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Conditional knockout of retinal determination genes in differentiating cells in *Drosophila*

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Abstract

Conditional gene knockout in post-mitotic cells is a valuable technique which allows the study of gene function with spatiotemporal control. Surprisingly, in contrast to its long-term and extensive use in mouse studies, this technology is lacking in *Drosophila*. Here, we use a novel method for generating complete loss of *eyes absent (eya)* or *sine oculis (so)* function in post-mitotic cells posterior to the morphogenetic furrow (MF). Specifically, genomic rescue constructs with flippase recognition target (*FRT*) sequences flanking essential exons are used to generate conditional null alleles. By removing gene function in differentiating cells, we show that *eya* and *so* are dispensable for larval photoreceptor differentiation, but are required for differentiation during pupal development. Both *eya* and *so* are necessary for photoreceptor survival and the apoptosis caused by loss of *eya* or *so* function is likely a secondary consequence of inappropriate differentiation. We also confirm their requirement for cone cell development and reveal a novel role in interommatidial bristle (IOB) formation. In addition, *so* is required for normal eye disc

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Conflicts of Interest

Baylor College of Medicine requires G.M. and R.C. to disclose a significant financial interest which results in a financial conflict of interest with *GenetiVision*. For more information please contact coi@bcm.edu.

Author Contributions

G.M. and M.J. conceived the project, designed the experiments and wrote the manuscript. M.J. performed the experiments and analyzed the data. R.C. and G.M. also analyzed the data. A.E. and M.J. generated the *GRs*. M.P. and S.C. performed the SEM.

morphology. This is the first report of a knockout method to study *eya* and *so* function in post-mitotic cells. This technology will open the door to a large array of new functional studies in virtually any tissue and at any stage of development or in adults.

Keywords

eyes absent; *sine oculis*; conditional gene knockout; *Drosophila*; eye development

Introduction

Conditional gene knockout (cKO) allows the study of gene function at a specific developmental stage and/or specific tissue. Such technology is necessary to study essential genes that cause early lethality when mutated. Although the *Cre-loxP* system has been widely used in the mouse and has greatly facilitated our understanding of gene function at different developmental stages [1], our ability to effectively study complete loss of gene function in post-mitotic cells in *Drosophila* has been prevented by the absence of appropriate technology [2, 3]. The most commonly used cKO technique in *Drosophila* has been genetic mosaic analysis, which employs the induction of mitotic recombination [4]. However, this approach has several limitations. First, mitotic recombination is cell division-dependent and therefore cannot be used in post-mitotic cells. Second, mosaic analysis requires that mutant cells are viable and able to proliferate, precluding the rigorous study of many essential genes. Finally, since many genes act pleiotropically, early loss in clones of mutant cells can often obscure or confound interpretation of later phenotypes. For example, although *eya* and *so* have been shown to be required for retinal determination [5, 6], little is known about their function in differentiating cells.

To study the postmitotic roles of *eya* and *so* posterior to the MF, two key retinal determination (RD) genes which regulate both growth and pattern formation during eye development [7], we developed a cKO method in differentiating cells in *Drosophila*. *eya* and *so* begin to be expressed in the eye during the second instar stage. After MF initiation [8], Eya and So are co-expressed within and posterior to the MF, as well as in a zone immediately anterior to it [5]. In the absence of *eya* or *so* function, cells die before differentiation and the MF cannot progress through large mutant clones. Therefore, it has been difficult to study the role of *eya* and *so* in differentiating cells posterior to the furrow. However, previous studies suggest that *eya* and *so* may be required posterior to the MF during retinal differentiation [9-11]. Specifically, loss of *eya* or *so* in single cells posterior to the furrow suggests that they are necessary for photoreceptor survival or/and differentiation [10]. In addition, *eya* or *so* hypomorphic mutant cells that survive to the late third instar stage have defective photoreceptor axon projections [11, 12]. Moreover, posterior expression of *RNAi* constructs targeting either *so* or *eya*, or an *eya* genomic rescue (GR) construct deficient for a posterior enhancer, resulting in development defects in lens-secreting cone cells and pigment cells [9]. However, none of these approaches cause complete and specific loss of gene function posterior to the furrow. Here, we present a new method to conditionally and completely knock out *eya* or *so* function at the DNA level in differentiating cells that allows study of null phenotypes specifically posterior to the MF for the first time.

