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Position Dependent Responses to Discontinuities in the Retinal Determination Network

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Summary

The development of any cell and/or tissue is dependent upon interconnections between several signaling pathways and myriad transcription factors. It is becoming more apparent that these inputs are best studied, not as individual components, but rather as elements of a gene regulatory network. Over the last decade several networks governing the specification of single cells, individual organs and entire stages of development have been described. The current incarnations of these networks are the products of the continual addition of newly discovered genetic, molecular and biochemical interactions. However, as currently envisaged, network diagrams may not sufficiently describe the spatial and temporal dynamics that underlie developmental processes. We have conducted a developmental analysis of a sub circuit of the Drosophila retinal determination network. This sub circuit is comprised of three genes, two (*sine oculis* and *dachshund*) of which code for DNA binding proteins and one (*eyes absent*) that encodes a transcriptional co-activator. We demonstrate here that the nature of the regulatory relationships that exist between these three genes changes as retinal development progresses. We also demonstrate that the response of the tissue to the loss of any of these three RD genes is dependent upon the position of the mutant cells within the eye field. Depending upon its location, mutant tissue will either overproliferate itself or will signal to surrounding cells instructing them to propagate and compensate for the eventual loss through apoptosis of the mutant clone. Taken together these results suggest that the complexities of development are best appreciated when spatial and temporal information is incorporated when describing gene regulatory networks.

Keywords

retinal determination; sine oculis; eyes absent; dachshund; groucho; Notch signaling; Drosophila; compensatory proliferation; gene regulatory networks; GRN

Introduction

The last decade has played witness to the revelation that the specification of tissues and organs, are regulated, not by simple linear cascades, rather by complicated interconnected gene regulatory networks (GRNs). The influence of such networks can be limited to a single context or can extend to multiple developing tissues. Such is the case for the retinal determination (RD) network, which, in addition to the eye, regulates the fate of a number of

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tissues in both insect and vertebrate systems. First identified in flies, this network also controls the development of learning and memory centers of the brain, several mesodermal derivatives, the gonads and select cells within the central nervous system (Bai and Montell, 2002; Bonini et al., 1998; Callaerts et al., 2001; Chang et al., 2003; Fabrizio et al., 2003; Kammermeier et al., 2001; Kurusu et al., 2000; Mardon et al., 1994; Niimi et al., 1999; Noveen et al., 2000). In addition to its role in vertebrate eye development, the RD network regulates ear, nose, kidney and muscle specification (Brodbeck and Englert, 2004; Gong et al., 2007; Hammond et al., 1998; Hanson, 2001; Heanue et al., 1999; Kalatzis et al., 1998; Laclef et al., 2003; Relaix and Buckingham, 1999; Simpson and Price, 2002; Xu et al., 2003). Over the years members of seven gene families have been identified to function within the RD network. In *Drosophila* these include the Pax6 genes *eyeless (ey)* and *twin of eyeless (toy)*, the Pax6(5a) genes *eyegone (eyg)* and *twin of eyegone (toe)*, the Six family members *sine oculis (so)* and *optix*, the founding member of the Eya family of transcriptional co-activators *eyes absent (eya)*, a distant relative of the Ski/Sno family of proto-oncogenes *dachshund (dac)*, the Meis1 homolog *homothorax (hth)* and the zinc finger transcription factor *teashirt (tsh)* (reviewed in (Kumar and Moses, 2001b; Treisman, 1999; Treisman and Heberlein, 1998; Weasner et al., 2004).

