© 2022. Published by The Company of Biologists Ltd | Development (2022) 149, dev199634. doi:10.1242/dev.199634

RESEARCH ARTICLE

The timing of cell fate decisions is crucial for initiating pattern formation in the *Drosophila* eye

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ABSTRACT

The eye-antennal disc of *Drosophila* is composed of three cell layers: a columnar epithelium called the disc proper (DP); an overlying sheet of squamous cells called the peripodial epithelium (PE); and a strip of cuboidal cells that joins the other two cellular sheets to each other and comprises the outer margin (M) of the disc. The M cells play an important role in patterning the eye because it is here that the Hedgehog (Hh), Decapentaplegic (Dpp) and JAK/STAT pathways function to initiate pattern formation. Dpp signaling is lost from the margin of *eyes absent* (*eya*) mutant discs and, as a result, the initiation of retinal patterning is blocked. Based on these observations, Eya has been proposed to control the initiation of the morphogenetic furrow via regulation of Dpp signaling within the M. We show that the failure in pattern formation surprisingly results from M cells prematurely adopting a head epidermis fate. This switch in fate normally takes place during pupal development after the eye has been patterned. Our results suggest that the timing of cell fate decisions is essential for correct eye development.

KEY WORDS: Eyes absent, Eye-antennal disc, Morphogenetic furrow, *Drosophila*, Retina, Cell fate

INTRODUCTION

A functioning tissue or organ depends upon the appropriate coordination of several developmental processes, including specification, growth and patterning. At its simplest, each organ type must be constructed to function correctly, must be generated in appropriate numbers, and must be placed in correct locations within and along the body. Failure to execute any of these processes accurately can be disastrous and results in congenital disorders, such as anencephaly, holoprosencephaly, spina bifida and anophthalmia, to name but a few. These disorders affect the mammalian brain, head, spinal cord and eye, respectively. As such, elucidating the developmental and cellular mechanisms underlying specification and pattern formation is crucial for understanding how development goes awry when transcriptional networks, signaling pathways and epigenetic complexes are disrupted. Here, we focus on how the timing of cell fate specification impacts later patterning events.

The eye-antennal disc of the fruit fly, *Drosophila melanogaster*, is an excellent model system for understanding how organs and tissues are specified and patterned. Each larva contains two eye-antennal discs and, together, they give rise to nearly all external structures of the adult head, including the compound eyes, ocelli, antennae, maxillary palps, head epidermis and bristles (Weismann, 1864; Birmingham, 1942; Haynie and Bryant, 1986). Like all other imaginal discs, the eye-antennal disc is a sac-like structure that is composed of three cell layers: the disc proper (DP), the peripodial epithelium (PE), and the outer margin (M) (Fig. 1A-C; Krafka, 1924; Chen, 1929). The DP is a pseudo-stratified epithelium that comprises tall columnar-shaped cells. The PE is identical in shape and size to the DP, is composed of flat squamous cells, and lies atop and juxtaposed to the DP. These two epithelial sheets are joined together along their edges by a strip of cuboidal M cells, which themselves are derived from the PE (Fig. 1C; Chen, 1929; Pilkington, 1942). Initially, the DP and PE physically contact each other, but as development proceeds, a small lumenal space forms and separates the two layers from each other (Auerbach, 1936; Gibson et al., 2002). Disruption of any of the three cell layers and/or the gene regulatory networks (GRNs) that function within them results in the failure of the adult head to be specified and/or patterned correctly (Birmingham, 1942; Milner and Haynie, 1979; Milner et al., 1983; Cho et al., 2000; Gibson and Schubiger, 2000; Atkins and Mardon, 2009; Weasner et al., 2020).

Specification of the eye takes place within the DP and is under the control of an evolutionarily conserved set of transcription factors that are collectively referred to as the retinal determination (RD) GRN (Kumar, 2010; Davis and Rebay, 2017). Embedded within this network is the Pax6-Six-Eya-Dach core module, which specifies the fate of multiple tissues in both flies and vertebrates (Wawersik and Maas, 2000; Hanson, 2001; Davis and Rebay, 2017). Relevant for this discussion is that these genes function as master regulators of eye development in all seeing animals (Gehring and Ikeo, 1999). Flies harboring loss-of-function mutations are often eyeless, whereas forced expression of these genes converts portions of non-ocular tissues, such as the antenna, legs, wings, halteres and genitals, into eyes (Kumar, 2010). The Pax6 homologs *eyeless* (*ey*) and *twin of eyeless* (*toy*) initiate their expression during embryogenesis, whereas transcription of the remaining core members *sine oculis* (*so*), *eyes absent* (*eya*) and *dachshund* (*dac*) is initiated sequentially during the first and second larval instars (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Czerny et al., 1999; Weasner et al., 2016). The stepwise activation of the RD network canalizes a portion of the disc until it commits to adopting an eye fate during the latter half of the second larval instar (Kumar and Moses, 2001a,b).

At the beginning of the third and final larval instar, a wave of morphogenesis initiates at the posterior 'margin' of the eye field and sweeps anteriorly until it reaches the eye/antennal border. The M is defined as comprising the cuboidal M cells and a small strip of adjoining cells from both the PE and DP. This broader M domain is the focus of this study. The leading edge of the differentiating wave is visualized as a dorsoventral groove in the epithelium and is called the morphogenetic furrow. As the furrow migrates across the eye field, it gradually transforms a sea of undifferentiated cells into

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Handling Editor: Cassandra Extavour
Received 25 March 2021; Accepted 9 December 2021

Development (2022) 149, dev199634. doi:10.1242/dev.199634

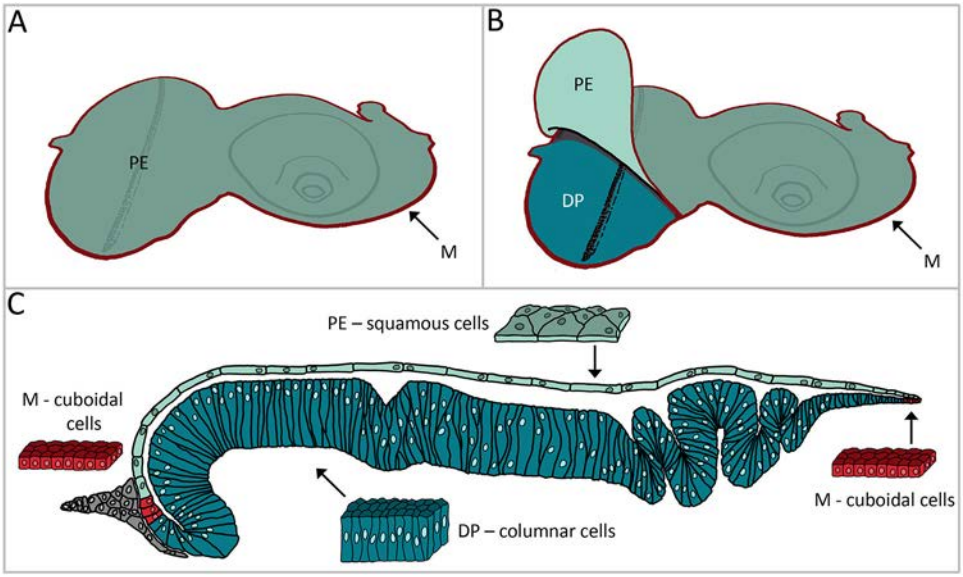

Fig. 1. The eye-antennal disc comprises three cell layers. (A) Schematic of the eye-antennal disc showing the PE (light green) overlying the DP (dark green). (B) The PE is peeled away to reveal the DP. (C) Cross-section of the eye-antennal disc revealing all three cell layers. The M is shown in red.

an ordered array of 750 unit eyes called ommatidia (Ready et al., 1976). The combined activities of the JAK/STAT, Hedgehog (Hh), Decapentaplegic (Dpp), Epidermal growth factor receptor (Egfr) and Notch pathways are important for initiating the furrow from the posterior M (Heberlein et al., 1993; Ma et al., 1993; Chanut and Heberlein, 1997; Domínguez and Hafen, 1997; Kumar and Moses, 2001a,b; Ekas et al., 2006). If these signaling pathways are disrupted, then the furrow fails to leave the M and eye development ceases (Heberlein et al., 1993; Ma et al., 1993; Jarman et al., 1994; Chanut and Heberlein, 1997; Domínguez and Hafen, 1997; Hazelett et al., 1998; Kumar and Moses, 2001a,b).

Once the furrow has departed the M, several signaling pathways are required for its continued propagation across the eye field. This forward movement again requires the activity of Hh and Dpp pathways within both the DP and PE. Within the DP, Hh is secreted from photoreceptor neurons and captured by cells in the furrow. These cells then produce and secrete Dpp, which is trapped by cells that lie immediately ahead of the furrow. These cells transiently enter a furrow-like state before being transformed into new photoreceptor clusters. These new ommatidia produce Hh and the cycle repeats itself until the furrow is propelled across the entire eye field. Reducing Hh and Dpp signaling arrests the furrow, blocks photoreceptor differentiation and reduces the size of the compound eye (Heberlein et al., 1993; Ma et al., 1993; Chanut and Heberlein, 1997; Domínguez and Hafen, 1997; Curtiss and Mlodzik, 2000). Hh, Dpp and Notch pathway ligands are also trafficked from the PE to the DP through cellular structures called translumenal extensions. If signaling from the PE is disrupted, the furrow also stops (Cho et al., 2000; Gibson and Schubiger, 2000; Gibson et al., 2002).