Results

Conditional *eya* and *so* alleles function efficiently *in vivo*

We have previously reported that a wild-type *eya* genomic rescue construct (*eya*⁺*GR*) can fully rescue viability and eye development in an *eya* null mutant background [9, 13-15]. In this report, we generated another genomic rescue construct, *so*⁺*GR*, which contains a 35 kb fragment encompassing the entire *so* gene and flanking regions (Figure 1A). To test *so*⁺*GR* function, we performed rescue assays in a *so* null background [6] and found that one copy of *so*⁺*GR* fully rescues eye formation and viability in the *so* null background (Figure 1B).

In order to precisely remove gene function with spatiotemporal control, we placed direct tandem *FRT* sequences on either side of several essential exons within each *GR* construct such that *FLP*-mediated recombination will generate a null allele (Figure 2A). This method is analogous to *Cre-loxP* technology except that we have inserted the *FRT*s within *eya* and *so* *GR* constructs (named *eya*^{FGRF} and *so*^{FGRF}) and introduced the conditional alleles by site-specific transgenesis [16]. We found that a single copy of *eya*^{FGRF} or *so*^{FGRF} also fully rescues the corresponding loss-of-function and null mutants (Figure 2C-F), indicating that the *FRT* insertions do not disrupt normal gene function. We then tested the recombination efficiency of *eya*^{FGRF} and *so*^{FGRF} by using heat-shock inducible *FLP* (*hs-FLP*). As expected, in an *eya* or *so* mutant background, a one hour heat shock efficiently causes a complete loss of larval Eya or So expression, respectively (Figure 2H,K), and the resulting adult flies emerge with no eyes (Figure 2I,L). These observations indicate that the *FRT* insertions recombine well and do not disrupt normal gene function in the genomic rescue constructs.

eya and *so* are dispensable for larval photoreceptor differentiation, but are required for differentiation after puparium formation

Although *eya* and *so* play pivotal roles during *Drosophila* eye development [5, 6, 12, 17], little is known about their function in differentiating cells posterior to the MF owing to limitations in previously existing methodology. To overcome this challenge, we crossed *GMR-FLP* into *eya*^{cllIID}; *eya*^{FGRF} or *so*^{3/Df(2L)BSC263}; *so*^{FGRF} backgrounds, which lack endogenous *eya* or *so* function, respectively. The only source of gene function comes from the *GR* with flanking *FRT*s. *GMR* is an eye-specific enhancer that is expressed only after cells enter the MF [18]. Therefore, placing *FLP* recombinase under the control of *GMR* restricts *FLP* expression to regions posterior to the MF [10]. As expected, the resulting progeny (termed *eya*^{GMRcKO} and *so*^{GMRcKO}) have dramatically reduced Eya or So expression posterior to the furrow (Figure 3B and 4B). Intriguingly, third instar larval eye discs of *so*^{GMRcKO} flies are misshapen and have a large outgrowth, resulting in a protrusion of the posterior region of eye discs (Figure 4C,D). This unusual dysmorphology is not observed with conditional loss of *eya* or *RNAi* targeting of *so* [9]. Consistent with previous findings that *eya* and *so* are required in single cells posterior to the MF [10], adult eyes of *eya*^{GMRcKO} and *so*^{GMRcKO} flies are smaller and glazed, indicating that loss of either gene posterior to the furrow causes massive disruption of normal eye development (Figure 3D and 4F). Plastic sections show severely disorganized ommatidia with few or no rhabdomeres (Figure 3E and 4G), suggesting that *eya* and *so* may be required for photoreceptor survival.

Indeed, cleaved Caspase 3 [19] staining is strongly increased in *eya^{GMRcKO}* and *so^{GMRcKO}* pupal eye discs (Figure 5).

