. *UAS-toy* RNAi efficiently blocks Toy protein production and any endogenous Toy protein is turned over within 12 h of RNAi expression onset (Zhu et al., 2017). Loss of Toy in the PE does not appear to affect the compound eye but instead results in the loss and/or mis-positioning of the three ocelli that sit at the vertex of the fly head (Fig. S10B-D). This is consistent with earlier reports demonstrating that Toy, but

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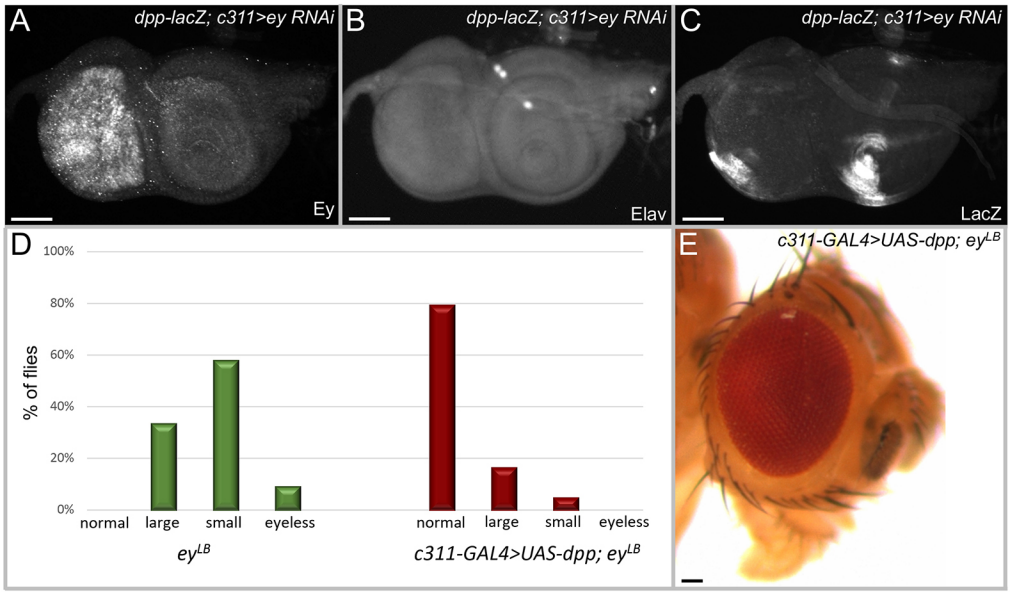

Fig. 8. Ey within the peripodial epithelium regulates *dpp* expression and pattern formation of the eye field. (A-C) Third instar eye-antennal discs in which *ey* expression has been removed from the peripodial epithelium while maintaining it within the eye disc proper. (D) Distribution of eye sizes in ey^{LB} mutants alone (n=111) and when *dpp* expression is added to the peripodial epithelium in an ey^{LB} mutant background (n=43). (E) ey^{LB} adult eye that has been completed rescued by the expression of *dpp* within the peripodial epithelium. Anterior is to the right. Scale bars: 50 µm.

not Ey, controls development of the ocelli (Blanco et al., 2010; Brockmann et al., 2011).

Finally, we set out to determine the critical period during which Ey functions to control *dpp* expression and the initiation of the furrow. To do this, we introduced a temperature-sensitive isoform of the GAL80 protein into our RNAi experiments (*tub-GAL80^{ts}; ey-GAL4>UAS-ey* RNAi). GAL80 is capable of inhibiting GAL4 activity. At the permissive temperature of 18°C, GAL80^{ts} is functional, inhibits GAL4, and blocks expression of the RNAi line. At the non-permissive temperature of 30°C, GAL80^{ts} is inactive thereby allowing GAL4 to drive expression of the RNAi line. By toggling back and forth between the two temperatures we can temporally control *ey* expression. We first kept flies at either of the two temperatures throughout development to test for the efficacy of the GAL80^{ts} protein. At 18°C (active GAL80^{ts}) all flies appear wild type in appearance and at 30°C (inactive GAL80^{ts}) the retinas appear very similar to those of ey^{LB} mutants. We then kept embryos/larvae at 30°C for defined periods of time before transferring them to 18°C. From these experiments, it appears that the critical period for Ey starts in the middle of the first larval instar and extends to the middle of the second larval instar. If Ey is removed during this developmental window then we recover flies that lack the compound eyes and we see a range of eye sizes in the remaining flies. If we remove Ey either before or after this window, we are then unable to recapitulate *ey* mutant retinal phenotypes. We note that the critical window of Ey activity follows the reported onset of *ey* and *dpp* expression within the PE and margin, respectively (Bessa and Casares, 2005; Won et al., 2015).

