

## Pleiotropy in *Drosophila* organogenesis: Mechanistic insights from Combgap and the retinal determination gene network

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### ABSTRACT

Master regulatory transcription factors cooperate in networks to shepherd cells through organogenesis. In the *Drosophila* eye, a collection of master control proteins known as the retinal determination gene network (RDGN) switches the direction and targets of its output to choreograph developmental transitions, but the molecular partners that enable such regulatory flexibility are not known. We recently showed that two RDGN members, Eyes absent (Eya) and Sine oculis (So), promote exit from the terminal cell cycle known as the second mitotic wave (SMW) to permit differentiation. A search for co-factors identified the ubiquitously expressed Combgap (Cg) as a novel transcriptional partner that impedes cell cycle exit and interferes with Eya-So activity specifically in this context. Here, we argue that Cg acts as a flexible transcriptional platform that contributes to numerous gene expression outcomes by a variety of mechanisms. For example, Cg provides repressive activities that dampen Eya-So output, but not by recruiting Polycomb chromatin-remodeling complexes as it does in other contexts. We propose that master regulators depend on both specifically expressed co-factors that assemble the combinatorial code and broadly expressed partners like Cg that recruit the diverse molecular activities needed to appropriately regulate their target enhancers.

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### Master regulatory function depends on interactions with other transcription factors

Developing animal cells progress through sequences of cellular behaviors to assemble functional adult organs. Cells that take on different fates share identical genomic information and basal gene regulatory machinery, so they specialize their developmental paths by expressing unique subsets of proteins. Transcription factors provide this discrimination by controlling which loci each cell transcribes. One uniquely powerful category of regulators, the master control transcription factors, operates in small networks that continuously adapt their transcriptional interactions to adjust the selection and regulation of downstream genes over time and drive the cellular transitions that generate the target organ [2,13,15,31]. This rewiring strategy is thought to contribute to the astonishing developmental potency of master regulatory networks [13]: it both underlies their ability to drive the sequence of cellular transitions that generate the target organ during normal development and enables their

mis-expression to redirect cells through an inappropriate developmental trajectory. A classic example is that of the retinal determination gene network (RDGN) of *Drosophila*, which orchestrates normal retinal development and can hijack the genetic machinery of cells in the primordial wings, legs, or antennae to generate ectopic eyes [6,10,17,38,44].

Flexible regulation of cellular events by the archetypal RDGN hierarchy illustrates the importance of master control networks rewiring to propel development. Four transcription factors comprise the core network and are expressed in an overlapping sequence that drives retinogenesis. Eyeless (Ey) is first expressed early in the development of the larval eye precursor, the eye-antennal imaginal disc, where it establishes regional identity, promotes tissue growth, and suppresses differentiation [4,18]. Later, it reverses the latter regulation by initiating expression of Eyes absent (Eya) and Sine oculis (So) [18,35,36,39], which form a bipartite transcription factor that activates *dachshund* (*dac*) transcription [10,37,38]. Together, the four

network members reinforce one another's expression and choreograph the first steps of retinal specification and differentiation in a domain known as the morphogenetic furrow (MF) [4,12,16,25,48,55]. Once differentiation begins, Eya-So and Dac switch their effect on *ey* to terminate its expression [2,23], and Eya-So directs subsequent specification and differentiation events [24,26].

Two related mysteries cloud our understanding of the RDGN: what biochemical changes reshape network activities to initiate these developmental transitions, and how does this flexibility contribute to network function during normal and ectopic development? The answers must lie in the way master control transcription factors interact with additional co-factors and how these extra-network interactions influence network relationships to orchestrate appropriate changes in target gene selection and regulation. Consistent with this idea, both negative and positive regulators can tune RD transcriptional output, but taking Eya-So as an example, only a handful of co-factors are known and none have been assigned rigorously to a specific transcriptional event that directs a developmental outcome (Table 1) [1,8,10,14,23,30,32,34,38,45,54]. Even in the

best understood example, where a switch in RD transcriptional output from activating to repressive at the *ey* locus is required to initiate differentiation, neither the composition of the different Eya-So-Dac-containing transcriptional complexes that assemble nor how they produce activating versus repressive outputs is known [2].