Based on the timing of *GMR-FLP* mediated loss of So or Eya expression posterior to the MF, we predicted that gene expression anterior to and within the MF would be largely normal. As expected, Eyeless (Ey), Dachshund (Dac) and dpERK are all normal (Figure 6). Unexpectedly, however, we find that neither *so* nor *eya* are required for larval photoreceptor differentiation during larval stages. In particular, expression of Elav, a marker for differentiating photoreceptors [20], appears normal throughout larval development in *eya^{GMRcKO}* and *so^{GMRcKO}* animals (Figure 3F,G and 4A',B'). Likewise, expression of the neuronal marker Futsch (22C10) [21] is normal (Figure 3H,I and 4H,I). Moreover, the R8 photoreceptor marker Senseless (Sens), which is used to highlight the MF, is normally expressed (Figure 3A',B'). Although there is a lag between the onset of *GMR-FLP* expression and a complete knockout at the protein level, or there may be some perdurance of transcript or protein, our staining results show rapid loss of Eya or So expression immediately posterior to the MF (Figure 3B and 4B). Taken together, our findings suggest that *eya* and *so* are dispensable for differentiation during larval stages, but are necessary for photoreceptor survival. Nevertheless, many photoreceptors and support cells do survive and differentiate, suggesting that cell death is a secondary consequence of inappropriate differentiation. Consistent with this hypothesis, expression of the Egr ligand Spitz (Spi), which drives the progressive differentiation of multiple retinal cell types [22], is gradually reduced in the posterior half of eye discs following loss of *eya* or *so* (Figure 7A-F). Eye discs from *eya^{GMRcKO}* and *so^{GMRcKO}* animals at the white puparium (WP) stage show disorganized expression of Elav (Figure 7G-I). Moreover, Sens expression at 24 hrs after puparium formation (APF) is disorganized but without an obvious decrease in the level of expression, while it is highly reduced at 48 hrs APF (Figure 8).

Loss of *eya* or *so* function in post-mitotic cells posterior to the MF causes defects in cone cell development

Adult eyes of *eya* or *so* conditional knockout animals show a glazed appearance, consistent with a cone cell defect [23]. In addition, our previous studies using *GMR-GAL4>UAS-RNAi* to knock down *eya* or *so* in eye discs suggested that both genes play a role in cone cell specification [9]. Nevertheless, the variable and incomplete nature of knockdowns and potential off-target effects with *RNAi* precluded any rigorous conclusions. Therefore, in this report we used our new *FGRF* system to further explore the function of *eya* and *so* posterior to the MF. Cone cell specification was assayed using the transcription factor Cut as an early marker for cone cell fate [24]. We found little or no Cut expression in third instar larval eye discs *eya^{GMRcKO}* and *so^{GMRcKO}* flies (Figure 3K and 4K), confirming the requirement for *eya* and *so* for cone cell differentiation.

eya and *so* play important roles in interommatidial bristles formation

Cut also serves as an early and specific marker of sensory organ precursor (SOP) development [24]. Since interommatidial bristles are derived from SOPs [25], our Cut staining results suggests that *eya* and *so* are required at early stages of IOB differentiation. This is consistent with the external morphology of adult eyes of *eya^{GMRcKO}* and *so^{GMRcKO}*

animals (Figure 9). *eya^{GMRcKO}* animals show strong loss of ventral but not dorsal IOBs and *so^{GMRcKO}* animals have very few interommatidial bristles across the entire eye. Such IOB defects are not observed in *eya* or *so RNAi* knockdown experiments [9]. Remarkably, this work is the first report of a role of Eya and So in IOBs formation.

Discussion

As key members of the retinal determination network, both Eya and So regulate multiple aspects of eye development for a wide range of higher eukaryotes [26, 27]. However, little is known about their function posterior to the MF during eye development in *Drosophila*. Here, we report a new method for rigorous study of *eya* or *so* function in differentiating cells. By knocking out gene function in differentiating cells posterior to the MF, we find that *eya* and *so* are dispensable for differentiation during larval stages. During pupal stages, loss of *eya* or *so* causes inappropriate or failed differentiation, resulting in massive apoptosis. In addition, both genes are required for normal development of cone cells and interommatidial bristles.

Based on Caspase 3 expression in pupal eye discs and plastic sections of adult eyes from *eya^{GMRcKO}* and *so^{GMRcKO}* animals, extensive apoptosis is readily apparent. However, expression of the differentiation markers Elav and Futsch, appear normal in *eya^{GMRcKO}* and *so^{GMRcKO}* late third instar eye discs. These data indicate that neither *eya* nor *so* is required for early photoreceptor differentiation. In contrast, we find decreased and disorganized expression Elav during WP stages and later. Moreover, reduced expression of Spi, the ligand for Egfr, in the posterior half of larval eye discs is observed following loss of *eya* or *so*. In the developing eye, Spi is required for the recruitment of cells into developing ommatidia after the specification of the founding R8 photoreceptor cell [22]. These findings suggest that the apoptosis caused by *eya^{GMRcKO}* and *so^{GMRcKO}* is a secondary consequence of inappropriate or aborted differentiation. The relatively late loss of Spi expression is likely to block normal differentiation of most accessory cells [28], which then die by apoptosis.