Several RD network members are expressed within the PE/M, but there is very little information surrounding their role in these tissues (Atkins and Mardon, 2009). We recently demonstrated that depletion of Ey just within the PE/M results in the loss of *dpp* expression within the M and a failure of the furrow to initiate patterning (Baker et al., 2018). *so* and *eya* mutant discs are characterized by a similar loss of *dpp* expression (Pignoni et al., 1997; Hazelett et al., 1998). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of transcription factor occupancy indicates that both Ey and the So-Eya complex bind to several positions within the *dpp* locus (Jusiak et al., 2014; Jin et al., 2016; Yeung et al., 2018). Together, these observations suggest an appealing model in which the RD network functions within the PE/M to control expression of a key signaling ligand that is important for initiation of the furrow.

Although this is an attractive mechanism, several observations call into question whether the RD network directly regulates Dpp signaling and/or the morphogenetic furrow. First, Dpp signaling has been reported to lie upstream, rather than downstream, of *so*, *eya* and *dac* during eye development (Chen et al., 1999; Curtiss and Mlodzik, 2000). Second, both Ey and the So-Eya complex bind to multiple positions outside the *blink* enhancer. This regulatory element directs expression within the eye (Blackman et al., 1991; Jusiak et al., 2014; Jin et al., 2016; Yeung et al., 2018). Third, forced expression of either Ey or the So-Eya complex fails to activate *dpp* transcription (Chen et al., 1999; Kango-Singh et al., 2003; Salzer and Kumar, 2010). Last, the restoration of Dpp signaling to the margin of either *so* or *eya* mutant discs does not rescue the observed patterning defects (Weasner and Kumar, 2013). As such, it is unlikely that the RD network directly regulates either *dpp* expression or the initiation of the morphogenetic furrow.

We make the unexpected discovery that Eya functions within the M of the disc to control the timing of cell fate decisions. In normal development, M cells that surround the eye are transformed into head epidermis during late pupal development, significantly after pattern formation has been completed. These cells then generate a gradient of Wingless (Wg) signaling, which establishes cellular fates along the periphery of the retina. The result is a clear and smooth transition from ommatidia to bristle-laden head epidermis (Tomlinson, 2003; Kumar et al., 2015). We show that, in *eya* mutant discs, this transformation occurs prematurely during larval development. Our findings suggest that this precocious change in cellular fate is the underlying reason for the collapse of *dpp* expression and for the failure of the morphogenetic furrow to initiate from the posterior M. We propose that a cardinal role for Eya (and potentially the entire RD network) is to control the timing of cell and tissue fate decisions.

RESULTS

Eya expression within the PE/M is required for eye development

Studies so far have described *eya* expression as being limited to the DP of the eye disc, where it promotes eye specification, tissue

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growth, photoreceptor cell fate and axon guidance (Bonini et al., 1993; Pignoni et al., 1997; Hsiao et al., 2001; Jemc and Rebay, 2007; Xiong et al., 2009; Weasner and Kumar, 2013; Karandikar et al., 2014; Lopes and Casares, 2015; Davis and Rebay, 2017). Although Eya is thought to participate in the initiation of the morphogenetic furrow (Pignoni et al., 1997; Hazelett et al., 1998), its expression has not been directly observed in cells of the PE/M. We found that, in addition to the DP, Eya was, in fact, distributed within both the PE and M layers. Eya was present at low levels in a subset of cells in posterior regions of the PE (Fig. 2A,B, purple asterisks). It was also expressed in all M cells along the posterior-lateral M (Fig. 2C-F, green asterisks).

To understand how Eya influences eye development from the PE/M, we removed it specifically from these tissues using RNAi. We first confirmed the efficacy of *UAS-eya* RNAi lines by combining them with the *eya^{comp}-GAL4* driver. With the exception of the ocellar region, this GAL4 line recapitulates the endogenous *eya* expression pattern within the eye-antennal disc (Fig. 3A,D; Weasner et al., 2016). As expected, the compound eyes were missing from adult flies and Eya was absent from the entire eye field except for the ocellar region (Fig. 3B,C,E,F). These phenotypes mimic those of *eya^{1}* and *eya^{2}* loss-of-function mutants, both of which harbor deletions of an eye-specific enhancer element (Bonini et al., 1993; Zimmerman et al., 2000). In these mutants, the loss of eye development results from an increase in apoptosis, a reduction in cell proliferation and a homeotic transformation of the eye into head epidermal tissue (Bonini et al., 1993; Weasner and Kumar, 2013). We show below that a portion of *eya^{comp}-GAL4, UAS-eya* RNAi discs was transformed into head epidermal tissue. Given that these discs were also of the same size and shape as the loss-of-function mutants, it is likely that increased cell death and reduced proliferation also contribute to the loss of the eye.

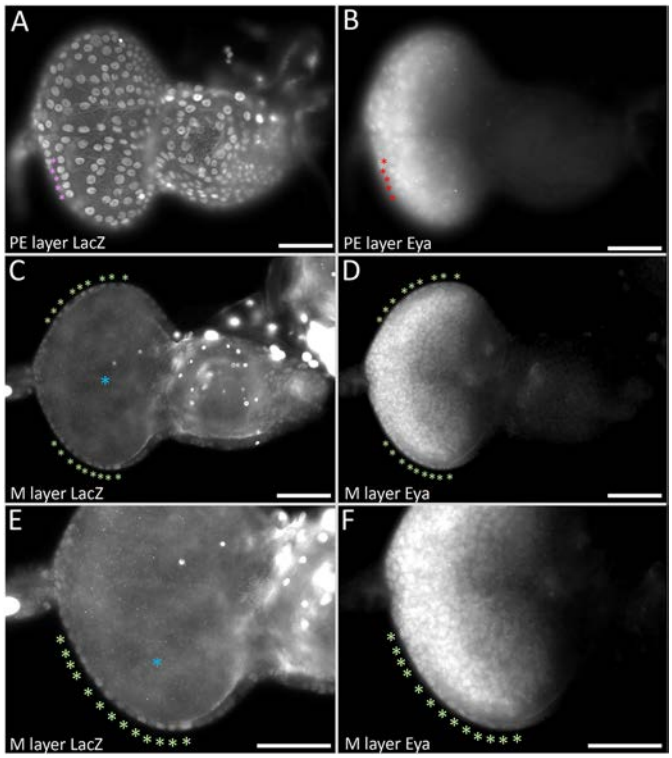

Fig. 2. Eya is distributed within the PE and M layers of the eye-antennal disc. (A-F) *c311-GAL4, UAS*-lacZ eye-antennal discs at 72 h AEL. (A,B) Focal plane has been adjusted to view the PE. (A) A subset of cells of the PE expresses the *c311-GAL4* driver (purple asterisks). (B) These same cells also express Eya (red asterisks). (C-F) The focal plane has been adjusted to view the M cells at the edge of the disc. (C,E) Expression from the *c311-GAL4* driver can be seen within the M cells (green asterisks) but not within the DP (blue asterisk). Scale bars: 25 µm. *n*=30 discs.

We then depleted Eya specifically from the PE/M by combining the *UAS-eya* RNAi line with GAL4 drivers that direct expression specifically within these two tissues. We began with the *c311-GAL4* driver (Manseau et al., 1997) because its specificity for the PE/M has been confirmed in two published studies (Fig. 2A,C and Fig. 3G; Gibson and Schubiger, 2000; Baker et al., 2018). In the first publication, the authors demonstrated that *c311-GAL4* drives expression of a reporter solely within the PE/M of third-larval instar eye-antennal discs (Gibson and Schubiger, 2000). We extended this observation by using the G-trace lineage-tracking system (Evans et al., 2009) to show that *c311-GAL4* was never expressed within the DP during larval development. We also used a *UAS*-lacZ reporter to confirm that *c311-GAL4* was limited to the PE/M even at the earliest stages of larval development. Lastly, we showed that, whereas Ey protein levels were reduced to below detectable levels in the PE/M, they remained robust within the DP of *c311-GAL4, UAS-ey* RNAi discs (Baker et al., 2018).

Surprisingly, removing *eya* expression only within the PE/M with *c311-GAL4* was sufficient to eliminate the compound eyes (Fig. 3I). Even more astonishing was that *eya* expression was also non-autonomously eliminated from the DP (Fig. 3H). This suggests that Eya is required within the PE/M to either establish or maintain *eya* expression within the DP. To distinguish between these two possibilities, we examined *eya* expression in *c311-GAL4*, *UAS-eya* RNAi discs throughout development. We found that Eya expression was never initiated in the eye field, indicating that it is probably required in the PE/M to establish *eya* expression within the DP (Fig. S1A-L).

We then combined the *UAS-eya* RNAi line with an additional 19 enhancer-GAL4 lines that are annotated in Flybase, Janelia Flylight and published studies as being expressed within the PE/M of the eye-antennal disc (Table 1). Some GAL4 lines drove expression broadly throughout the entire PE/M, whereas others showed more-restricted patterns of expression (Fig. S2A-P). In one subset of these *enhancer-GAL4/UAS-eya* RNAi combinations, both *eya* expression and retinal development were completely lost (Table 1, Fig. S3A-L). In a second group of *enhancer-GAL4/UAS-eya* RNAi combinations, *eya* expression was present in variable patches within the disc. These adult flies had inconsistent amounts of retinal tissue and the complete loss of eye development was of variable penetrance (Table 1; Fig. S4A-R). Overall, these results support the proposition that Eya is required in the PE/M to control both *eya* expression and retinal development non-autonomously within the DP of the eye disc.