### A screen for Eya-binding proteins identifies Combgap as a novel co-factor that limits activating output from Eya-So

In an effort to identify co-factors that modulate RDGN output to effect specific developmental transitions, we performed a yeast two-hybrid screen using Eya as bait. Transcription factors that interfered with Eya-So's ability to promote gene expression were of particular interest, given that repression by this complex is poorly understood. Among the most intriguing hits was the C<sub>2</sub>-H<sub>2</sub> zinc finger transcription factor Combgap (Cg) [14]. Initial characterization of the genetic relationship between *cg* and *eya* revealed antagonism, such that halving *cg* dosage strongly suppressed *eya* loss-of-function phenotypes at multiple

**Table 1.** Known binding partners of *Drosophila* Eya and So.

Eya Binding Partner	Symbol	Yeast two-hybrid	<i>in vitro</i> binding	Co-IP	References
Combgap	Cg	Positive	Positive	Negative	[10]
Dachshund	Dac	Positive	Positive	Negative	[2-4]
Eyeless	Ey	Untested	Untested	Positive	[4]
Eyes absent	Eya	Untested	Untested	Positive	[6,8]
Groucho	Gro	Untested	Positive	Negative	[5,8]
I-κB kinase β	IKKβ	Untested	Untested	Positive	[11]
Optix	Optix	Negative	Untested	Positive	[1,5]
Relish	Rel	Untested	Untested	Positive	[11]
Sine oculis	So	Positive	Positive	Positive	[1,2,4-8]
So Binding Partner	Symbol	Yeast two-hybrid	<i>in vitro</i> binding	Co-IP	References
Eyeless	Ey	Untested	Positive	Untested	[9]
Eyes absent	Eya	Positive	Positive	Positive	[1,2,4-8]
Groucho	Gro	Positive	Untested	Positive	[5,8]
Optix binding protein	Opbp	Positive	Positive	Untested	[5]
Sine oculis binding protein	Sobp	Positive	Positive	Untested	[5]
TBP-associated factor 1	Taf1	Positive	Positive	Untested	[5]

We considered yeast two-hybrid, direct *in vitro* binding assays, and co-immunoprecipitation experiments to be evidence of complex formation. (A) Proteins that bind Eya. (B) Proteins that bind So. <sup>1</sup>Anderson, A. M., Weasner, B. M., Weasner, B. P. and Kumar, J. P. (2012). Dual transcriptional activities of SIX proteins define their roles in normal and ectopic eye development. *Development* 139, 991–1000. <sup>2</sup>Bui, Q. T., Zimmerman, J. E., Liu, H. and Bonini, N. M. (2000). Molecular analysis of *Drosophila* eyes absent mutants reveals features of the conserved Eya domain. *Genetics* 155, 709–20. <sup>3</sup>Chen, R., Amoui, M., Zhang, Z. and Mardon, G. (1997). Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. *Cell* 91, 893–903. <sup>4</sup>Jin, M. and Mardon, G. (2016). Distinct Biochemical Activities of Eyes absent During *Drosophila* Eye Development. *Sci. Rep.* 6, 23228. <sup>5</sup>Kenyon, K. L., Li, D. J., Clouser, C., Tran, S. and Pignoni, F. (2005). Fly SIX-type homeodomain proteins Sine oculis and Optix partner with different cofactors during eye development. *Dev. Dyn.* 234, 497–504. <sup>6</sup>Mutsuddi, M., Chaffee, B., Cassidy, J., Silver, S. J., Tootle, T. L. and Rebay, I. (2005). Using *Drosophila* to decipher how mutations associated with human branchio-oto-renal syndrome and optical defects compromise the protein tyrosine phosphatase and transcriptional functions of eyes absent. *Genetics* 170, 687–95. <sup>7</sup>Pignoni, F., Hu, B., Zavitz, K. H., Xiao, J., Garrity, P. a and Zipursky, S. L. (1997). The eye-specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. *Cell* 91, 881–91. <sup>8</sup>Silver, S. J., Davies, E. L., Doyon, L. and Rebay, I. (2003). Functional Dissection of Eyes absent Reveals New Modes of Regulation within the Retinal Determination Gene Network. *Mol. Cell. Biol.* 23, 5989–5999. <sup>9</sup>Zhang, T., Ranade, S., Cai, C. Q., Clouser, C. and Pignoni, F. (2006). Direct control of neurogenesis by selector factors in the fly eye: regulation of atonal by Ey and So. *Development* 133, 4881–9. <sup>10</sup>Davis, T. L. and Rebay, I. (2017). Antagonistic regulation of the second mitotic wave by Eyes absent-Sine oculis and Combgap coordinates proliferation and specification in the *Drosophila* retina. *Development*. <sup>11</sup>Liu, X., Sano, T., Guan, Y., Nagata, S., Hoffmann, J. A. and Fukuyama, H. (2012). *Drosophila* EYA Regulates the Immune Response against DNA through an Evolutionarily Conserved Threonine Phosphatase Motif. *PLoS One* 7, e42725.