The evidence that prompted the placement of these genes into a functional network is principally drawn from loss-of-function mutant phenotypes (Bonini et al., 1993; Cheyette et al., 1994; Jang et al., 2003; Mardon et al., 1994; Quiring et al., 1994; Serikaku and O'Tousa, 1994), overlapping expression patterns (Bessa et al., 2002), direct transcriptional activation of one gene by another (Czerny et al., 1999; Niimi et al., 1999; Ostrin et al., 2006; Pauli et al., 2005), protein-protein interactions amongst selected network members (Chen et al., 1997; Pignoni et al., 1997) and the unique ability of these genes to induce ectopic eyes in non-retinal tissues (Bonini et al., 1997; Czerny et al., 1999; Halder et al., 1995; Pan and Rubin, 1998; Seimiya and Gehring, 2000; Shen and Mardon, 1997; Weasner et al., 2007). As additional experimental evidence is gathered, new positive or inhibitory arrows are added resulting in a network with ever increasing complexity. Similar GRNs with equal or greater complexity have been identified in a number of systems including the fly wing and ventral furrow (Aracena et al., 2006; Guss et al., 2001); mouse stem cell, B lymphocyte and brain (Li et al., 2007; Medina et al., 2004; Wang et al., 2007; Zhou et al., 2007); *Xenopus* mesoendoderm (Loose and Patient, 2004); vertebrate neural crest (Sauka-Spengler and Bronner-Fraser, 2008a; Sauka-Spengler and Bronner-Fraser, 2008b; Sauka-Spengler et al., 2007); *Arabidopsis* flower development (Espinosa-Soto et al., 2004) and sea urchin embryogenesis (Davidson et al., 2002; Oliveri et al., 2002; Oliveri and Davidson, 2004a; Oliveri and Davidson, 2004b) just to name a few. However, as is the case with any complex system, no single regulatory model can fully describe all of the spatial and temporal events that occur during develo pment (Flores et al., 2000) to produce the final adult tissue.

Eye specification in *Drosophila* begins during embryogenesis when a small group of cells are set aside to give rise to the future compound eye (Cohen, 1993). Upon emerging as a larva, these cells become organized into a monolayer epithelium called the eye-antennal imaginal disc. During the first two larval instars the eye disc undergoes massive proliferation to generate the large numbers of cells that are required to produce the approximately 800 unit eyes or ommatidia that comprise the adult compound eye. At the start of the third and final instar, pattern formation is initiated at the posterior margin of the epithelium. The wave of morphogenesis can be visualized by a dorso-ventral groove in the epithelium referred to as the morphogenetic furrow (Ready et al., 1976). As the furrow passes, the pool of undifferentiated cells are organized into periodic clusters of developing ommatidia (Ready et al., 1976; Wolff and Ready, 1991). Within each cluster are approximately twenty cells that adopt either photoreceptor or non-neuronal accessory cell fates (Cagan and Ready, 1989; Tomlinson and Ready, 1987a; Tomlinson and Ready,

1987b). These decisions involve complex, stereotyped patterns of gene expression (Dickson and Hafen, 1993; Doroquez and Rebay, 2006; Flores et al., 2000; Kumar and Moses, 1997; Nagaraj and Banerjee, 2007; Voas and Rebay, 2004). Ultimately, the several hundred ommatidia are organized into a precise hexagonal array characteristic of the adult retina.

In the developing fly retina *ey* is one of the first RD genes to be expressed. Along with *toy, ey* directly activates the transcription of several downstream targets including itself and three other network genes: *so, optix* and *eya* (Halder et al., 1998; Niimi et al., 1999; Ostrin et al., 2006). So and Eya proteins form a composite transcription factor with So contributing a DNA binding domain and Eya providing an activation domain (Pignoni et al., 1997). The So-Eya complex, in turn, activates a number of target genes that play crucial roles in cell proliferation (*string*, (Jemc and Rebay, 2007), pattern formation (*hedgehog*, (Pauli et al., 2005) and cell fate specification (*lozenge*, (Yan et al., 2003). Additionally, So-Eya feeds back to regulate the transcription of the upstream gene *ey* (Pauli et al., 2005) and the downstream target *dac* (Pappu et al., 2005). It is this last interaction that is the central focus of this report, as it highlights an instance in which the totality of experimental evidence is not represented by the most current network models.