DISCUSSION

Ever since *Drosophila* Ey was shown to be homologous to the murine and human Pax6 genes [previously named 'small eye' (*Sey*) and 'aniridia' after their phenotypes] (Quiring et al., 1994), there has been intense interest in understanding how Pax6 controls eye development. These interests only intensified once it was shown that Pax6 is interchangeable across the entire animal kingdom and that its forced expression is sufficient to induce ectopic eye formation in non-ocular tissues (Callaerts et al., 1997; Chow et al., 1999; Halder et al., 1995). Such findings led to the view that Ey/Pax6 is the 'master control gene' for the eye. Once this title was granted to Ey then all loss- or gain-of-function phenotypes were, and continue to be, viewed through this singular lens. Here, we have described several unexpected findings that suggest that Ey/Pax6 also contributes to eye development in additional and unexpected ways.

The prevailing view is that, as Ey is the master regulator of the eye, then its loss must result in a complete collapse of the underlying eye/lens gene regulatory network, halting the development of the compound eye. It has been widely assumed that the continued presence of retinal tissue in *ey* mutant stocks is due to the accumulation of second site suppressor mutations in downstream or parallel acting genes. Indeed, outcrossing can lead to strains with smaller, more consistently sized eyes. However, continued outcrossing and selection over a dozen generations does not increase the frequency of 'eyeless' flies and the variability in eye size returns almost immediately once the outcrossing is stopped. The sudden return of variability in eye size clearly suggests that an accumulation of suppressor mutations is not a viable explanation.

Here, we provide an alternative model by showing that, in the absence of Ey, a second Pax6 protein, Toy, is able to weakly activate downstream targets of Ey. As a consequence, eye development continues to be supported to varying and reduced degrees in each individual eye-antennal imaginal disc. In support of this finding is the observation that removing one copy of Ey target genes *so*, *eya* and *dac* leads to a dramatic increase in the percentage of *ey* mutant flies that lack the compound eyes. As Ey and Toy arose through a duplication event relatively late in insect evolution (only holometabolous insects contain both Ey and Toy) (Czerny et al., 1999), it is somewhat surprising that Toy is incapable of fully substituting for Ey during eye development. However, if one considers the combined activity of Ey and Toy then the developing *Drosophila* eye might be more in line with vertebrates that only have a single *Pax6* gene. Dosage effects are associated with vertebrate Pax6 as heterozygote mice and human patients suffer from eye malformations whereas homozygotes die before birth with severe encephalopathy (Glaser et al., 1994; Glaser et al., 1992; Hogan et al., 1986; Schmahl et al., 1993). In *Drosophila*, the loss of *ey* (while maintaining *toy*) yields the eye defects described here whereas removal of both genes from the eye-antennal disc leads to the complete loss of this tissue and all head structures that are derived from it (Wang and Sun, 2012; Zhu et al., 2017). We propose

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that Ey and Toy should be considered together as carrying out the equivalent functions of the single vertebrate *Pax6* gene.

It is intriguing that in *ey* mutants the RD network continues to be expressed, the fate of the eye field is not altered, cell proliferation is not directly affected, and the number of dying cells does not significantly contribute to the smaller eye size. These are all important features of more downstream RD mutants such as *so*, *eya* and *dac*. We suggest that such differences are due to the continued presence of Toy, which is capable, albeit weakly, of activating the remaining RD network. If the RD network is not completely lost and if the tissue is not transformed into head epidermis, then how does the no-eyed phenotype arise? We show that Ey promotes eye development, in part, by activating *dpp* expression at the posterior margin of the retinal field and this in turn leads to the initiation of the morphogenetic furrow (Fig. 9). Our findings suggest that the no-eyed phenotype is caused by a failure of the eye field to be patterned. We can rescue this phenotype by simply restoring Dpp back to the eye field (while still inhibiting *ey* expression) suggesting that the eye disc is still fated to adopt a retinal identity in the absence of Ey. This result is consistent with observations that ectopic eye induction by Ey in imaginal discs takes place almost exclusively at the A/P border, where *dpp* is normally expressed (Salzer and Kumar, 2010). It is also supported by data showing that ectopic eyes can be induced outside the A/P boundary only if Dpp and Ey are simultaneously expressed (Chen et al., 1999; Kango-Singh et al., 2003). Ey might trigger eye formation in non-ocular tissues through alterations in fate specification and the generation of new morphogenetic furrows. This would suggest that Ey also functions as a patterning molecule in addition to a being a fate determinant. Several other signaling pathways, such as Hh, JAK/STAT, Epidermal growth factor receptor and Notch, have been shown to promote initiation of the furrow (Kumar, 2013); thus, it is possible that Ey also regulates the expression of ligands for these pathways at the margin of the eye disc.