We used the expression patterns of the GAL4 drivers listed in Table 1 as a guide to determine whether there is a specific region of the PE/M in which *eya* expression is absolutely required for normal eye development. GAL4 drivers that produced a no-eye phenotype in 100% of progeny (Table 1; Fig. S3A-L) were robustly expressed in the M of the disc (Fig. S2A-F). In contrast, if GAL4 expression was either absent or weakly expressed within the M (Fig. S2G-P), then the severity of the adult eye reduction was variable and less than 100% penetrant (Fig. S4A-R). This is intriguing because the M is an important source of signaling molecules for initiating pattern formation (Heberlein et al., 1993; Ma et al., 1993; Chanut and

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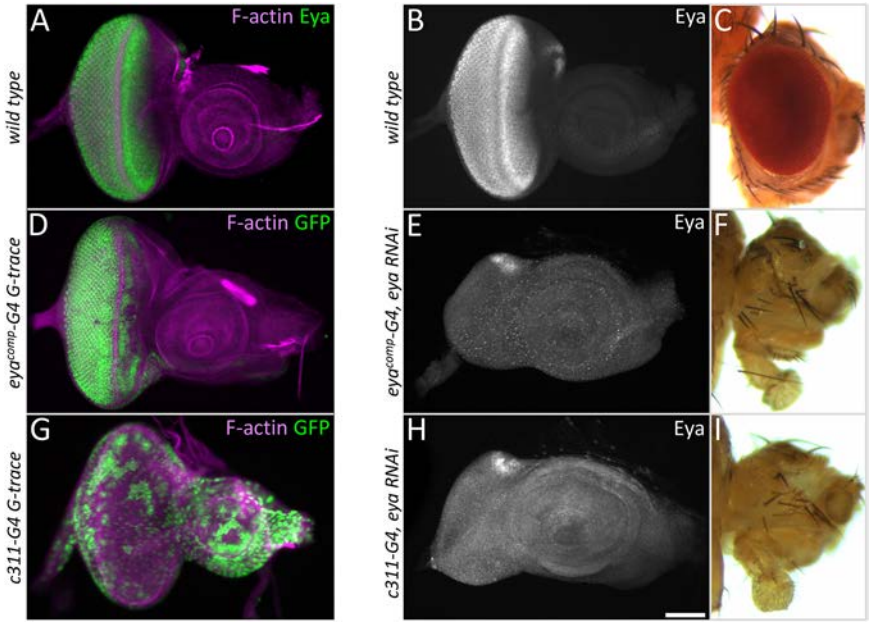

Fig. 3. Removal of Eya from the PE/M abrogates eye development. (A,B) Distribution of Eya protein within a wild-type third-larval instar eye-antennal disc. (C) Light microscope image of a wild-type adult compound eye. (D) Historical expression pattern of the *eya*^{comp}*-GAL4* line (described by Weasner et al., 2016) using the G-trace method (Evans et al., 2009). (E) Eya protein is lost throughout the disc (except for the ocelli) when *eya*^{comp}*-GAL4* is combined with an *UAS-eya* RNAi line. (F) The compound eye is eliminated in *eya*^{comp}*-GAL4, UAS-eya RNAi* adults. (G) Historical expression pattern of the *c311-GAL4* driver, particularly its expression within the PE and M cells. (H) Eya is removed from the entire disc (except for the ocelli) in *c311-GAL4, UAS-eya* RNAi larvae. (I) The compound eye fails to develop in *c311-GAL4, UAS-eya RNAi* adults. Scale bar: 25 µm. *n*=30 discs or 100 adult eyes.

Heberlein, 1997; Ma and Moses, 1995; Treisman and Rubin, 1995; Domínguez and Hafen, 1997; Kumar and Moses, 2001a,b; Ekas et al., 2006). Particularly relevant is that *dpp* expression was lost within the posterior M of *eya* mutant discs (Pignoni et al., 1997; Hazelett et al., 1998).

We wanted to determine whether the loss of *eya* specifically within M cells was responsible for the elimination of the compound eyes. However, there are no GAL4 drivers that, on their own, are expressed only within the M cells. In addition, intersectional tools that could limit expression of the *UAS-eya* RNAi line to the M cells do not currently exist. Thus, we relied on drivers that removed Eya within different regions of the broader M zone. To begin, we combined the *UAS-eya* RNAi line with the *dpp*^{blk}*-GAL4* driver. This driver is expressed robustly along the posterior-lateral margins of the eye disc (Fig. 4A; Staehling-Hampton and Hoffman, 1994). As expected, the compound eyes were eliminated in a majority (74%) of adults (Table 1, Fig. 4C). More notable was our observation that robust Eya protein levels were still present within the DP, whereas *eya* expression was completely absent from the ventral margin (Fig. 4B).

We then selected two additional GAL4 drives with strong expression within the margin and combined them with the *UAS-eya* RNAi line. *E132-GAL4*, an insertion within the *unpaired1* (*upd1*) locus, drives expression at the point at which the midline meets the posterior M (Fig. 4D; Pignoni and Zipursky, 1997), and *GMR28E03-GAL4*, which contains an enhancer from the h*h* locus, drives expression along the M in a similar pattern to *dpp*^{blk}*-GAL4* (Fig. 4G; Jory et al., 2012). Removal of *eya* from these

Table 1. Removal of Eya from the PE/M affects development of the compound eye

GAL4 driver*	Source	% eyeless	Sample size
c311-GAL4	Gibson and Schubiger (2000)	100	100 eyes
c784-GAL4	Hrdlicka et al. (2002)	100	100 eyes
dally-GAL4	Jory et al. (2012)	100	100 eyes
EcR-GAL4	Jory et al. (2012)	100	100 eyes
Egfr-GAL4	Jory et al. (2012)	100	100 eyes
eya^{comp}*-GAL4*	Weasner et al. (2016)	100	100 eyes
fru-GAL4	Jory et al. (2012)	100	100 eyes
hh-GAL4	Jory et al. (2012)	100	100 eyes
osa-GAL4	Jory et al. (2012)	100	100 eyes
tsh^{md621}*-GAL4*	Pallavi and Shasidhara, 2003	83	100 eyes
upd-GAL4	Pignoni et al. (1997)	79	100 eyes
dpp^{blk}	Staehling-Hampton and Hoffman (1994); Pignoni et al. (1997)	74	100 eyes
en-GAL4	Jory et al. (2012)	73	100 eyes
c855a-GAL4	Hrdlicka et al. (2002)	55	100 eyes
T100-GAL4	Harrison et al. (1995)	34	100 eyes
T98-GAL4	Hrdlicka et al. (2002)	24	100 eyes
Dl-GAL4	Jory et al. (2012)	0	100 eyes
Gug-AGiR-GAL4	Gibson et al. (2002)	0	100 eyes
lab-GAL4	Jory et al. (2012)	0	100 eyes
stg^{GMR31F05}*-GAL4*	Jory et al. (2012)	0	100 eyes
stg^{GMR32F08}*-GAL4*	Jory et al. (2012)	N/A‡	N/A‡

*Each GAL4 driver was combined with a *UAS-eya* RNAi responder. Please see Figs 3 and 4 and Fig. S2 for the expression pattern of each GAL4 driver.
‡Embryonic lethal.

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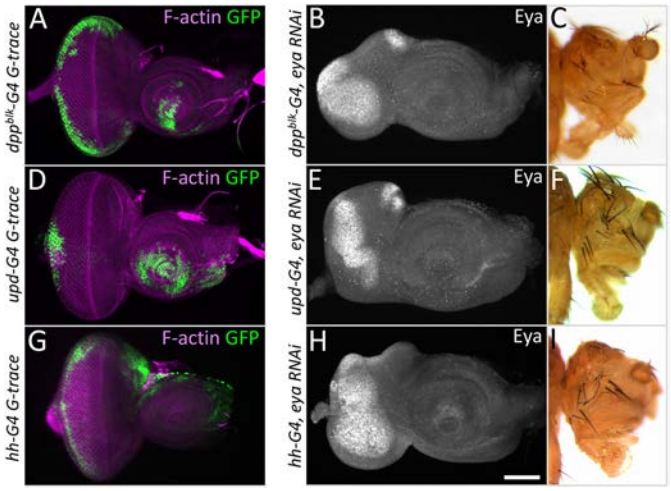

Fig. 4. Removal of Eya from the M abrogates eye development. (A,D,G) The *dpp^{blk}-GAL4*, *upd-GAL4* and *hh-GAL4* drivers are expressed within subsets of M cells during larval development. (B,E,H) If each of the GAL4 drivers in A,D,G are combined with an *UAS-eya* RNAi line, then Eya expression is removed from the M cells. Eya is still present at robust levels within the DP. (C,F,I) In all three instances, adult flies lack compound eyes, suggesting that Eya is required within the M cells of the disc to support eye development. Scale bar: 25 µm. *n*=30 discs or 100 adult eyes.