Consistent with their roles as obligate partners, So and Eya proteins are distributed in completely overlapping expression patterns in the developing eye. Both are expressed in a swathe of undifferentiated cells ahead of the advancing morphogenetic furrow and in all cells posterior to the furrow (Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Dac protein distribution ahead of the furrow overlaps that of So and Eya. However, posterior to the furrow *dac* expression is maintained for approximately eight rows where it is restricted to only a subset of photoreceptors and then quickly tapers off (Mardon et al., 1994). Two enhancers responsible for the activation of *dac* expression in the retina are under the partial control of both *so* and *eya* (Pappu et al., 2005). As the So-Eya complex is still present and functioning in the more posterior cells it is intriguing that *dac* expression ceases. The seminal experiments that established the regulatory relationships among the RD genes were based in large measure on immunohistochemical assays completed in entirely mutant eye discs in which a furrow failed to initiate (Anderson et al., 2006; Chen et al., 1997; Halder et al., 1998; Pappu et al., 2005) and in ectopic eye assays in which the distribution of RD proteins were measured in response forced expression of either individual or combinations of genes (Bonini et al., 1997; Chen et al., 1999; Czerny et al., 1999; Halder et al., 1995; Shen and Mardon, 1997; Weasner et al., 2007). These experiments have been critical to our understanding of the regulatory interactions that take place during nascent phases of eye development and within the anterior compartment of the developing retina. Several regulatory relationships, first established genetically, have been supported by evidence of protein-protein interactions and direct transcriptional regulatory relationships (Chen et al., 1999; Czerny et al., 1999; Michaut et al., 2003; Niimi et al., 1999; Ostrin et al., 2006; Pauli et al., 2005; Pignoni et al., 1997).

A distinct disadvantage to this historical approach is that interactions taking place along the margins, at the D/V and A/P boundaries, and in cells posterior to the furrow cannot be assessed and thus have largely been neglected. This is particularly true of *so, eya* and *dac*, which are the only three RD genes to be expressed posterior the furrow (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Serikaku and O'Tousa, 1994). All three genes are required for furrow initiation and the So-Eya complex is required in the R1, R6 and R7 photoreceptors (Mardon et al., 1994; Pignoni et al., 1997). However it is unclear if the regulatory relationships existing among the three genes in anterior regions of the eye also exist along the posterior regions where pattern formation initiates and in differentiating photoreceptor neurons. In order to verify existing interactions or identify new regulatory relationships among *so, eya* and *dac*, we generated randomly distributed retinal mosaic

clones for each gene and determined the effect that loss of each gene had on the expression of the other two factors.

Here we show that the response of the eye to discontinuities in the retinal determination network is not static across the eye field but rather is dynamic and position dependent. In particular we demonstrate that, unlike regions anterior the furrow, removal of *so* and *eya* in posterior positions of the eye lead to an attempt by these cells to reinitiate the retinal determination program by expressing RD genes that are normally found exclusively in the anterior compartment. This attempt fails and is then followed by cell suicide via programmed cell death but not before the *so* and *eya* mutant cells non-autonomously signal through the Notch pathway to adjacent undifferentiated cells instructing them to compensate for their loss by activating *dac* expression and proliferating. These surrounding cells, which are not competent to properly execute the RD program neither adopt a retinal fate nor die, therefore they assume a default head cuticle fate. We also demonstrate that the loss of either *so* or *eya* at the margins of the eye epithelium results in a different developmental path. In these cases, the mutant cells themselves will autonomously overproliferate thereby bypassing any requirement for communication with adjacent cell populations. Consistent with this, the adjacent undifferentiated cells do not activate Notch signaling, express *dac* or proliferate. The conclusion that we draw from these observations is that the gene regulatory networks governing early specification and patterning decisions are not static sets of connections but rather are temporally and spatially dynamic.

Materials and Methods

Fly Stocks

The following stocks were used to generate retinal mosaic clones: *w; FRT40A dac^{E462}* (gift from Graeme Mardon), *w; FRT42D so^{3}* (gift from Francesca Pignoni), *w; FRT42D eya^{2}*, and *w;; FRT82B gro^{E48}* (gift from Janice Fischer) with the following FRT lines: *w; FRT40A Ubi-GFP*, *w; FRT 40A Pw^{+}*, *w; FRT42D Ubi-GFP*, *w; FRT42D Pw^{+}*, *w;; FRT82B Ubi-GFP RpS3* and *yweyflp*. The following stocks were used for generalized and flpout over-expression assays; *ey-G4*, *GMR-G4*, *ywhsflp^{22}*, *act5C>yellow>GAL4*, in conjunction with *UAS-so/CyO*, *UAS-gro* (gift from Albert Courey), *UAS-dac* (gift from Graeme Mardon), *UAS-hid* (gift from Andreas Bergmann), and *UAS-N^{ICD}* (gift from Sujin Bao). Unless noted otherwise, the above stocks are available from the Bloomington Drosophila Stock Center.