We also provide a developmental explanation for why the retinas of *ey* mutants are smaller than those of wild type. We have shown previously that whereas simultaneous loss of both *Drosophila* Pax6 genes leads to a dramatic reduction in cell proliferation, the loss of individual Pax6 genes does not affect cell division or survival (Zhu et al., 2017). It has been a conundrum as to why the retinas of *ey* mutants are smaller than normal. Here, we demonstrate that in *ey* mutants, the D/V axis is mis-positioned along the posterior margin. This results in an uneven starting point for growth of the two compartments and this leads to the development of smaller eyes. We have provided an example of a small *ey* mutant adult eye that is composed of almost only dorsal tissue. In these retinas, the D/V midline is shifted so far along the posterior margin that the ventral compartment comprises only a fraction of its normal size. This is relevant for vertebrate eye development as smaller eyes and lenses are also seen in *Sey* mutants and patients with aniridia (Washington et al., 2009). As alterations in the establishment and positioning of the D/V axis in the optic vesicle leads to smaller and defective neural retinas and pigment epithelia (Uemonsa et al., 2002), it is interesting to consider the possibility that Pax6 might play a role in setting up the axes of the vertebrate eye.

Lastly, we present the astonishing finding that all *ey* loss-of-function phenotypes can be recapitulated if Ey is removed only from the overlying peripodial epithelium (while maintaining Ey in the eye field itself; Fig. 9). Ey is expressed in cells at the margin of the disc, which themselves are derived from the PE. It remains unknown whether Ey controls *dpp* expression at the margin or remotely from the PE proper. It is also not clear at this point whether this regulation requires the So-Eya complex or if Ey directly controls *dpp* transcription. A parallel example exists in vertebrate eye/lens development. If Pax6 is removed from the vertebrate lens, then multiple neural retinas are generated (Ashery-Padan et al., 2000). Also, if BMP4 (a member of the TGFβ superfamily) is removed from the lens then the eye begins to express genes that are specific to the telencephalon (Pandit et al., 2015). The results indicate that both Pax6 and BMP signaling are working non-autonomously from the developing lens to influence the neural retina. We have discovered that an analogous situation appears to be taking place in the *Drosophila* eye-antennal disc. Our data suggest that Pax6 and TGFβ/Dpp direct pattern formation remotely from the adjacent peripodial epithelium. Although the fly peripodial epithelium and the vertebrate lens are non-homologous structures, it is exciting to think that the non-autonomous functions of Pax6 might be conserved across 500 million years of evolutionary history.

MATERIALS AND METHODS

Fly stocks

The following fly stocks were used in this study: ey^{2} [Bloomington *Drosophila* Stock Center (BDSC)], ey^{LB} (this report), so^{1} (Larry Zipursky,