developmental time points. This relationship appeared to limit transcriptional activation by Eya-So, as lowering *cg* levels improved misexpressed *eya*'s ability to initiate ectopic Dac expression, and overexpressing *cg* attenuated Eya-So output in S2 cell-based transcription assays. Coupled with *in vitro* confirmation that Cg can participate in the Eya-So complex, these data established Cg as the first bona fide inhibitor of Eya-So transcriptional function.

Pinpointing a specific developmental transition regulated by Cg-Eya interactions proved more challenging, as *cg* loss did not overtly disrupt eye formation. However, we noticed that deleting *cg* reduced the number of mitotic retinal precursors in a zone immediately posterior to the MF. This swath of proliferation, known as the second mitotic wave (SMW), comprises the final division of unspecified precursors in the retina and ensures that the larva generates sufficient cells with which to assemble an adult eye [3,53]. Although RDGN activity was known to regulate proliferation-differentiation transitions anterior to the MF [4,7,22,33], its role in the SMW had not been studied.

Further experiments revealed that Cg and Eya-So antagonize one another to choreograph the SMW cell cycle and prepare retinal precursors for differentiation. Knocking down *eya* or *so* caused cells that should have exited the SMW to re-enter another S phase, indicating that the Eya-So complex limits unspecified precursors to a single division after the MF. Cg and Eya-So's opposing inputs at the SMW cell cycle suggested that their antagonism calibrates the mitotic rate prior to differentiation. Consistent with this hypothesis, *eya* or *so* heterozygosity restored a nearly normal number of mitoses to *cg* mutant SMWs, while reducing *cg* dosage largely eliminated ectopic cell division when *eya* was knocked down. Based on these data, we proposed that Cg and Eya-So co-regulate transcription at one or more loci in the genetic circuitry surrounding the cellular decision to continue dividing or permanently exit the cell cycle.