Reagents

The following primary antibodies were used in this study: mouse anti-Dac (1:5), mouse anti-Eya (1:5), rat anti-Elav (1:10), mouse anti-2B10 (1:100), mouse anti-Delta (1:10), and mouse anti-Notch^{ICD} (1:4) all of which are available from the Developmental Studies Hybridoma Bank. The guinea pig anti-So (1:200), rabbit anti-Hth (1:500) and rabbit anti-Tsh (1:3000) antibodies are kind gifts from Ilaria Rebay, Richard Mann and Steve Cohen, respectively. The rabbit anti-Cleaved Caspase-3 (1:100) antibody is from Cell Signaling Technology and Phalloidin-Cy5 (1:1000) is from Molecular probes. The following secondary antibodies from Jackson Laboratories were used in this study at 1:100 dilutions: goat anti-mouse TRITC, donkey anti-mouse TRITC, goat anti-rat TRITC, donkey anti-rat TRITC, donkey anti-rat CY5, goat anti-rabbit TRITC, goat anti-guinea pig TRITC.

Microscopy

Imaginal discs were dissected in phosphate buffer, fixed in 4% paraformaldehyde, washed in wash buffer (0.2% Triton), and then incubated in primary antibody overnight. Secondary antibody incubations lasted 2–3 hours after which tissues were further dissected in wash buffer then mounted on slides in Vectashield (Vector Laboratories). Tissues were examined

using a Zeiss Axioplan2 with Apotome and imaged using a Zeiss Axiocam MRm camera. Adult flies with clones were either imaged live using a Nikon SMZ1500 microscope equipped for fluorescence, frozen at −80°C for 20 minutes then imaged using Zeiss Discovery scope with color camera, or prepared for SEM by drying through a series of ethanol dilutions and HMDS treatments.

Results

Dac is Repressed by So-Eya in the Posteror Eye

Based on the expression profiles of the eye specification genes, the developing eye can be divided into five zones (Fig. 1A, (Bessa et al., 2002; Bonini et al., 1993; Cheyette et al., 1994; Czerny et al., 1999; Mardon et al., 1994; Pan and Rubin, 1998; Quiring et al., 1994; Seimiya and Gehring, 2000; Serikaku and O'Tousa, 1994). The cells that constitute zone 1 reside at the most anterior regions of the eye and express the two eye specification genes, *tsh* and *hth*, in addition to *cut*, which antagonizes the retinal determination pathway. Zone 2 is located adjacent to zone 1 and extends to the morphogenetic furrow. Every eye specification gene, with the exception of *hth*, is expressed within this region. Zone 3 lies posterior to the furrow and essentially is defined by the expression pattern of *dac*. Additionally, *so* and *eya*, are expressed in these cells. Zone 4 begins where *dac* expressions ceases and extends to the posterior edge of the eye field. Finally, the posterior-lateral margins of the eye field constitute zone 5. Two eye specific enhancers within *dac* are under the partial control of the *so* and *eya* (Fig. 1B; Ostrin et al., 2006), however, *dac* expression is activated in only a subset of cells that contain the So-Eya transcription factor (Fig. 1C–E; (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Serikaku and O'Tousa, 1994). We generated retinal mosaic clones mutant for either *so, eya* or *dac* with the intent of understanding the complexity of their regulatory relationship throughout the five defined expression zones of the developing eye.

A prediction of the current RD network model is that removal of either *so* or *eya* should result in the cessation of *dac* expression. As expected, in regions of the eye where Dac protein is normally distributed (zones 2 and 3) removal of either *so* and *eya* leads to a halt in *dac* expression (Fig. 2A,B, blue arrow). This is consistent with earlier reports on whole mutant discs in which *dac* expression is drastically reduced but not eliminated from so^1 and eya^2 mutants (Anderson et al., 2006; Pappu et al., 2005). However, removal of either gene within zone 4 has the effect of non-autonomously activating dac expression in cells surrounding the mutant patch (Fig. 2A,B, purple arrow). This is surprising as our *a priori* expectation was that the loss of either *so* or *eya* in non-*dac* expressing cells would have no appreciable effect on Dac transcription. Our results indicate that, contrary to such expectations, *so* and *eya* function early to first activate *dac* expression but then later reverse course to suppress it (see model in Fig. 8C). We also find that cells along the margin react differently to the loss of So-Eya activity than the nearby cells that populate the interior of the retina. Mutant clones at the margins (zone 5) are unable to induce *dac* expression in neighboring cells (Fig. 2A,B, pink arrow).