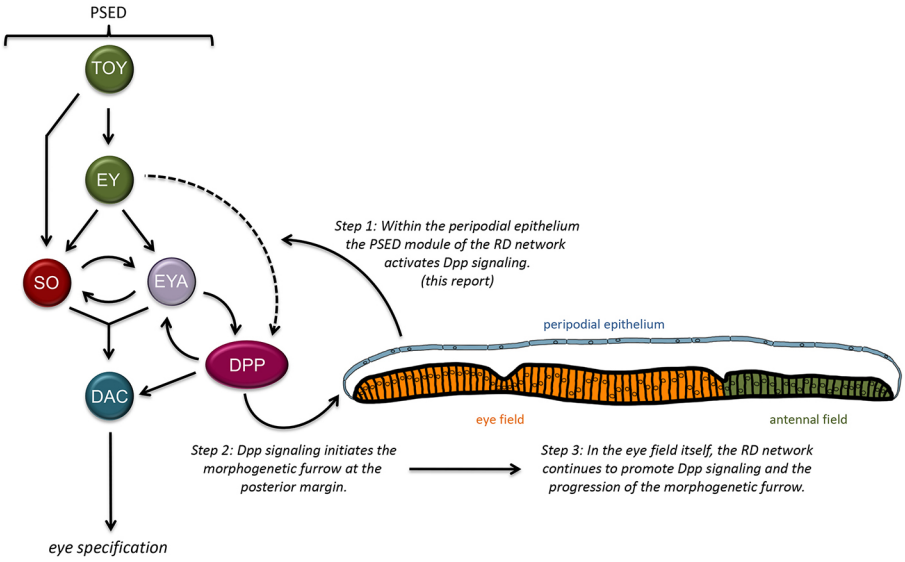

Fig. 9. A new model for how Ey/Pax6 regulates eye development. We propose that Ey (and other members of the PSED core unit of the RD network) function within the peripodial epithelium to activate Dpp signaling. In turn, as shown by many laboratories, Dpp signaling at the posterior margin (cuboidal cells) cooperates with other pathways such as Hh, Jak/STAT, Egfr and Notch to initiate the morphogenetic furrow. The RD network then, as currently envisioned, promotes the progression of the furrow by regulating Dpp signaling within the eye field itself. We further propose that in the absence of Ey, the other Pax6 protein, Toy, is unable to robustly activate the downstream PSED members and, as a result, the size of retina is reduced and variable. In animals that completely lack the compound eyes, the primary defect appears to be the loss of Dpp expression and a failure of the morphogenetic furrow to initiate from the posterior margin.

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UCLA, Los Angles, CA, USA), *eya*^{2} (Nancy Bonini, University of Pennsylvania, Philadelphia, PA, USA), *hh-lacZ* (BDSC), *so-lacZ* (Graeme Mardon, Baylor College of Medicine, Houston, TX, USA), *dpp-lacZ* (Kevin Moses, Emory University, Atlanta, GA, USA), *salm-lacZ* (BDSC), *mirror-lacZ* (Kwang Choi, KAIST, Seoul, Korea), *fj-lacZ* (Ken Irvine, Rutgers University, Piscataway, NJ, USA), *upd-lacZ* (Henry Sun, Academia Sinica, Taipei, Taiwan), *slp-lacZ* (BDSC), *emc-GFP* (Michael Buszczak, UTSW, Dallas, TX, USA), *eyg-GFP* (Henry Sun, Academia Sinica, Taipei, Taiwan), *ey-GAL4* (Georg Halder, Katholic University, Leuven, Belgium), *ey-GAL4* (BDSC), *dpp-GAL4* (Graeme Mardon, Baylor College of Medicine, Houston, TX, USA), *c311-GAL4* (BDSC), *dorsal eye-GAL4* (Georg Halder, Katholic University, Leuven, Belgium), *tub-GAL80*^{ts} (BDSC), *UAS-P35* (BDSC), *UAS-DIAP1* (Bruce Hay, California Institute of Technology, Pasadena, CA, USA), *UAS-toy* (Walter Gehring, University of Basel, Basel, Switzerland), *UAS-toy* (Weasner et al., 2009), *UAS-toy* (this report), *UAS-dpp* (Kristi Wharton, Brown University, Providence, RI, USA), *UAS-GFP* (BDSC), *G-TRACE w[*]; Pw[+mC]=UAS-RedStinger6, Pw[+mC]=UAS-FLP.Exel3, Pw[+mC]= Ubi-p63E(FRT.STOP)Stinger15F2* (BDSC), *ey-GFP* (BDSC), *y*^{1} *M(vas-int.Dm)ZH-2A w*; PBac (y*$^{+}$ *attP-3B)VK00033* (BDSC), *y*^{1} *M(vas-int. Dm)ZH-2A w*; PBac (y*$^{+}$ *attP-9A)VK00022* (BDSC), *y*^{1} *M(vas-Cas9.S)ZH-2A w*^{1118} (BDSC), Df(4)G/ln(4)ci^{D}, ci^{D} pan^{ciD} sv$^{spa\text{-}pol}$ (BDSC), w^{1118}; Df(2R)Exel6055, PXP-UExel6055/CyO (BDSC), *w*^{1118}*; Df(3R)Exel6201, PXP-UExel6201/TM6B, Tb*^{1} (BDSC), *w*^{1118}*; Df(3L)Exel6279, PXP-UExel6279/TM6B, Tb*^{1} (BDSC), *w*^{1118}*; Df(2R)Exel7138/CyO* (BDSC), *w*^{1118}*; Df(2L)BSC150/CyO* (BDSC), *w*^{1118}*; Df(2L)BSC151/CyO* (BDSC), *w*^{1118}*; Df(2R)BSC264/CyO* (BDSC), *w*^{1118}*; Df(2L)BSC354/CyO* (BDSC), *w*^{1118}*; Df(3L)BSC380/TM6C Sb*^{1} *cu*^{1} (BDSC), *w*^{1118}*; Df(3L)BSC479/TM6C Sb*^{1} *cu*^{1} (BDSC) and *y*^{1} *w*; ey*$^{15.71}$*/ln(4)ci*^{D}*, ci*^{D} *pan*^{ciD} *sv*$^{spa\text{-}pol}$ (BDSC). All fly stocks were maintained at 25°C unless otherwise noted. The list of all genotypes for each figure panel in this manuscript is provided in Table S3.