### What are Combgap's transcriptional functions?

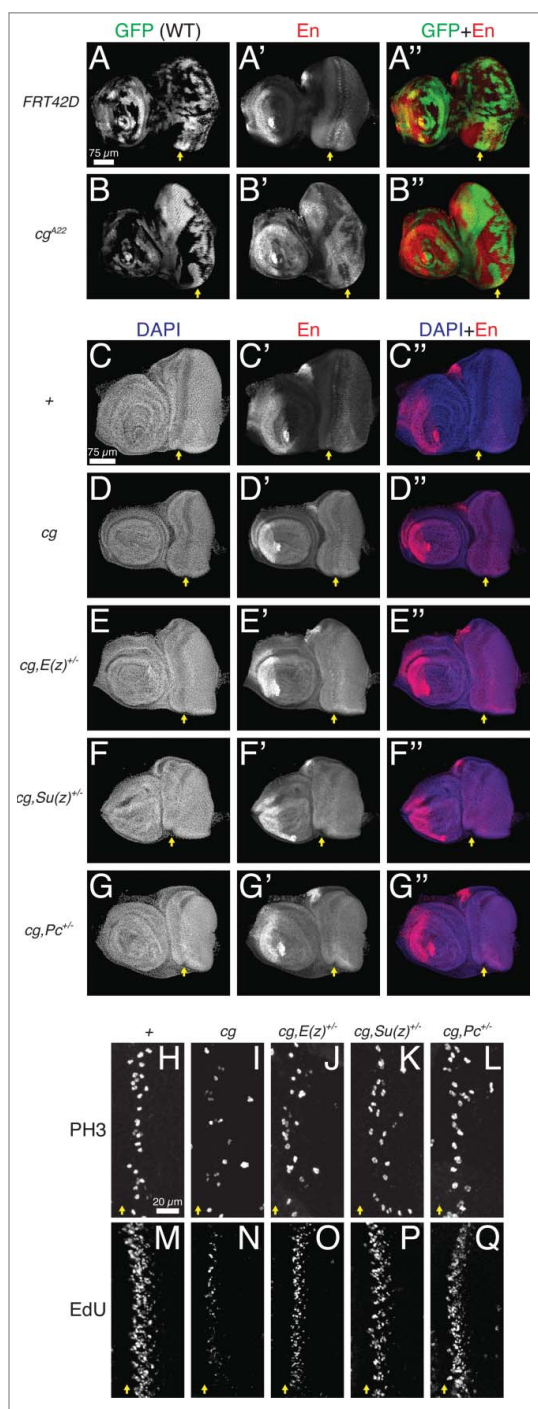
This work not only described novel developmental roles for the Eya-So complex and Cg, but also uncovered a conspicuous paradox. Our prediction had been that Eya-So co-factors that orchestrate specific developmental transitions would be expressed in restricted

patterns consistent with their function. Cg fulfilled the first expectation, in that its retinal requirement and genetic interaction with Eya and So appeared dedicated to a single cell cycle, but defied the accompanying prediction, as it is expressed ubiquitously and at uniform levels throughout the eye imaginal disc [14]. In fact, Cg is present broadly in all imaginal discs and many other tissues in the fly, and has long been known to control myriad developmental processes [9,46,47]. *cg* loss is not lethal until puparium formation [14,40], and amorphic and hypomorphic alleles produce pleiotropic defects throughout the larva and adult, respectively [9,14,21,40,46,47,49-51]. Together, these observations argue that Cg does not provide the spatial information that rewires Eya-So activity to promote cell cycle exit after the SMW.

If Cg does not determine specificity, then what activity might it contribute to the gene regulatory program that controls the transition from proliferation to differentiation? Coupled with its broad expression pattern, Cg's spatially restricted loss-of-function phenotype in the eye suggests that it interprets the combinatorial code, rather than supplementing it, to limit Eya-So output at target genes in cells at the SMW. Thus, while the factors that confer specificity to this regulatory circuit remain unknown, we propose that Cg recruits the repressive activity that switches Eya-So output at the SMW.

Two molecular activities could explain this role. First, work from the Gilboa laboratory determined that Cg both recruits the EcR transcription factor to target enhancers and organizes the three-dimensional configuration of these loci [21], raising the possibility that Cg could limit Eya-So output by preventing enhancer-promoter contacts that favor transcription. Second, the Kassis group showed that Cg recruits and provides DNA-binding specificity to Polycomb Group (PcG) repressive complexes [40], suggesting that Cg could help install repressive chromatin modifications that dampen activation from Eya-So.