As *dac* expression is under the control of both *so* and *eya* (Anderson et al., 2006; Pappu et al., 2005), we set out to determine if the de-repression of *dac* in zone 4 is primarily due to either *so*, *eya*, or both genes equally. We generated loss of function clones mutant for either *so* or *eya* and examined the distribution pattern of the other protein. We observe that loss of *eya* always results in the elimination of *so* expression, irrespective of geographical location within the eye field (Fig. 2C, blue, purple arrows). In contrast, there is only a reciprocal requirement of *so* for *eya* expression within zone 2 and zone 5 (Fig. 2D, blue arrow). It should be noted that when large clones span the furrow, the cells respond as zone 2 cells. In so mutant cells located strictly posterior to the furrow (zones 3 and 4), Eya protein levels

appear to be unaffected (Fig. 2D, purple arrow). Thus it appears that in zone 4 the ectopic activation of *dac* results primarily from the loss of *so*.

dac has been shown to be regulated by *dpp* prior to the initiation of the morphogenetic furrow (Curtiss and Mlodzik, 2000). Loss of either *dpp* or *dac* at the posterior margin (zone 5) inhibits the initiation of the morphogenetic furrow (Chanut and Heberlein, 1997; Mardon et al., 1994). In addition, loss of either *so* or *eya* in zone 5 also leads to an arrest in pattern formation (Pignoni and Zipursky, 1997). We sought to determine if *so, eya* and *dac* function at the same level or if a feedback step from *dac* to *so* and *eya* exists during furrow initiation. We generated *dac* mutant clones and observed that both *so* and *eya* are absent in a narrow strip of cells with the mutant patches that contact zone 5 (Figs, 2E,F, pink arrow) indicating that at least at the point of initiation *dac* does, in fact, feedback and regulate the expression of both *so* and *eya*. Interestingly, both So and Eya proteins are still present within the center of the mutant patches suggesting that these cells may still retain their anterior fate (Fig. 2E,F, white arrow). We also observed that removal of *dac* in cells completely within the eye disc proper appears to have no detrimental effect on the expression of either *so* or *eya* (Fig. 2E,F, blue, purple arrows). From these results we draw the conclusion that the regulatory relationships that exist among the *so, eya* and *dac* genes is dynamic across the developing eye field. We also propose that the differential requirement for *dac* in furrow initiation and progression (Mardon et al., 1994) may be the result of *dac* regulating *so* and *eya* at the margin but not within the eye field.

So-Eya Mutant Cells Reinitiate the RD program

As the So-Eya complex regulates *dac* expression we turned our attention to zone 4 where the loss of either gene unexpectedly leads to the non-autonomous de-repression of *dac* in cells bordering the mutant patch (Fig. 3A). We stained clones with antibodies against a number of molecular markers in an attempt to determine the molecular identity of the dac expressing cells bordering the clones and the mutant cells themselves. The cells experiencing the non-autonomous de-repression of *dac*, express neither the neuron specific protein ELAV nor the cone cell marker Cut (Fig B,C). These cells are also unlikely to be pigment cells, as this cellular fate is not assigned until the pupal stage (Cagan and Ready, 1989; Wolff and Ready, 1993). Therefore, these cells most probably belong to the undifferentiated pool of cells that are present within the developing eye disc at this stage. Consistent with earlier reports on the role that *so* and *eya* play in cell fate decisions (Pignoni et al., 1997), cells mutant for so fail to express neuronal and cone cell specific markers (Fig 3B,C). We wanted to determine the developmental paths that these two cell populations follow.