Antibodies and light microscopy

The following primary antibodies were used in this study: chicken anti-βgal (1:800, AB134435, Abcam), guinea pig anti-Toy (1:500, Henry Sun), mouse anti-Ey [1:100, anti-eyeless, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Cut (1:100, 2B10, DSHB), mouse anti-Eya (1:5, eya10H6, DSHB), mouse anti-Dac (1:5, mAbdac1-1, DSHB), rabbit anti-GFP (1:1000, A-11122, Invitrogen/Life Technologies), rabbit anti-DCP1 (1:100, 9578S, Cell Signaling Technologies), rabbit anti-Tsh (1:3000, Stephen Cohen, University of Copenhagen, Denmark), rabbit anti-Lim1 (1:1000, Juan Botas, Baylor College of Medicine, Houston, TX, USA), mouse anti-Dll (1:500, Dianne Duncan, Washington University, St. Louis, MO, USA), rat anti-ELAV (1:100, Rat-Elav-7E8A10 anti-elav, DSHB), rat anti-Al (1:1000, Gerard Campbell, University of Pittsburgh, PA, USA), guinea pig anti-Ss (1:100, Robert Johnston, Johns Hopkins University, Baltimore, MD, USA), mouse anti-Wg (1:800, 4D4, DSHB), rabbit anti-Otd (1:650, Tiffany Cook, Wayne State University, Detroit, MI, USA). Secondary fluorophore-conjugated antibodies and phalloidin-fluorophore conjugates (for detection of F-actin) were from Jackson ImmunoResearch and Life Technologies, respectively. Hoechst 33342 was from Invitrogen/Life Technologies and was used at 1:2000. Imaginal discs were dissected and prepared for immunostaining and microscopy as described by Spratford and Kumar (2014). Low to medium magnification images of whole eye antennal discs were taken on a Zeiss Axioplan II compound microscope. Cross-sectional images were taken on a Leica SP8 scanning confocal microscope and *z*-stacks were analyzed using ImageJ (NCBI) and/or Leica LASX software. For light microscopy of adult eyes, flies were collected into empty glass vials, incubated at −80°C for 10 min, and then imaged using a Zeiss Discovery light microscope.

Scanning electron microscopy and ommatidial numbers

Thirty adult female flies from each specified genotype were randomly selected for imaging. Flies were prepared for the scanning electron microscopy by serial incubation for 24 h in each of the following solutions: (1) 25% ethanol, (2) 50% ethanol, (3) 75% ethanol, (4) 100% ethanol, (5) 50% ethanol-50% HMDS and (6) 100% HMDS. The right eyes were imaged and, for determining the average number of ommatidia, the number of unit eyes within the right eye was counted three times.

Categorization of eye size

Adult *ey*^{LB} mutants were placed into bins based on the overall size of their eyes. Eyes smaller than 50% of wild type were considered to be ‘small and those that were larger than 50% of wild type were categorized as being ‘large’. For an eye to be considered wild type or for it to be considered rescued, it had to be indistinguishable from a set of control eyes. The right and left eyes of an *ey* mutant fly are often asymmetric in size. Flies were placed into the category of the more severely affected eye. Flies that lacked either both compound eyes or were missing just one eye were both categorized as being ‘eyeless’.