To test the latter idea, we asked whether *cg* and PcG components interact synergistically to promote SMW proliferation. We first confirmed that Cg can support epigenetic repression in the developing retina by showing that deleting *cg* de-repressed the classic PcG target Engrailed (En) in this tissue (Fig. 1A,B) [27,28]. Lowering the dosage of PcG components in the *cg* mutant background did not further increase En levels, arguing that all modes of PcG-mediated *en* repression

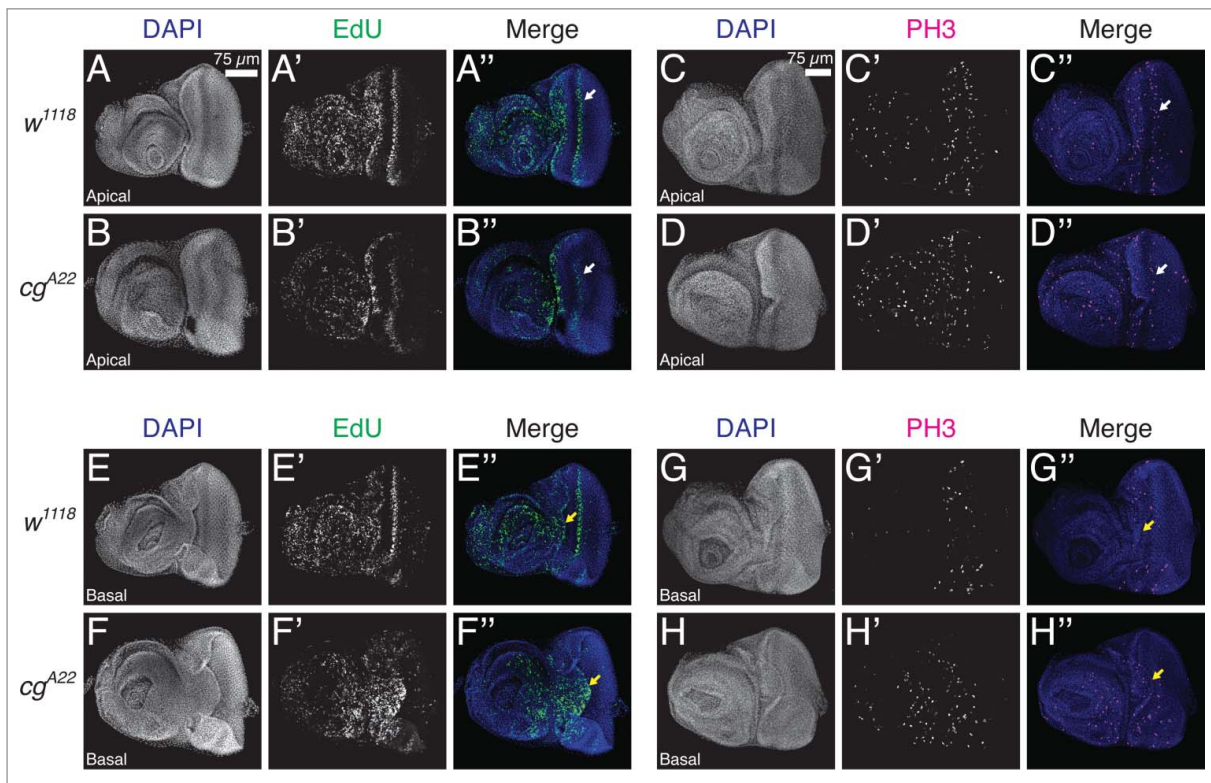


**Figure 1.** Cg may not be dedicated to repressive Polycomb group recruitment in the eye-antennal imaginal disc. All images are maximum confocal projections of late third instar eye-antennal imaginal discs oriented anterior left and dorsal up, with yellow arrows marking the MF. (A-B) Mitotic *cg* clones de-repress En in the antenna and retina. (C-G) Lowering the dosage of PRC1 or PRC2 components does not exacerbate En de-repression in *cg* null discs. (H-Q) Heterozygosity for PRC1 (*Pc*) or PRC2 (*E(z)* and *Su(z)12*) components suppresses the reduced mitosis and S phase entry of *cg* mutants. Each panel is a zoomed view centered on a representative SMW.

require Cg (Fig. 1C-G). In contrast, when we turned to SMW regulation, we found that halving the dosage of genes encoding Polycomb Repressive Complex 1 or 2 proteins strongly suppressed the *cg* phenotype, restoring nearly normal numbers of nuclei in S or M phase (Fig. 1H-Q). This unexpected result suggested that Cg might prevent rather than promote PcG recruitment to target genes used in SMW regulation.