Our results suggest that *eya* and *so* play important roles in cone cell differentiation. Cut expression is severely reduced in both *eya^{GMRcKO}* and *so^{GMRcKO}* late third instar eye discs. Interestingly, Eya and So have been shown to repress Cut expression and antennal fate in the early eye field [29], suggesting context-dependent repression and activation of the same target, as we have previously shown for So regulation of *ey* [30]. Since we do not observe So ChIP-Seq enrichment at the *cut* locus [31] and So directly regulates *lozenge* (*lz*) [32], which is required for Cut expression via *Shaven* (*sv*) (which does show So ChIP-Seq enrichment), it is possible that an Eya/So complex indirectly regulates *cut* via *lz* and/or *sv*. While a regulatory pathway among *lz*, *sv*, and *cut* involving both activation and repression has been proposed [33], the mechanism of RD gene regulation of this pathway will require further study.

This is the first report that Eya and So are involved in IOB formation. During IOB development in *Drosophila*, the proneural genes *achaete* (*ac*) and *scute* (*sc*) are expressed in SOPs, which divide twice to form the four mechanosensory precursor cells, including bristle cells [25]. However, the primary targets of *ac* and *sc* remain largely unknown. Eya and So are possible primary targets of *ac* and *sc* since loss of *eya* or *so* function also results in a

severe reduction of IOBs. However, since the loss of both *ac* and *sc* function causes a complete absence of IOBs, it is likely that other targets exist. Along with a previous study showing that *sens* is a primary target of *ac* and *sc* [34], *eya*, *so* and *sens* may function together to regulate IOB differentiation.

This is also the first study to examine the complete loss of *eya* or *so* function in differentiating cells in *Drosophila*. This is in large part due to the limitations of previously available methods. First, experiments based on traditional mitotic recombination only allow the study of gene function in proliferating cells. Second, loss of many genes causes cell lethality and/or blocks proliferation, preventing traditional clonal or mosaic analysis. Third, while hypomorphic mutants may allow examination of phenotypes after the lethal phase, they cannot reveal the null phenotype, an essential step in the elucidation of gene function in any tissue. Fourth, *RNAi* knockdown of genes is generally incomplete and often lacks specificity. Fifth, the previously reported cKO approach targeting FRT, loxP, or attB/attP sites to endogenous loci in *Drosophila* requires multiple steps and *in vivo* homologous recombination [35, 36]. Our approach only requires a null mutant allele, which is now relatively easy to obtain using CRISPR/Cas9 technology. Finally, engineering FRTs at endogenous loci via CRISPR/Cas9 (or any other current method) is more complicated and difficult than engineering FRTs in BAC clones in *E. coli*. Given the availability of BAC clones tiling the entire genome, the ease of using recombineering to introduce FRTs, and current technologies for introduction of large BACs by site-specific integration, our *FGRF* system efficiently bypasses each of these deficiencies. Since there are a large number of *GAL4* lines that can be easily combined with *UAS-FLP*, it is now possible to remove gene function in a wide variety of cells and time points, allowing new tests to rigorously explore mechanisms of development in post-mitotic cells in a manner not previously possible.

Materials and Methods

Construction of *so*⁺*GR*, *eya*^{FGRF} and *so*^{FGRF} and *Drosophila* transgenesis

We cloned a 35 kb fragment encompassing the *so* gene and flanking regions into the *attB*-*P[acman]-ApR* vector using recombineering [37]. To make *eya*^{FGRF} and *so*^{FGRF}, plasmid PL451 was used as a template to insert the first *FRT* within each *GR* using recombineering. Then we used plasmid PL452 to place the second *FRT* in each *GR*. All recombineering reagents were provided by Biological Resources Branch, NCI.

For transgenic fly generation, each construct was injected into a docking site at 68A (P2) using ΦC31 integrase [16]. Transgenic flies were generated by *GenetiVision* Corporation. Correct integration events were identified by genomic PCR by standard methods. Primer sequences are available on request.