In order to determine the developmental path of so mutant cells and neighboring dac expressing cells, we used antibodies against several proteins that are expressed in zones 1 and 2 (Fig. 1B). Our analysis of Tsh, Hth and Cut distribution is that *so* and *eya* mutant cells express each of these genes while the surrounding cells do not (Fig. 3D–F). It should be noted here that the Cut protein that is shown within Fig. 3C is in cells that are not within the cone cell layer. Rather, the cells are at a more basal position, consistent with the position of the *cut* positive cells located ahead of the furrow and within the antenna in zone 1. As Tsh is normally present in zone 2 and both Hth and Cut are distributed in zone 1, it appears that cells mutant for either RD gene adopt an anterior fate that approximates but does not exactly recreate the molecular environment ahead of the furrow. Interestingly, while Dac, So and Eya are present within the undifferentiated cells surrounding the clones (Fig. 3A, data not shown) they do not express other anterior genes. We conclude that the two cell populations are molecularly distinct with the mutant cells adopting a hybrid zone 1–2 identity and the surrounding cells assuming a fate that is most closely associated with zone 3. What is the ultimate fate of these two cell populations in the eye?

So-Eya Mutant Cells Commit Suicide

We noticed that *so* clones residing in zone 4 are typically smaller than those that are located in more anterior zones. Since recombination is induced early in eye development, the difference in clone size, several days later, could be attributed to programmed cell death. We stained *so* and *eya* mutant discs with an antibody that recognizes activated cleaved Caspase-3 (Cas-3), a marker for cells that are undergoing apoptosis. We observe that cells mutant for *so* are, in fact, undergoing cell death (Fig. 4A). This was an unexpected finding as we often observe cuticular outgrowths emanating from the adult compound eye (Fig. 4B). Our initial assumption was that these outgrowths were descended from the *so* and *eya* mutant clones. However, the elevated levels of Cas-3 in the clone coupled with its absence in the surrounding cells suggests that the outgrowths are derived from another pool of cells. We attempted to determine which cell population gives rise to the cuticular outgrowths. Normally, when clones are generated and assayed in the adult retina, the mutant tissue is identified by the absence of red pigment, which results from the loss of a mini-white construct during recombination. However, since the cells of interest are comprised of head cuticle and not retinal tissue, eye pigmentation is not a useful marker. Instead we made use of a GFP reporter that is under the control of the ubiquitously activated Ubi-63E promoter, thus, as in the eye disc, the presence of GFP can be used to mark wild type tissue in the adult (compare Fig. 4C–F). The cuticular outgrowths in adult flies express GFP indicating that they are wild type. As the undifferentiated, Dac expressing cells surrounding *so* clones are the only cells in the tissue that have not yet adopted a retinal fate, we believe that it is this population that gives rise to the cuticular outgrowths (Fig. 4G,H). This is consistent with the elevated levels of Cas-3 in mutant clones and also consistent with the fact that the *dac* positive cells are as yet undifferentiated. We next sought to determine whether decisions to proliferate and express *dac* are a response to transmitted signals from the mutant tissue to the surrounding cell population.

So-Eya Mutant Tissue Instruct Surrounding Cells Via Delta-Notch

Our expectation is that the two populations of cells do, indeed, communicate with each other. As the Notch signaling pathway plays roles in both eye specification (Kumar and Moses, 2001a; Kurata et al., 2000) and cell proliferation (Baonza and Freeman, 2005; Chao et al., 2004; Dominguez et al., 2004; Ferres-Marco et al., 2006; Reynolds-Kenneally and Mlodzik, 2005; Singh et al., 2006) we stained discs containing either *so* mutant clones with antibodies against the intracellular domain of the Notch receptor (N^{ICD}) and the ligand, Delta (Dl). We observe an up-regulation of Dl within the mutant tissue (Fig. 5A) and activated Notch in the surrounding, undifferentiated *dac* positive cells (Fig. 5B). These results suggest that cells mutant for the So-Eya complex, prior to their ultimate demise, signal to the surrounding undifferentiated cells via Notch signaling. This signaling has the effect of inducing compensatory proliferation and dac expression in surrounding cells. As Notch signaling has already been shown to induce cell proliferation when ectopically expressed (Reynolds-Kenneally and Mlodzik, 2005) we set out to determine if ectopic Notch signaling posterior to the furrow was sufficient, on its own to induce *dac* expression. We used the "flp-out" technique to over-express N^{ICD} in clones within zone 4. We observe the expression of N^{ICD} was indeed sufficient to induce *dac* expression both autonomous and non-autonomously (Fig. 5C). We then looked to see if the compensatory proliferation that we observe is mediated by *dac* or if it is a distinct effect of Notch signaling. Expression of *dac* either in flp-out clones or using a GMR-GAL4 driver in zones 3 and 4 failed to induce cell proliferation (data not shown). We conclude from these results that the Delta-Notch signal from cells mutant for either *so* or *eya* is sufficient to induce both dac expression and cell proliferation but that these two outputs are separable and thus distinct from each other.