Given that PcG heterozygosity did not modify *eya* loss-of-function phenotypes in the SMW (data not shown), we speculate that the SMW target genes influenced by PcG-Cg interactions are not those regulated by Eya-So, and that at Eya-So targets, Cg repressive influence occurs via an alternate mechanism, perhaps by influencing 3D chromatin interactions. Genes that would prevent re-entry into S phase after the SMW, such as *Rbf*, are prime candidates for shared Cg-Eya-So regulation [14]. One model is that Cg organizes these loci into repressive configurations during the SMW, but that as cells exit this cell cycle, Eya-So directly binds Cg, disrupts Cg's interactions with proteins or DNA binding sequences that enforce the repressive 3D architecture, and installs enhancer-promoter contacts that lead to transcriptional activation. More detailed mechanistic exploration of the relationship between Cg-Eya binding, specific chromatin arrangements, and transcriptional output will elucidate how Cg and Eya-So schedule cell cycle phases during the SMW.

Considering Cg's ability to control either the chromatin environment or three-dimensional conformation of its targets in light of the idea that it selectively deploys these activities in response to the combinatorial code may explain the long-observed context specificity of its effects on gene expression. For example, Cg promotes or inhibits *Cubitus interruptus* expression in the two compartments of the larval wing imaginal disc [9,47] and potentiates or dampens expression of Dpp signaling targets in separate regions of the larval brain [46]. While these gene expression outcomes have not yet been assigned to direct transcriptional regulation, one model is that Cg recruits PcG complexes to loci where it represses transcription and promotes favorable chromatin conformations at genes that it activates. Cg also associates with at least one ubiquitously expressed protein complex that communicates directly with the basal transcriptional machinery [52], hinting that it may possess additional means



**Figure 2.** *cg* has opposite effects on the cell cycle in different regions of the eye-antennal imaginal disc. All panels are confocal projections of four optical sections centered on the apical or basal domains of late third instar eye-antennal imaginal discs. Anterior is to the left and dorsal is up. (A-D) *cg* promotes S and M phase entry at the SMW (white arrows). (E-H) *cg* limits proliferation in the presumptive head cuticle (yellow arrows).