Drosophila Genetics

All flies were maintained with standard medium at 25°C. To test the *FRT*-mediated recombination efficiency of *eya*^{FGRF} and *so*^{FGRF}, we set crosses as follows: *y w hs-FLP*; *eya*² × *w*; *eya*²; *eya*^{FGRF} and *y w hs-FLP*; *so*¹ × *w*; *so*¹; *so*^{FGRF}. Heat shocks were performed

at 37°C for 1 hr 48 hrs after egg laying (AEL) [38]. *GMP-FLP* was used to eliminate Eya or So expression from regions posterior to the MF.

Histology, scanning electron microscopy (SEM) and immunohistochemistry

Plastic sections and SEM of adult eyes were performed as described [34, 39]. Staining of larval imaginal discs and imaging were conducted as described previously [30]. Immunohistochemistry on 24 and 48 hr pupal eye discs was performed as previously described [40]. Primary antibodies used were 1:300 mouse anti-Eya, 1:400 rat anti-Elav, 1:100 mouse anti-Cut, 1:150 mouse anti-Dlg, 1:10 mouse anti-22C10 (Developmental Studies Hybridoma Bank), 1: 100 rabbit anti-cleaved Caspase 3 (Cell Signaling Technologies), 1:100 mouse anti-dpErk (M8159, Sigma), 1:2000 guinea pig anti-So (a gift from Dr. Ilaria Rebay, Chicago, IL, USA) and 1:2000 guinea pig anti-Sens (a gift from Dr. Hugo Bellen, Houston, TX, USA). Secondary antibodies used (all 1:500) were Alexa Fluor 488 goat anti-mouse, anti-guinea pig, anti-rabbit and anti-rat (Molecular Probes) and Cy3 goat anti-mouse and anti-rat (Jackson ImmunoResearch).

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Abbreviations

eya	eyes absent
so	sine oculis
FRT	flippase recognition target
cKO	conditional gene knockout
MF	morphogenetic furrow
RD	retinal determination
GR	genomic rescue
Ey	Eyeless
Dac	Dachshund
Sens	Senseless
Spi	Spitz
Sv	Shaven
Lz	Lozenge
ac	achaete

sc	scute
WP	white puparium
APF	after puparium formation
AEL	after egg laying
SOP	sensory organ precursor
IOB	interommatidial bristle
SEM	scanning electron microscopy

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Figure 2. Conditional *eya* and *so* alleles function efficiently *in vivo*.

(A) The *eya* and *so* genomic loci, with the location of *FRT* insertions indicated (green triangles). “FRTed” genomic rescue constructs (*FGRFs*) extend ~10 kb on either side of the transcription units shown. (B-F) The *eya^{FGRF}* and *so^{FGRF}* transgenes fully rescue their cognate loss-of-function and null mutant phenotypes. External and internal morphology of rescued adult eyes are indistinguishable from wild-type *Canton S*. (G-I) *hs-FLP*-mediated recombination causes a complete loss of Eya staining (H) and eyeless adult flies (I). Eya expression in ocelli (H) is endogenous Eya still present in an *eya²* mutant background. (J-L) Similar results are observed for *yw hsFLP/+; so¹; so^{FGRF/+}* animals. Scale bars: 100 μm (adult eyes), 10 μm (plastic sections) and 50 μm (eye discs).

Figure 3. Post-mitotic loss of *eya* function posterior to the furrow disrupts normal eye development.

(A-E) Complete loss of *eya* function in the differentiating cells posterior to the furrow was induced using *GMR-FLP* and *eya^{FGRF}*. Eya (A,B) and Sens (A',B') expression in *eya^{cli}*; *eya^{FGRF}/+* and *GMR-FLP/+*; *eya^{cliIID}*; *eya^{FGRF}/+* (*eya^{GMRcKO}*) third instar larval eye imaginal discs is shown. (A''-B'') merge of channels. Blue arrows indicate the approximate position of the MF based on the Sens expression pattern. (D) Adult eyes of *eya^{GMRcKO}* animals are smaller, have a glazed appearance, and IOB defects. (E) Ommatidial architecture in *eya^{GMRcKO}* flies is highly disorganized. Occasionally, rhabdomeres remain in abnormal ommatidia (yellow arrows). Third instar eye discs from *eya^{cli}*; *eya^{FGRF}/+* and *eya^{GMRcKO}* were stained with anti-Elav (F,G), anti-Futsch (H,I) and anti-Cut (J,K). Although ELAV and Futsch expression are normal, loss of *eya* results in a strong loss of Cut staining. Scale bars: 100 μ m (adult eyes), 10 μ m (plastic sections) and 50 μ m (eye discs).