Groucho Represses dac Expression in the Retina

Our results suggest that *dac* expression is repressed in more posterior regions (zone 4) of the retina by a complex containing So and an unidentified transcriptional co-repressor. One candidate, *groucho* (*gro*), is a member of the TLE family of transcriptional repressors (Fisher and Caudy, 1998; Parkhurst, 1998; Paroush et al., 1994; Stifani et al., 1992). Gro functions within the Enhancer of split complex (Delidakis et al., 1991; Ziemer et al., 1988) whose products are downstream components of the Notch signaling cascade (reviewed in (Artavanis-Tsakonas and Simpson, 1991; Campos-Ortega, 1993; Campos-Ortega and Jan, 1991). The main role for *gro* in the eye appears to be in the specification of photoreceptor cell fates although its loss can also be accompanied by extensive overproliferation of imaginal disc tissue (Chanut et al., 2000; Fischer-Vize et al., 1992). Additionally, So and other members of the SIX family form composite transcriptional repressors with Gro and its vertebrate homologs (Kenyon et al., 2005; Kobayashi et al., 2001; Lopez-Rios et al., 2003; Silver et al., 2003; Zhu et al., 2002). We generated mutant clones of *gro* and observed that *dac* expression was ectopically activated in zone 4 cells surrounding the clone as well as in some, but not all, cells within the clone (Fig. 5D arrowhead, arrow). Therefore it is possible that Gro functions both in a cell autonomous and a cell non-autonomous manner in zone 4 to repress *dac* transcriptional activation in both photoreceptor neurons and undifferentiated cells (Fig. 7B).

To test whether *gro* is sufficient to repress *dac* transcription we used a GMR-GAL4 driver (Hay et al., 1994) to over-express it in all cells behind the furrow. Our expectation was that if Gro is able to repress *dac* transcription, we should see Dac protein levels drop immediately behind the furrow zone 3. As expected, we see a reduction within the photoreceptors that normally express dac in this region (Fig. 5E, bracket). However, more strikingly, we observe ectopic Dac expression in all undifferentiated of cells behind the furrow (Fig. 5E Note the absence of ELAV stain in Dac positive nuclei).These data suggest that Gro overexpression influences Dac expression differently in photoreceptors vs. undifferentiated cells. Interestingly, over-expression of *so* using GMR-GAL4 results in an increase in *dac* expression predominantly in a different cell population; namely the developing photoreceptor neurons and accessory cone cells (Fig. 5F Note that nuclei ectopically expressing Dac also are ELAV positive). These results suggest that the So-Eya and So-Gro complexes play critical and complex roles in regulating dac expression (Fig. 7A,B).

Distinct Responses to RD Gene Loss at the Eye Field Margin

It had been shown previously that loss of particular RD genes along the margins of the eye field not only resulted in a block in pattern formation but also induced cell proliferation (Mardon et al., 1994). We set out to determine if the mechanisms underlying cell proliferation that accompanies RD gene loss along the margin (zone 5) are similar to or distinct from those governing internal regions (zone 4). Similar to the loss of *dac*, removal of *so* and/or *eya* along the margins leads to a block in the initiation of pattern formation (Fig. 2A,B; (Pignoni et al., 1997) and to an increase in cell proliferation (Fig. 6A). It appears that the mutant cells are themselves proliferating as the cuticular outgrowths that we see along the margins of the adult eye are derived from the mutant tissue as these cells do not express the GFP reporter that we use for marking wild type cells (Fig. 6D,E; compare arrow to arrowhead). This result suggests that, unlike zone 4, mutant cells along the margin (zone 5) proliferate autonomously. Consequently, we do not see upregulation of either Dl within the clone or Notch in adjacent cells or Cas-3 within the clone (data not shown). It should be noted here that the posterior-lateral margin of the retinal field appears to be the only location within the eye disc in which *dac* feeds back and positively regulates that expression of both *so* and *eya* (Fig. 2E,F; 7C).