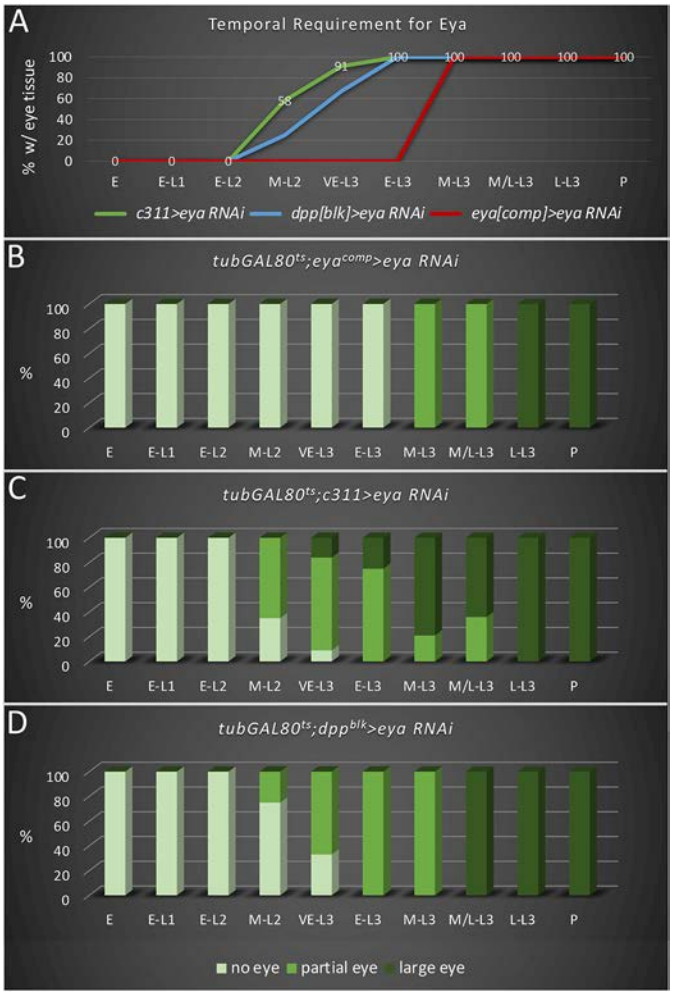

Fig. 5. Eya is required at the M of the disc during the second larval instar. (A-D) Summaries of experiments in which Eya was removed from the eye disc at different developmental periods using the TARGET system. Eya was removed by combining an *UAS-eya* RNAi line with the (B) *eya^{comp}-GAL4*, (C) *c311-GAL4* and (D) *dpp^{blk}-GAL4* drivers. In each case, if Eya was removed prior to, and during, the mid-second larval instar from the M, then the compound eye failed to form in a majority of animals. However, if Eya was removed after the mid-second larval instar, then eye development proceeded. In D, the *UAS-eya* RNAi line is expressed specifically within the M. The data suggest that Eya is required at the M prior to the mid-second larval instar. *n*=100 adult eyes for each of the 30 time-shift experiments. E, embryogenesis; E-L1, early first larval instar; E-L2, early second larval instar; M-L2, middle second larval instar; VE-L3, very early third larval instar; E-L3, early third larval instar; M-L3, mid third larval instar; M/L-L3, mid to late third larval instar; L-L3, late third larval instar; P, pupal stage.

expression domains resulted in a significant proportion of adult flies being completely eyeless (Table 1, Fig. 4F,I). Similar to *dpp^{blk}-GAL4, UAS-eya* RNAi flies, *eya* expression was also maintained within the DP but absent from the M (Fig. 4E,H). Taken together, these findings support prior contentions that Eya is required at the M to promote the launch of the furrow. However, the experiments described below demonstrate that Eya influences the initiation of retinal patterning by controlling the timing of cell fate decisions.

Eya is required prior to the initiation of the morphogenetic furrow

We used the TARGET system (McGuire et al., 2003) to determine whether the temporal window of Eya activity within the margin coincides with the initiation of the morphogenetic furrow (Fig. 5A-D); see Materials and Methods for a description of the system. The *eya^{comp}-GAL4* line serves as a useful control because it drives expression throughout the normal *eya* expression pattern (Weasner et al., 2016). The eye failed to be specified or to initiate pattern formation when Eya was removed from the entire eye disc using this driver at any point prior to the beginning of the third larval instar (Fig. 5A,B). This is consistent with its early onset of expression and its known roles in eye specification (Pignoni et al., 1997; Weasner et al., 2016). With the TARGET system, there is a lag between the onset of RNAi expression and the ultimate degradation of the Eya protein. We determined how long it took for Eya to drop below detectable levels so that we could accurately determine the phenocritical period for its activity. To do this, we combined *tub-GAL80^{ts}* with the *DE-GAL4* driver and the *UAS-eya* RNAi line. Given that the *DE-GAL4* driver is expressed solely within the dorsal-anterior quadrant of the eye field during the third larval instar (Morrison and Halder, 2010), Eya levels in this region can be compared with those in the rest of the eye field. Once larvae reached the mid-third larval instar stage, *eya* RNAi expression was activated for 6, 8, 10, 12, or 24 h. Eya was visibly lower in the dorsal-anterior quadrant after 8 h of continuous RNAi expression and below detectable levels at 12 h compared with the other time points (Fig. 5SA-F).

Removing Eya from the PE/M (using *c311-GAL4*) at different times in development allowed us to determine that it is required in these cells until the middle of the second larval instar (Fig. 5A,C). We confirmed that Eya falls below detection limits within 12 h during this crucial window (Fig. S5G-H). Considering the data from the *eya^{comp}-GAL4* and *c311-GAL4* lines together, one can conclude that, after the mid-second larval instar, Eya is mainly required within the DP (Fig. 5A-C). We then removed Eya only from the margin (using *dpp^{blk}-GAL4*) at different developmental times and found an identical temporal requirement (Fig. 5A,D). As with the *c311-GAL4* driver, Eya fell below detectable levels by 12 h during the crucial

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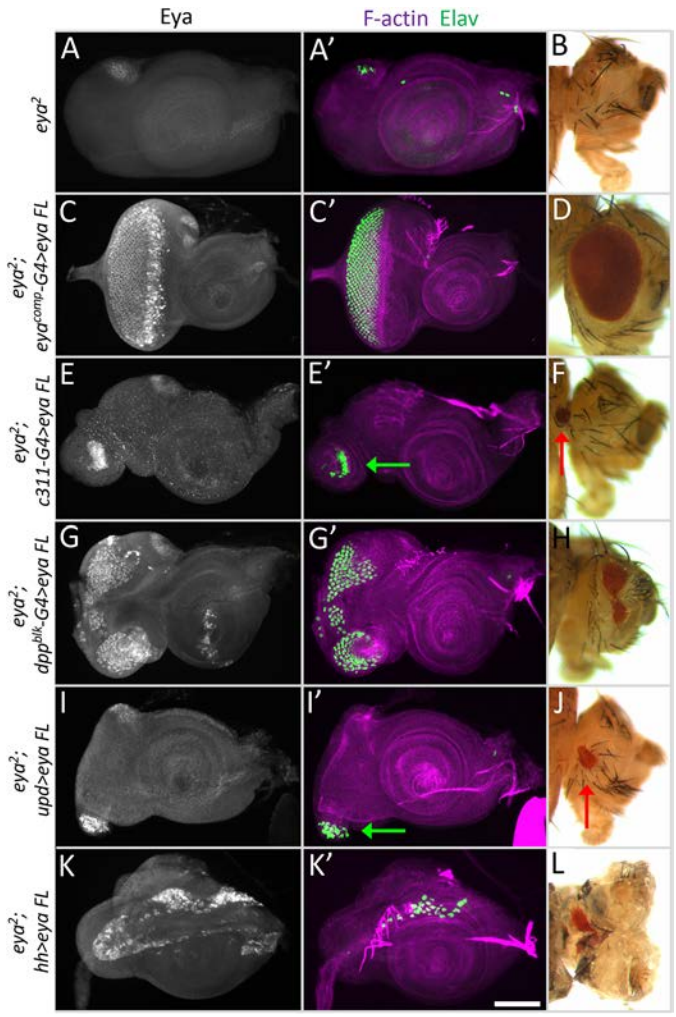

Fig. 6. Restoration of Eya to the PE/M is sufficient to rescue eye development. (A) In an *eya*^{2} mutant, Eya protein is lost from the compound eye but is still present within the ocelli. (A′,B) Without *eya* expression, photoreceptor specification is inhibited and the compound eye fails to form. (C-D) Expression of Eya under the control of the *eya*^{comp}*-GAL4* driver completely restores photoreceptor development and rescues the structure of the compound eye. (E-F) Eya expression within the PE/M with *c311-GAL4* only partially restores photoreceptor development (green and red arrows). (G-H) The *dpp*^{blk}*-GAL4* driver is expressed along almost the entire posterior lateral margin. As such, expression of Eya with this driver results in a larger degree of photoreceptor development. (I-J) Expression of the *upd-GAL4* driver is restricted to the 'firing point', which is the point at which the midline of the disc meets the posterior M. Expression of Eya, only at that point, results in few photoreceptors and a small compound eye (green and red arrows). (K-L) The expression pattern of the *hh-GAL4* driver is altered in *eya*^{2} mutants; therefore, very few photoreceptors develop within the DP when Eya is expressed with this driver. Scale bar: 25 µm. *n*=30 discs or 100 adult eyes.

time window in *dpp*^{blk}*-GAL4, UAS-eya* RNAi discs (Fig. S5I,J). When Eya was eliminated after the mid-second larval instar (using either *c311-GAL4* or *dpp*^{blk}*-GAL4*), retinal development was initiated in most flies (Fig. 5A,C,D). Interestingly, the requirement for Eya was significantly before the time at which the furrow initiates from the posterior M (Ready et al., 1976; Spratford and Kumar, 2013).