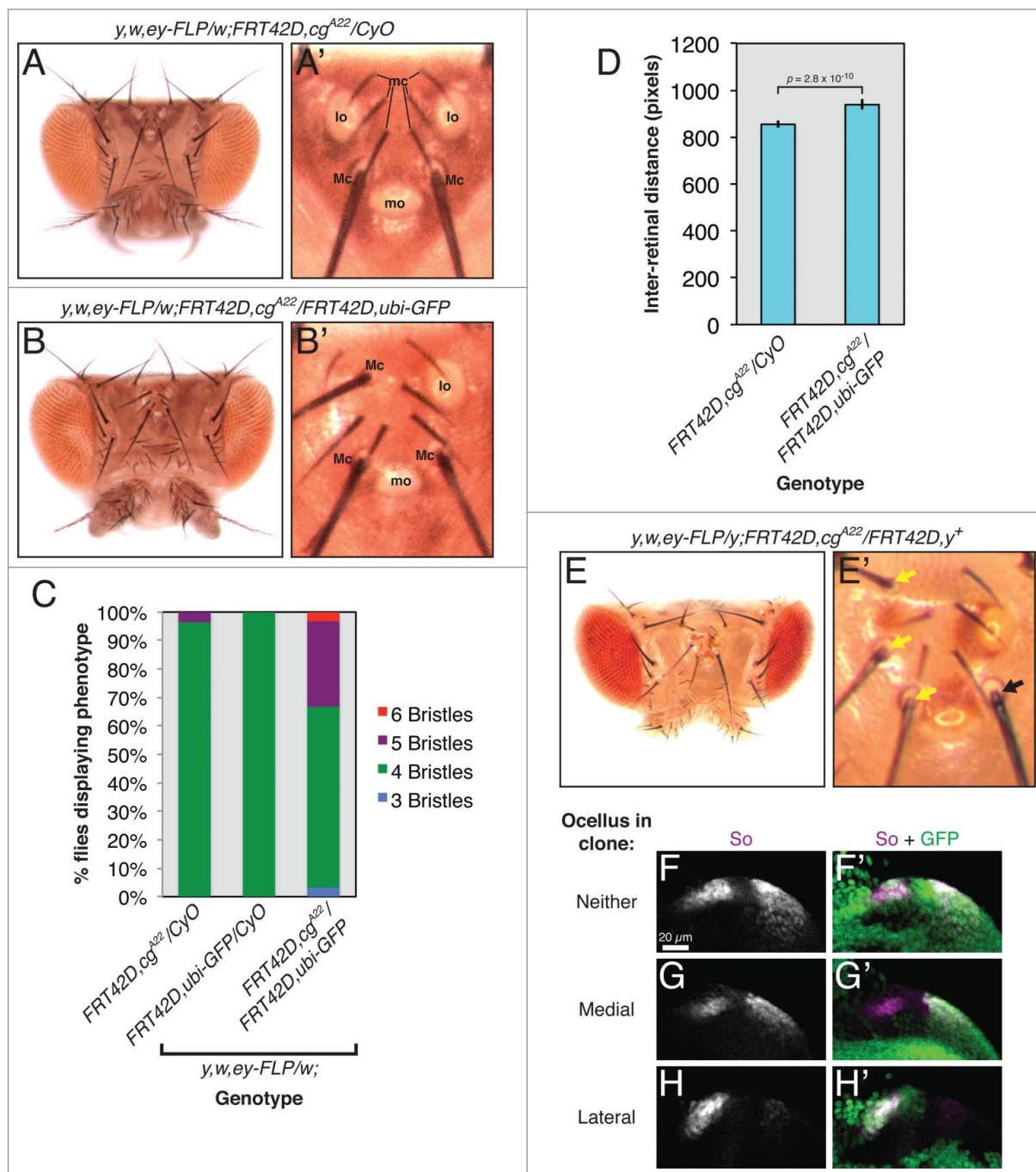
of controlling gene expression. More broadly, we speculate that *Cg* interfaces with numerous transcription factors, uniquely interprets the combinatorial code at shared target enhancers, and recruits biochemical activities according to the developmental requirements of each cell.

### Combgap's regulation of development is highly context-dependent

As we examined *cg* loss-of-function animals in more detail, we uncovered additional phenotypic complexities that support this model. First, we noted strong regionalization of the null phenotype, such that *cg* oppositely regulates the same cellular processes in different portions of larval tissue. In the most striking example, *cg* both promotes and inhibits proliferation in the eye-antennal imaginal disc. *cg* is required for the SMW (Fig. 2A-D) [14], but has no effect on proliferation in other portions of the developing retina. By contrast, removing *cg* in the adjacent presumptive head cuticle induced hyperplasia, such that this normally small patch of tissue extended basally under the eye (Fig. 2E-

H) [14]. Over-proliferation likely caused this defect, as cells in this ectopic flap synthesize DNA and undergo mitosis at a higher rate than in the wild type (Fig. 2E-H). While the transcriptional targets mediating *Cg*'s regulation of the cell cycle are not yet known, the published observation that *Cg* oppositely controls expression of putative targets in the larval wing and brain [9,46,47] hint that similar switching of transcriptional output, perhaps based on tissue-specific expression of transcriptional binding partners like *Eya*, could underlie *Cg*'s functions in the eye-antennal disc. Alternatively, *Cg* may co-occupy one set of cell cycle loci with *Eya*-So at the SMW, but engage a different collection of targets and transcription factors in the head cuticle.