Developmental delay

Wild-type and *ey*^{LB} embryos were collected 1 h AEL and placed at 25°C. The number of eclosed flies was then determined at 24 h intervals. We recorded a developmental delay of 24 h in eclosion rates and determined that this delay takes place during the third larval instar stage. In normal development, several rows of photoreceptors are seen at 84 h AEL (Spratford and Kumar, 2013). Because there is a 24 h delay in *ey*^{LB} mutants all imaginal discs shown in this manuscript (unless otherwise stated) were dissected at 108 h. At this time point there should be several ommatidial rows, therefore, if an *ey*^{LB} disc lacked ELAV-positive cells at 108 h then it was considered to be a ‘no-eyed’ disc.

GAL80 control of RNAi induction

tub-GAL80^{ts}*; ey-GAL4>ey-RNAi* embryos were collected for 1 h at 30°C (restrictive temperature) and were then incubated at this temperature for defined periods of time before being transferred to the permissive temperature of 18°C for the remainder of development. Eye-antennal imaginal discs were then dissected from late wandering third instar larvae.

RT-qPCR

RNA from wild type (control), *ey*^{2}, *ey*^{LB} and *c311-GAL4>UAS-ey* RNAi eye-antennal discs was isolated as described by Weasner et al. (2016) and subjected to RT-qPCR analysis as described by Ihry et al. (2012). For each genotype, RNA isolation, cDNA synthesis and qPCR were performed on three separate biological replicates with each sample consisting of approximately 50 third larval instar eye-antennal imaginal discs. Primers that were used for each RD gene are listed in Table S2. Relative Expression Software Tool (REST) was used to calculate standard error using confidence intervals centered on the median. This allows the error bars to reflect asymmetric tendencies in the data.

Generation of the *ey*^{LB} allele

Quiring et al., (1994) identified the position of a transposable element within the *ey* gene. This insertion was shown by Hauck et al. (1999) to disrupt an eye-specific enhancer element. We used the CRISPR/Cas9 system to delete the enhancer element (from wild-type flies) and generate the *ey*^{LB} mutant allele. The *ey*^{LB} allele is named after the first author, Luke Baker. The CRISPR Optimal Target Finder program was used to locate CRISPR target sites that flank the enhancer element. The sequence of the 5′ guide is 5′-CCGCAAATACTCTCGCCATGGCC-3′ and the sequence of the 3′ guide is 5′-CCGCCCCATGGCCGATGTGGCCC-3′. These guide RNA targeting sequences were individually cloned into the pU6-Bbsl-gRNA plasmid (Kate O’Connor-Giles, University of Wisconsin, Madison, WI, USA). To construct the 5′ homology/repair arm we used PCR to amplify 1647 bp upstream of the 5′ end of the enhancer (647 bp to the 5′ PAM site +an additional 1000 bp) and to generate the 3′ homology/repair arm we used PCR to amplify 3698 bp downstream of the 3′ end of the enhancer (2698 bp to the 3′ PAM site+an additional 1000 bp). Both homology arms were cloned into the pHD-DsRed-attP donor plasmid (Kate O’Connor-Giles). A mixture of gRNA plasmids and the donor plasmid was injected into *Drosophila* embryos that harbor the vas-Cas9 (BDSC) construct within its genome. Putative deletions of the enhancer element were selected based on the expression of DsRed in the adult compound eyes. Deletion of the enhancer was confirmed by DNA sequencing. It is important to note here that this strategy results in the deletion of the 212 bp enhancer that was identified by Hauck et al. (1999) while leaving the rest of the *ey* gene intact. The sequence of the deleted enhancer element is provided in Fig. 1.

Generation of *UAS-toy*

A full-length toy cDNA was cloned into pUAS.g.attB plasmid. The *UAS-toy* construct was stably integrated into the *PBac (y$^+$ attP-3B)VK00033* third chromosome landing site using PhiC31-mediated integration. Proper site-specific integration was confirmed by PCR with attP/attB primers. DNA sequencing was used to verify the *UAS-toy* sequence after it was integrated into the genome.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.P.K.; Formal analysis: L.R.B., B.M.W., S.D.N., J.P.K.; Investigation: L.R.B., B.M.W., A.N., S.D.N.; Writing - original draft: J.P.K.; Writing - review & editing: L.R.B., B.M.W., A.B., J.P.K.; Supervision: J.P.K.; Project administration: J.P.K.; Funding acquisition: A.B., J.P.K.

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Supplementary information

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