If, as our results suggest, Eya is required at the M, then restoring Eya to this domain should reinitiate eye development in the *eya*^{2} mutant (Fig. 6A,B). To test this hypothesis, we combined a *UAS-eya* FL construct with four GAL4 lines that drive expression within the M of the disc (Table S1). The degree to which retinal development was rescued in these instances was compared with *eya*^{2}*; eya*^{comp}*-GAL4, UAS-eya* flies, which have a fully restored compound eye (Fig. 6C,D; Table S1; Weasner et al., 2016). When compared with the complete rescue of eye formation in *eya*^{2}*; eya*^{comp}*-GAL4, UAS-eya* adults, retinal development was re-established to lesser and varying degrees when PE/M-specific drivers were used (Fig. 6E-L; Table S1). The failure to rescue eye formation completely was expected because Eya was only being returned to a small portion of its normal expression domain (Fig. 6E, G,I,K). We also note that the loss of *eya* causes changes in the fate of the eye field (Weasner and Kumar, 2013). The change in fate appeared to alter significantly the expression of several GAL4 lines (Fig. S6A-F). This likely had an impact on the degree to which eye development was restored to the *eya*^{2} mutant. Similarly, the lack of significant amounts of Eya within the DP must also affect the degree to which patterning can be sustained and propagated across the eye field. Despite these caveats, any restoration of eye development when Eya is expressed only within the PE/M clearly suggests that Eya is functioning within these tissues to initiate patterning of the retina.

Eya maintains the fate of cells along the margin of the eye disc during larval development

Given that the So-Eya complex is not predicted to bind to the *dpp* blink enhancer, we investigated whether an alternate mechanism, which does not involve Eya regulation of *dpp* transcription, may explain the loss of patterning in *eya* mutants. We had previously demonstrated that the eye field undergoes a homeotic transformation into head epidermis when the RD network is disturbed (Weasner and Kumar, 2013). The changes in the spatial patterns of GAL4 drivers that are placed in *eya* mutants (Fig. S6A-F) were consistent with a role for Eya in establishing retinal fate. As such, we examined whether the failure to initiate the furrow is, in fact, caused by a change in the fate of cells at the M. We used the expression of the Cut transcription factor as a readout for whether the M was transformed into head epidermis or antennal tissue. In normal eye-antennal discs, *cut* is transcribed within these tissues (Fig. 7A; Blochlinger et al., 1993). However, it is ectopically activated within the eye field of *eya* mutants and, as a result, adult heads have epidermal tissue in place of the compound eyes (Fig. 7G; Salzer and Kumar, 2009; Wang and Sun, 2012; Weasner and Kumar, 2013). *cut* expression is a reliable readout for an eye-to-head epidermis transformation because other head epidermis/antenna genes, such as *Lim1*, *orthodenticle* (*otd*; also known as *ocelliless*, *oc*), and *wingless* (*wg*), are also ectopically expressed in the eye fields of RD network mutants (Weasner and Kumar, 2013).

Broad ectopic activation of *cut* within the eye field was observed in any instance in which the expression of the *UAS-eya* RNAi line resulted in substantial reduction in Eya (Fig. 7B,C; Fig. S7A-N). This included the *eya*^{comp}*-GAL4* and *c311-GAL4* drivers (Fig. 7B, C). By contrast, if the loss of Eya had minimal or no effect on the developing eye, then Cut protein was consigned to its normal domain (Fig. S7O-P). When we used the *dpp*^{blk}*-GAL4*, *upd-GAL4*, and *hh-GAL4* lines to remove Eya only from the margin, *cut* expression was ectopically activated within these cells (Fig. 7D-F). These findings clearly indicate that Eya normally functions at the M to prevent it from being prematurely transformed into head epidermis.

We then expressed *eya* in different spatial domains within *eya*^{2} mutant discs and observed that Eya can inhibit *cut* expression. For example, when Eya was restored to its endogenous spatial domain

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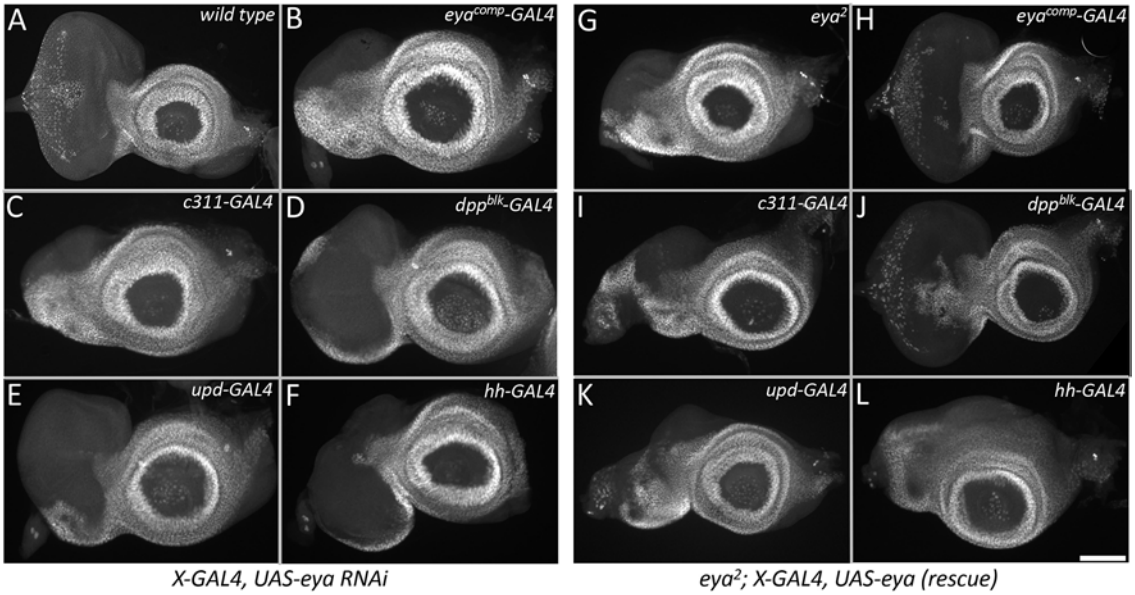

Fig. 7. The loss of Eya expression results in ectopic expression of *cut*, a member of the head epidermis GRN. (A-F) We examined *cut* expression in discs in which a *UAS-eya* RNAi line was combined with different GAL4 lines. (A) In a wild-type disc, *cut* is expressed broadly within the antennal portion and within the most anterior domain of the eye field. (B-F) Loss of *eya* results in the derepression of *cut* within the eye field. Cut is now expressed within the M cells. (G-L) In the second experiment, we monitored *cut* expression in eya^2 mutant discs in which we either fully or partially restored *eya* expression. (G) In the eya^2 mutant, *cut* expression is derepressed within the eye field and M. (H) If a *UAS-eya* transgene is driven with the eya^{comp}*-GAL4* driver, then *cut* expression is relegated to its normal expression pattern (see phenotypic rescue in Fig. 6). (I-L) *cut* expression is still present in eya^2 discs in which *eya* expression is driven by the *c311-GAL4*, dpp^{blk}*-GAL4*, *upd-GAL4* and *hh-GAL4* drivers. This is consistent with the partial rescue of eye development (see Fig. 6). Scale bar: 25 µm. *n*=30 discs.

(using eya^{comp}*-GAL4*), *cut* expression was relegated back to its normal spatial pattern (Fig. 7H) and eye development was completely rescued (Fig. 6C,D; Weasner et al., 2016). When we restored *eya* expression with *c311-GAL4*, dpp^{blk}*-GAL4*, *upd-GAL4* and *hh-GAL4*, c*ut* expression was repressed along the M to varying degrees (Fig. 7I-L). Given that *cut* remains expressed within significant portions of the DP and parts of the ventral margin, the degree and frequency of eye restoration was considerably less with these drivers than with eya^{comp}*-GAL4* (Table S1; Fig. 6F,H,J,L). The ability of Eya to prevent the activation of *cut* using the above four GAL4 lines supports our model that Eya is required to maintain the fate of the M and prevent it from prematurely adopting a head epidermis fate.

We next set out to identify the point in larval development when the margin prematurely transforms into head epidermis. To do this, we examined *cut* expression in eye-antennal discs of wild-type and dpp^{blk}*-GAL4, UAS-eya* RNAi mutants throughout larval development. In wild-type discs, *cut* expression was activated within the antennal disc by 72 h after egg lay (AEL; Fig. 8A). As development proceeded, *cut* expression was extinguished within the inner segments of the antenna while being activated within small regions anterior and ventral of the eye field (Fig. 8B-D, red asterisk and green arrows). These two domains of the eye field give rise to a portion of the head epidermis (Haynie and Bryant, 1986). Lastly, *cut* was activated within the non-neuronal glia and cone cells (Fig. 8E, green arrow). However, it was never activated in the posterior-lateral margins of the disc (Fig. 8A-E).