Figure 4. *so* function is required in differentiating cells during eye development.

(A-G) *FLP*-mediated *FGRF-FRT* recombination in *cis* was used to eliminate *So* expression in all cells posterior to the MF. Third instar eye discs from *so³/Df(2L)BSC263; so^{FGRF/+}* and *GMR-Flp/+; so³/Df(2L)BSC263; so^{FGRF/+} (so^{GMRcKO})* larvae were stained with anti-*So* (A,B) and anti-*Elav* (A',B') to mark photoreceptor cells posterior to the furrow; merged images are shown in A'' and B''. No overlap of *So* and *Elav* expression is observed in eye discs of *so^{GMRcKO}* animals, suggesting strong loss of *so* function posterior to the MF. The top-right inset shows a fragment of an eye disc from an *so^{GMRcKO}* animal to more clearly show *So* and *Elav* staining results. Blue arrows and yellow dashed lines indicate the approximate position of the MF based on morphology of the disc. (C) A bright field image of a fixed eye disc from an *so^{GMRcKO}* late third instar larva is shown. (D) A cartoon model of an *so^{GMRcKO}* larval eye disc (dorsal view). (F) After recombination, adult eyes show a disorganized morphology with reduced eye size, very few interommatidial bristles and black scars. (G) Plastic sections reveal a severely reduced number of and highly dysmorphic rhabdomeres (yellow arrows) and unusual ommatidial architecture compared with the normal trapezoidal array of rhabdomeres in wild-type animals. (H,I) *Futsch* expression in late third instar larval eye discs. A disc fragment (the top right insert) is shown to improve visualization of *Futsch* expression. (J,K) Eye discs of *so³/Df(2L)BSC263; so^{FGRF/+}* and *so^{GMRcKO}* animals were stained to reveal a severe loss of *Cut* expression in the absence of *so* function. *Cut* expression in the antennal disc is normal in all eye-antenna discs observed. Scale bars: 100 μ m (adult eyes), 10 μ m (plastic sections) and 50 μ m (eye discs).

Figure 5. *eya* and *so* posterior to the MF are required for cell survival.
 (A-C) Pupal eye discs from *Canton S*, *eya^{GMRcKO}* and *so^{GMRcKO}* at 24 hrs after puparium formation (APF) were stained with anti-cleaved Caspase 3 to assess apoptosis. (D-F) Immunohistochemistry (anti-cleaved Caspase 3) of pupal eye discs from flies with *eya* or *so* knockouts posterior to the MF at 48 hrs APF. Scale bar: 20 μ m.

Figure 6. Dac, Ey and dpErk expression are not affected by loss of *eya* or *so* in differentiating cells.

Expression of Dac (A-C), Ey (D-F) and dpErk (G-I) are normal in *Canton S*, *eya^{GMRcKO}* and *so^{GMRcKO}* eye discs. Scale bars: 50 μ m.

Figure 7. Post-mitotic loss of *eya* or *so* function posterior to the MF affects differentiation gradually.

(A-C) Spi expression in late third larval instar eye discs dissected from *Canton S*, *eya^{GMRcKO}* and *so^{GMRcKO}* animals. (D-F) Disc fragments are shown to improve visualization of reduced Spi expression (yellow arrows). (G-I) Expression of Elav in eye discs from *Canton S*, *eya^{GMRcKO}* and *so^{GMRcKO}* animals at the white puparium (WP) stage. Scale bars: 50 μ m.

Figure 8. Sens and Dlg expression at 24 and 48 hrs APF stages.

(A) Expression of Sens and Dlg in *eya^{cli};eya^{FGRF/+}* and *eya^{GMRcKO}* eye discs at 24 hrs APF.

(B) Pupal eye discs from 48 hrs APF were stained with anti-Sens and anti-Dlg to assess differentiation. Scale bar: 20 μ m.

Figure 9. *eya* and *so* are required for IOBs differentiation.

(A,B) SEM of a wild-type eye shows a regular array of ommatidia and bristles. (C,D) SEM of *eya*^{GMRcKO} adult eyes shows fused ommatidia and disorganized bristles. IOBs are disoriented, of variable length, and absent in the ventral regions of adult eyes. A yellow dashed line marks the dorsal/ventral boundary. (E,F) SEM of *so*^{GMRcKO} adult eyes. IOBs are almost completely absent and ommatidial shape is disrupted. Scale bars: 50 μm.