We also discovered regional specificity in *Cg*'s requirement for fate specification in the ocelli. Normal flies generate one medial and one lateral ocellar field in each of their eye-antennal imaginal discs, separated by presumptive interocellar cuticle [41,42]. During pupal stages, the medial fields from each imaginal disc fuse to produce a single mature ocellus, while the lateral fields form two separate ocelli, in a tissue known as the head vertex (Fig. 3A) [19,29]. During our investigation of *cg*'s



**Figure 3.** *cg* controls fate specification in the head vertex. Dorsal views of adult heads are oriented posterior up. For larval tissues, all images are zoomed views of the developing head vertex in late third instar eye imaginal discs, oriented anterior to the left and dorsal up. (A) The wild type arrangement of structures in the adult head vertex (zoomed view in A'). mc: microchaetae; lo: lateral ocellus; Mc: macrochaetae; mo: medial ocellus. (B) Lateral ocelli are lost and ectopic macrochaetae form in flies carrying *cg* clones. Note three macrochaetae, one protruding from between the microchaetae, in the zoomed view in B'. (C) The number of head vertex macrochaetae in control flies or those carrying *cg* clones.  $n > 28$  for each genotype. (D) The inter-retinal distance increases in flies carrying *cg* clones. (E) Ectopic macrochaetae arise from *cg* null tissue marked by yellow (*y*). Zoomed view in E'. Yellow arrows mark *y* macrochaetae. The black arrow marks a wild type *y<sup>+</sup>* bristle. (F) *So* expression marks the two ocellar fields in a wild type head vertex. Wild type cells are marked with GFP. (G) *So* expression is subtly reduced in the presumptive medial ocellus compared to the lateral field in *cg* null clones (marked by the absence of GFP). (H) *So* expression is lost in *cg* mutant lateral ocellar fields.

role in the retina, we noticed that animals carrying *cg* null mitotic clones frequently lost lateral ocelli, produced macrochaetae in their place, and had larger vertexes (Fig. 3A-D). We never observed wild type coloration of ectopic bristles when clones were marked by the *yellow* mutation (Fig. 3E), confirming that ectopic macrochaetae developed cell-autonomously from *cg* null tissue. To determine when during development *cg* controls lateral ocellar fate, we examined the levels of *So*, which marks both developing ocelli and is required for their specification [5,11,20,43,55]. Deleting *cg* ablated *So* expression in the larval lateral ocellar fields but did not affect *So* levels in the medial fields (Fig. 3F-H), confirming that *cg* is required only for lateral ocellar specification. In keeping with the theme of context specificity, *cg* biases the decision between ocellar and bristle fate in only one portion of the head vertex.

### Master regulators interface with complex transcriptional machinery

Returning to our goal of understanding how the RDGN integrates with the transcriptional machinery of the cell, *Cg*'s participation in the *Eya-So* complex presages complexity in the biochemical basis of master regulatory function. *Cg* can initiate repressive or activating chromatin states, controls numerous developmental events across the fly, and appears to tune its regulation of individual cellular processes according to context, implying unusual flexibility of function for such a broadly expressed transcription factor. Consequently, adding *Cg* to the repertoire of proteins that bind *Eya-So* hints that this complex may rely on interactions with multifunctional, pervasive adaptors to communicate with the basal transcriptional and epigenetic machinery. Moving forward, the field's challenge is to assign changes in the compositions of RD transcriptional complexes to the network rewiring that initiates transitions between cellular behaviors. We expect that identifying both specifically expressed co-regulators, which comprise the combinatorial code that interprets enhancer sequences, and ubiquitous platforms for recruiting activating or repressive biochemical activities, such as *Cg*, will be essential to assembling a complete model of master regulatory biology.

### Disclosure of potential conflicts of interest

Authors declare no conflict of interest and no competing financial interests.

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