In dpp^{blk}*-GAL4, UAS-eya* RNAi discs, *cut* expression initiated in a similar pattern to wild-type discs (Fig. 8F). However, it was ectopically activated along the M by 84 h AEL and within the DP of the eye field by 108 h AEL (Fig. 8G-J, green arrows). The ectopic onset of *cut* transcription along the ventral M of the disc was accompanied by the loss of *eya* expression (Fig. 8K-O, green arrows). This further suggests that a premature change in cell fate was taking place at the margin. We note that the onset of *cut* activation at the margin occurred after the temporal requirement for Eya had passed. This suggests that *cut* may not be a direct target of the So-Eya complex and instead functions at a lower level within the GRN, which controls head epidermis/bristle specification. Two additional lines of evidence support this contention. First, forced expression of the So-Eya complex within the antennal disc does not inhibit *cut* expression within the antennal field (Anderson et al., 2012). Second, although there are eight ChIP-seq So peaks within the *cut* locus, the index number that is assigned to each peak is significantly weaker than the indices that have been assigned to sites experimentally verified in other genes (Jusiak et al., 2014).

Ectopic expression of *cut* throughout the eye field is known to transform it into head epidermis, bristle and antennal tissue (Anderson et al., 2012). We investigated whether forced expression of Cut along the M would result in the loss of both *eya* expression and eye development. To do this, we combined a *UAS-cut* FL line with each of the PE/M GAL4 drivers that we used to downregulate *eya* expression at the beginning of the study. Most of these combinations resulted in embryonic or early larval lethality as a result of the ectopic expression of *cut* in crucial tissues (Fig. S8, chart). However, three combinations survived long enough to examine eye development in third-larval instar discs. In all three instances, *eya* expression was altered and pattern formation was severely impaired by the ectopic presence of Cut (Fig. S8A-F). As expected, when *cut* was expressed throughout the eye field with the eya^{comp}*-GAL4* driver, then eye development was blocked and *eya* expression was all but extinguished from the disc (Fig. S8A,B). Interestingly, when *cut* was forcibly expressed only along the M of the disc with the dpp^{blk}*-GAL4* driver, then furrow initiation was inhibited only along the ventral M. As a result, the ventral half of the eye failed to form (Fig. S8C,D).

The dpp^{blk}*-GAL4, UAS-cut* discs and adults appeared strikingly similar to *dpp* loss-of-function mutants in which the *blink* eye-

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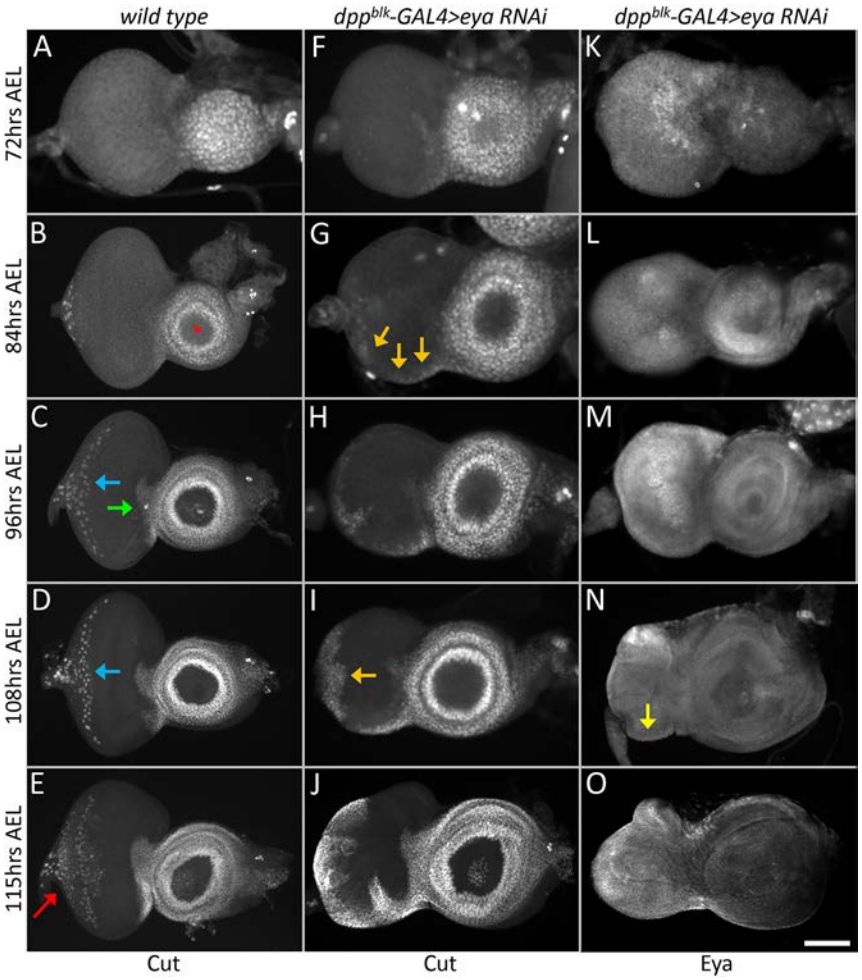

Fig. 8. Changes in Cut distribution correlate with a loss of *eya* transcription. (A-E) Wild-type eye-antennal discs showing the temporal and spatial evolution of *cut* expression during development. (A) At 72 h, *cut* is initially expressed throughout the entire antennal segment. (B) By 84 h, *cut* is lost within the A3 and aristal segments (red asterisk). (C,D) At 96 h, *cut* expression is derepressed within the most-anterior regions of the eye field (green arrow) and a subset of glial cells (blue arrows). (E) By 115 h, *cut* is expressed within the cone cells (red arrow). (F-J) When Eya levels are reduced along the M of the disc, *cut* expression is derepressed in this region (orange arrows). This suggests that retinal M cells are being converted into head epidermis. (K-O) Eye-antennal discs showing a corresponding loss of Eya from the M (yellow arrow). Scale bar: 25 µm. *n*=30 discs.

specific enhancer was deleted. In these mutants, the entire ventral eye and portions of the dorsal eye failed to form. When compared with wild-type eyes, all that remained of the retina of adult dpp^{blk} flies was a small portion of the dorsal eye (Fig. 3C and Fig. 9A; Chanut and Heberlein, 1997). The similarities between dpp^{blk}-*GAL4*, *UAS-cut* and dpp^{blk} prompted us to examine *eya* and *cut* expression in dpp^{blk} mutants. We note that dpp^{blk} mutants experience a 24 h developmental delay. As such, the time points that were analyzed were offset by 24 h compared with dpp^{blk}-*GAL4, UAS-eya* RNAi. At 96 h AEL, *eya* was still expressed broadly throughout the disc, whereas *cut* remained within its normal domain (Fig. 9B,E). However, by 120 h AEL, it was evident that *eya* expression failed to be maintained within the ventral- and dorsal-most regions of the disc (Fig. 9C,D). The loss of *eya* expression is consistent with previous studies placing the Dpp pathway upstream of Eya (Chen et al., 1999; Curtiss and Mlodzik, 2000). Although *eya* expression was inhibited along the ventral M of the disc, we observed a corresponding derepression of *cut* expression within the same domain (Fig. 9F, green arrow). By 144 h AEL, *cut* expression was also derepressed within the DP (Fig. 9G). This ectopic activation of *cut* in dpp^{blk} mutants mimics our observations in *eya* mutants (Weasner et al., 2016).

If a change in fate along the M is the underlying reason for why the eye is not completely patterned, then expression of Eya along the margins of dpp^{blk} mutants should revert the M back to its original identity, block the ectopic expression of *cut* and restore retinal development. Indeed, reinstating Eya to the PE/M with the *c311-GAL4* and *dpp-GAL4* drivers did partially rescue eye development, whereby the eyes contained higher numbers of ommatidia (Fig. 10A,B,D,E; Curtiss and Mlodzik, 2000). Interestingly, the eyes were still substantially smaller than wild type (Fig. 3C). We further observed that the ectopic expression of *cut* along the ventral margin was significantly reduced compared with dpp^{blk} mutant discs (Fig. 9F,G and Fig. 10C,F). Based on our findings, we propose that, in both *eya* and *dpp* mutants, the M of the eye field is prematurely transforming into head epidermis and this change in cell fate is the underlying cause of the failure of the morphogenetic furrow to initiate.

DISCUSSION

In this paper, we examined the role that the timing of cell fate decisions plays in the *Drosophila* eye. During development, the eye is surrounded by a strip of cuboidal (M) cells. These cells play multiple roles in development. First, during larval stages, these cells are the source of several signaling molecules that are required for the initiation and re-initiation of the morphogenetic furrow (Heberlein et al., 1993; Ma et al., 1993; Chanut and Heberlein, 1997; Domínguez and Hafen, 1997; Kumar and Moses, 2001a,b; Ekas et al., 2006). Then, during pupal development, the M is sequentially required for: the fusion of the eye-antennal discs; the elaboration of the head epidermis, which lies between the two compound eyes; the sensory bristles; the vibrissae; and the generation of a Wingless morphogen gradient that specifies peripheral fates within the retina (Milner and Haynie, 1979; Milner et al., 1983, 1984; Haynie and

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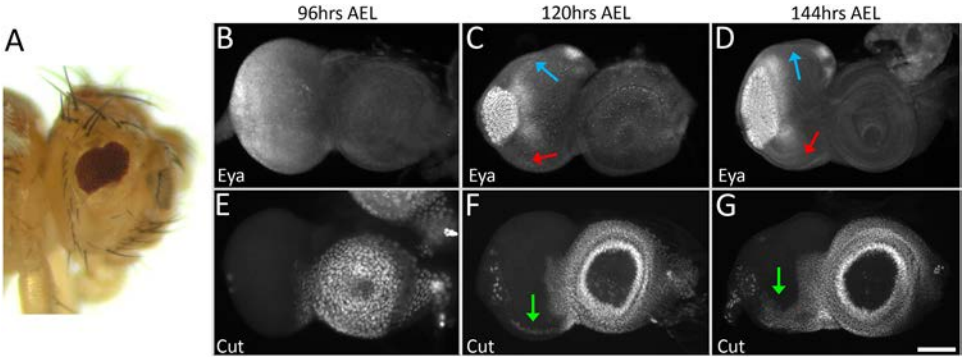

Fig. 9. The reduction in retinal development within *dpp^{blk}* mutants is caused by a loss of *eya* and a gain of *cut* expression at the M of the eye-antennal disc. (A) Light microscope image of an adult *dpp^{blk}* head showing that both ventral and dorsal eye development is abrogated. Only a small portion of the dorsal eye compartment remains compared with the wild-type eye in Fig. 3C. (B-D) In *dpp^{blk}* eye-antennal discs, *eya* expression is lost within the ventral (red arrows) and dorsal (blue arrows) M. (E-G) Light microscope images of *dpp^{bkl}* eye-antennal discs showing the corresponding gain in ectopic *cut* expression along the ventral M (green arrows). Scale bar: 25 µm. *n*=30 discs.

Bryant, 1986; Tomlinson, 2003; Kumar et al., 2015). In order for these diverse functions to be carried out, cells must first maintain their identity as retinal PE/M cells during larval development before transitioning towards a head epidermis fate later during pupal development. We discovered that, when Eya is removed from the M during larval life, this tissue is prematurely forced into adopting a head epidermis/bristle fate. Given that M cells no longer retain their retinal identity, they do not express key signaling molecules, such as Hh and Dpp, which are required to initiate pattern formation (Fig. 11; Baker et al., 2018). As a result, the morphogenetic furrow fails to initiate from the posterior margin and adult flies are eyeless.

Our findings shed light on the role that Eya plays in patterning the retina. The loss of *dpp* expression in *eya* mutant discs has traditionally been interpreted to mean that Eya directly regulates the initiation of pattern formation via activation of at least one key signaling pathway (Pignoni et al., 1997; Hazelett et al., 1998). This is mechanistically distinct from its earlier role in specifying the fate of the retina. In this context, Eya functions to activate the eye GRN while simultaneously preventing non-ocular GRNs from being inappropriately switched on within the eye field (Bonini et al., 1997; Pignoni et al., 1997; Salzer and Kumar, 2009; Weasner and Kumar, 2013). Here, we have shown instead that, by regulating signaling pathways during the initiation of pattern formation, Eya is, in fact, functioning at the M to control the timing of cell fate decisions. Specifically, Eya maintains the retinal-like identity of the M until the patterning of the eye field is complete. After this point, the M is free to transform into head epidermis/bristle during pupal development. Our findings indicate that the timing of cell fate decisions is important for ensuring that a tissue/organ can be appropriately patterned after it has been specified.

Our examination of the role that Eya plays in pattern formation has implications for the entire RD transcriptional network. Other members of the RD network are also thought to be used reiteratively within the eye-antennal disc to specify and pattern the eye field. Furthermore, their roles in these processes have been thought to mirror those of Eya: that is, to promote tissue fate early through activation and repression of GRNs and then to later control signaling pathways during pattern formation. A role for the RD network in activating/repressing entire GRNs during tissue specification is evidenced by the complete absence of retinal development and the replacement of the eye with other tissues, such as maxillary palps, antennae and head epidermis (Hoge, 1915; Milani, 1941; Ives, 1942; Sved, 1986; Mardon et al., 1994). Furthermore, the forced expression of a single RD network member is able to redirect completely the fate of non-ocular tissues, such as the antenna, legs, wings, halteres and genitals, into ectopic eyes (Halder et al., 1995; Shen and Mardon, 1997; Pignoni et al., 1997; Bonini et al., 1997; Pan and Rubin, 1998; Czerny et al., 1999; Seimiya and Gehring, 2000; Singh et al., 2002; Yao and Sun, 2005; Curtiss et al., 2007; Yao et al., 2008; Bessa et al., 2009; Datta et al., 2009). Later during development, the RD network is thought to control pattern formation via regulation of several signaling pathways. This view is based on the observation that expression of both *hh* and *dpp* is lost when the network is compromised (Pignoni et al., 1997; Hazelett et al., 1998; Pauli et al., 2005; Rogers et al., 2005; Baker et al., 2018). However, based on the observations made here, it is possible that the RD network promotes pattern formation via multiple mechanisms. Some RD network members may indeed directly regulate one or more signaling pathways at the PE/M, whereas others, such as Toy and Ey, may cooperate with Eya to maintain the

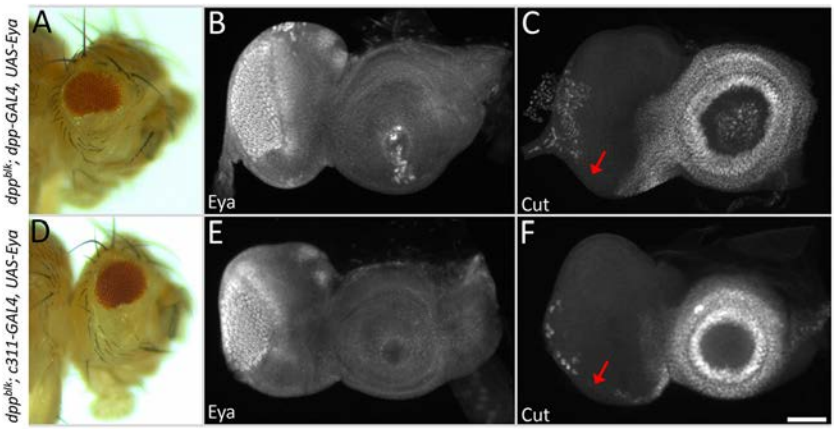

Fig. 10. Eya blocks *cut* expression in, and restores eye development to, *dpp^{blk}* mutants. (A,D) Adult heads showing partial restoration of photoreceptors when *eya* expression is restored to *dpp^{blk}* mutants via the *dpp^{blk}-GAL4* and *c311-GAL4* drivers, also shown by a comparison of the size of these eyes to those of the *dpp^{blk}* allele in Fig. 9A and the wild-type eye in Fig. 3C. (B,E) Restoration of Eya within the PE/M is sufficient to induce *eya* expression within the DP. This results in an increase in the number of photoreceptor clusters and a larger eye. (C,F) As *eya* expression is reinstated within the eye-antennal disc, *cut* is correspondingly repressed at the ventral M (red arrows). This results from the maintenance of a retina-like state at the M. Anterior is to the right. Scale bar: 25 µm. *n*=30 discs or 100 adult eyes.

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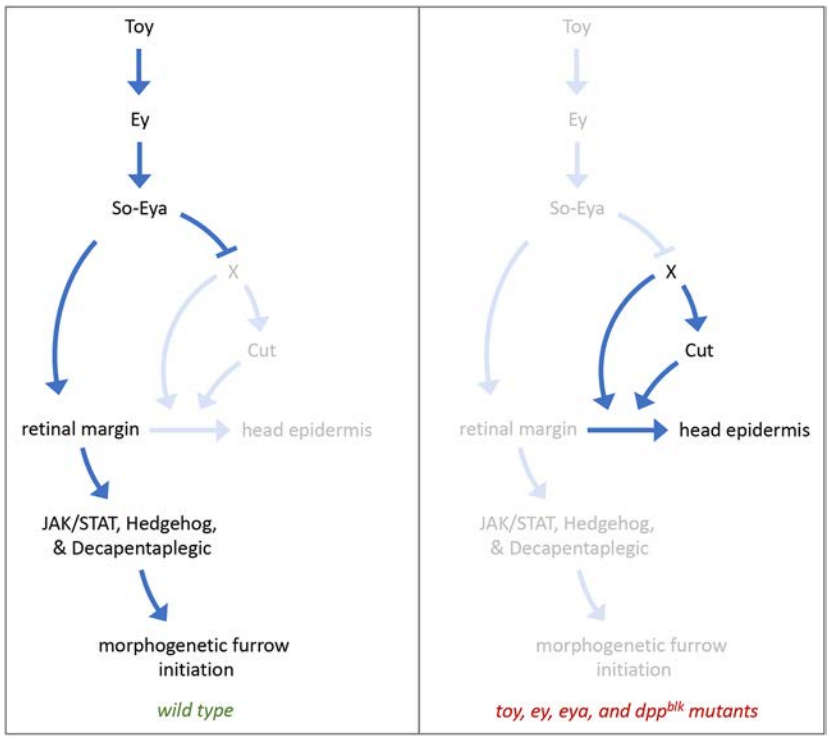

Fig. 11. Model of how the So-Eya complex regulates pattern formation. (Left panel) We propose that, during normal development, members of the retinal determination network, including Toy, Ey, and the So-Eya complex, repress the expression of one or more members of the head epidermis GRN. This inhibition ensures that the retinal M maintains its identity and can express ligands for the JAK/STAT, Hh and Dpp pathways. (Right panel) We have shown here that the loss of Eya leads to the activation of genes, such as *cut*, and that this results in a homeotic transformation of the retinal M into head epidermis. A consequence of this fate change is that key signaling pathways, which are needed for the initiation of the morphogenetic furrow, are never activated. The lack of pathway activation is not the result of direct regulation by the So-Eya complex but rather of a change in cellular fate.

fate of the PE/M. Together, these activities ensure that the morphogenetic furrow is released from the PE/M at the beginning of the third larval instar.

Finally, the results provided here are a cautionary note for inferring regulatory relationships based on changes in gene expression patterns. It is tempting to propose that one gene regulates another because the expression of the latter is altered in a loss-of-function mutant of the former. Results from molecular epistasis studies such as these often become the underpinnings of GRN models and influence how we view the molecular path that cells and tissues take en route to their final form. We have shown here that our understanding of how the fly retina is patterned has suffered from the incorrect assumption that Eya (and other members of the RD network) initiate the furrow via activation of *dpp* expression. Our findings indicate that the loss of *dpp* expression and the failure of pattern formation to initiate is, in fact, the result of a premature change in cell fate. Two studies have further observed that *dpp* expression is lost in both *so* and *eya* loss-of-function mutant clones that lie within the middle of the disc (Pignoni et al., 1997; Hazelett et al., 1998). Both papers concluded that Eya is also required for the furrow to progress across the eye field. However, it appears that, without an intact So-Eya complex, cells within the disc are transformed into head epidermis (Salzer and Kumar, 2009; Weasner et al., 2016). Based on these observations, it is possible that changes in cell fate are the underlying cause of many of the patterning defects that are observed when the RD networks is perturbed. As such, a re-examination of genetic networks, especially in situations in which evidence of a direct regulatory relationship is lacking, is worth undertaking.

MATERIALS AND METHODS

Fly strains

The following fly stocks were used in this study: (1) *bun-GAL4 GMR78D02* (Bloomington Drosophila Stock Center; BDSC); (2) *c311-GAL4* (BDSC); (3) *c784-GAL4* (BDSC); (4) *c855a-GAL4* (BDSC); (5) *dally-GAL4 GMR56G11* (BDSC); (6) *Dl-GAL4* (BDSC); (7) *dpp^{blk}-GAL4* (BDSC); (8) *E132/upd-GAL4* (BDSC); (9) *EcR-GAL4 GMR46E06* (BDSC); (10) *Egfr-GAL4 GMR23C11* (BDSC); (11) *en-GAL4 GMR94D09* (BDSC); (12) *eya^{comp}-GAL4* (Weasner et al., 2016); (13) *fru-GAL4 GMR22B06*; (14) *Gug-Agir-GAL4* (BDSC); (15) *hh-GAL4 GMR28E03* (BDSC); (16) *lab-GAL4 GMR27B01* (BDSC); (17) *osa-GAL4 GMR56H11* (BDSC); (18) *stg-GAL4 GMR31F05* (BDSC); (19) *stg-GAL4 GMR32F08* (BDSC); (20) *T98-GAL4* (BDSC); (21) *T100-GAL4* (BDSC); (22) *tsh^{md621}-GAL4* (BDSC); (23) *tub-GAL80^{ts10}* (BDSC); (24) *UAS-eya* (Weasner and Kumar, 2013); (25) *UAS-cut* (Chrysoula Pitsouli, University of Cyprus, Nicosia, Cyprus); (26) *G-TRACE w[*]; Pw[+mC]=UAS-RedStinger6, Pw[+mC]=UAS-FLP.Exel3, Pw[+mC]=Ubi-p63E(FRT.STOP)Stinger15F2* (BDSC); (27) *eya^{2}* (Nancy Bonini, University of Pennsylvania, Philadelphia, PA, USA); (28) *dpp^{blk}* (Jessica Treisman, New York University, New York, NY, USA); (29) *UAS-ct RNAi* (BDSC 29625); (30) *UAS-eya* RNAi (BDSC 28733, 57314, and 67853); and (31) *DE-GAL4*. *UAS-eya* (BDSC 57314) was used for all experiments depicted in the figures, whereas the other two lines (BDSC 28733 and BDSC 67853) were used to confirm mutant phenotypes.

Genetics

All screen, rescue and overexpression crosses were conducted at 25°C on standard Bloomington media. For time-course experiments using a temperature-sensitive GAL80 to control RNAi expression, adult flies were allowed to lay for 4 h at 25°C in vials with standard Bloomington media. The adults were then removed and vials were shifted to either 18°C (permissive) or 30°C (restrictive) for varying time periods. The effects of removing Eya were assayed in eye-antennal discs and adult heads. For time-course experiments that determined the temporal and spatial expression of *eya* and *cut*, adult flies were placed in collection chambers and allowed to lay eggs for 2 h on molasses-agar plates supplemented with active yeast paste. The adult flies were then discarded and the collection plates containing embryos were transferred to 25°C. After varying incubation periods, the eye-antennal discs were dissected and stained with anti-Eya and anti-Cut antibodies (see below).

Immunohistochemistry

The following primary antibodies were used in this study: mouse anti-Eya (1:5; eya10H6, Developmental Studies Hybridoma Bank; DSHB); (2) rat

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anti-Elav (1:100; 7E8A10, DSHB); mouse anti-Cut (1:100; 2B10, DSHB); (4) chicken anti-beta Galactosidase (1:250; 134435, Abcam); and mouse anti-beta Galactosidase (1:250; Z3781, Promega). Secondary fluorophore-conjugated antibodies [AffiniPure donkey anti-mouse IgG (H+L) Alexa Fluor 488 (715-545-151), AffiniPure donkey anti-mouse IgG (H+L) Cy3 (715-165-151), AffiniPure donkey anti-rat IgG (H+L) Alexa Fluor 488 (712-545-153), AffiniPure donkey anti-rat IgG (H+L) Cy3 (712-165-153), AffiniPure donkey anti-chicken IgG (H+L) Alexa Fluor 488 (703-545-155) and AffiniPure donkey anti-chicken IgG (H+L) Alexa Fluor 488 (703-165-155)] were sourced from Jackson ImmunoResearch Laboratories and were used at a concentration of 1:100. Phalloidin-fluorophore conjugates used to detect F-actin were from Thermo Fisher Scientific/Life Technologies and were used at a concentration of 1:100. Hoechst 33342, which is used to detect DNA, was from Thermo Fisher Scientific/Invitrogen and used at a concentration of 1:2000.

Microscopy

Imaginal discs were prepared for immunohistochemistry as described by Spratford and Kumar (2014) and viewed under a Zeiss Axioplan II compound microscope. Adult flies were frozen at −20°C and then viewed under a Zeiss Discovery light microscope.

TARGET system for determining the crucial window for Eya

The TARGET system, developed by McGuire et al. (2003), was used to determine the crucial window for Eya activity within the M of the eye disc. Three of the GAL4 lines listed in Table S1 (*eya^{comp}-GAL4*, *c311-GAL4* and *dpp^{blk}-GAL4*) were combined with the *UAS-eya* RNAi line and a *tub-GAL80^{ts}* construct. GAL80 blocks GAL4 activity by binding to, and interfering with, the ability of the activation domain of GAL4 to interact with the mediator complex. The temperature-sensitive version of GAL80 allowed us to use temperature as a means to control the onset of RNAi expression. At the permissive temperature of 18°C, the GAL80 protein is active, binds and inhibits GAL4, and prevents the *UAS-eya* RNAi line from being expressed. *eya^{comp}-GAL4, UAS-eya* RNAi flies raised at this temperature throughout development had completely normal eyes. In contrast, at the nonpermissive temperature of 30°C, GAL80 is inactive, which allows for the *UAS-eya* RNAi line to be transcribed robustly. Flies held constantly at this temperature during development completely lacked compound eyes. By toggling between these two temperatures, we were able to exert considerable control over the timing of *eya* RNAi expression during development and to identify the phenocritical period for Eya function. For each temperature shift experiment described in Fig. 5, we examined 50 adult flies for the presence or absence of compound eyes.

TARGET system to determine efficacy of Eya RNAi line

tub-GAL80^{ts}; DE-GAL4, UAS-eya RNAi larvae were held at 18°C until the middle of the third-larval instar stage, at which point they were shifted to 30°C for 6 h, 8 h, 10 h, 12 h, or 24 h. Given that DE-GAL4 is expressed within the anterior-dorsal quadrant of the eye, the presence or absence of Eya in this region was noted and compared with that of the anterior-ventral quadrant. Eya fell below detection levels after 12 h of continuous RNAi expression (Fig. S5). We then subjected both *tub-GAL80^{ts}; c311-GAL4, UAS-eya* RNAi and *tub-GAL80^{ts}; dpp^{blk}-GAL4, UAS-eya* RNAi larvae to similar temperature shifts during the phenocritical period. In both instances, Eya protein also fell below detection levels at 12 h. For each experiment, 30 eye-antennal imaginal discs were analyzed at each time point.

Quantifications

For each experiment, we analyzed 30 eye-antennal imaginal discs or 100 compound eyes from 50 adults. Percentages are presented in Fig. 5, Table 1 and Table S1.

Acknowledgements

We thank Chrysoula Pitsouli (University of Cyprus, Nicosia, Cyprus), Nancy Bonini (University of Pennsylvania, Philadelphia, PA, USA), Jessica Treisman (New York University, New York, NY, USA), the Bloomington Drosophila Stock Center and the Developmental Studies Hybridoma Bank for fly strains and antibodies. We also thank Brandon Weasner for the schematics of the eye-antennal disc in Fig. 1.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

Funding

The work was supported by the National Eye Institute (R01 EY030847 to J.P.K.). Deposited in PMC for release after 12 months.

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