Discussion

Here we have demonstrated that the so-eya-dac sub circuit of the retinal determination gene regulatory network is dynamically organized across the developing eye field. First, along the margins of the field (Fig. 1B, zone 5), where pattern formation is initiated, positive feedback loops link all three genes to each other (Fig. 7C, right panel). The So-Eya composite transcription factor is thought to partially regulate *dac* expression through two eye specific enhancers that contain putative So binding sites suggesting that the regulation may be direct (Pappu et al., 2005). Dac has been shown recently to also contact DNA (Kim et al., 2002): however, a consensus binding site has not been identified so it is unclear if the regulation of *so* and *eya* by *dac* is direct or goes though other RD gene intermediates such as ey. As the furrow sweeps across the epithelium the once vast pool of undifferentiated cells are canalized towards a retinal fate: cells in the most anterior regions are the least committed while cells in progressively posterior regions are funneled along a path of terminal differentiation. In regions straddling the advancing morphogenetic furrow (Fig. 1B, zones 2 and 3), where competent, undifferentiated cells are transformed into periodic clusters of photoreceptors, the character of the so-eya-dac sub circuit is altered and becomes unidirectional with So-Eya maintaining its regulation of dac but not visa versa (Fig. 7C, middle panel). In the most posterior portions of the eye (Fig. 1B, zone 4), where retinal cell fate differentiation is complete, the So-Eya complex appears to no longer activate *dac* transcription. Instead, a complex most likely composed of So and the Gro transcriptional co-repressor (So-Gro) represses *dac* (Fig. 7C, middle). Our interpretation of the results presented here is that static gene regulatory network maps, in many instances, may not accurately represent the shifting alliances amongst genes during development. It is more likely the case that relationships amongst genes will differ depending upon temporal and/or spatial context.

We also describe a second phenomenon in which cells that suffer from interruptions in the RD gene regulatory network appear to actively attempt several corrections. First, mutant cells will attempt to restart the retinal determination program by activating all of the RD genes that are normally expressed in more anterior segments of the eye primordium. As this attempt proves futile, the mutant cells, in a second attempt to correct this deficiency, will then communicate to adjacent undifferentiated cells via Delta-Notch signaling instructing them to both proliferate and attempt an initiation of retinal development. Coupled to this effort is a decision to commit cell suicide via programmed cell death, which clears the defective cells from the developing retina (Fig. 7A). We interpret these findings to suggest that programmed cell death may not, as is often implied, be the first option for cells that are genetically or molecularly compromised. However, it appears that cells mutant for components of gene regulatory networks go to considerable lengths to self-correct prior to their eventual decision to eliminate themselves from the developing tissue. While our analysis is limited to mutations within three different RD network genes, we suggest that this mechanism may not be limited but be a rather common response in other developmental contexts.

And finally our results suggest that within a single cell different transcriptional complexes, involving one common component, in this case a DNA binding protein, and at least two different co-factors, one a transcriptional co-activator and one a transcriptional co-repressor, can both regulate the transcription of a target gene. We have demonstrated that So, Eya and Gro are co-expressed in developing photoreceptor cells. However, in newly differentiated cells just adjacent to the morphogenetic furrow the So-Eya complex activates *dac* transcription. As these cells mature, a So-Gro repression complex shuts down expression of *dac* despite the continued presence of the Eya co-activator. We have also shown that hyper-expression of either *so* or *gro* affects dac expression in very different cell types although

both genes were expressed uniformly. It is likely that So-Eya and So-Gro exist as independent complexes and not as a larger super complex as it has been possible to biochemically isolate just the individual heterodimers (Jemc and Rebay, 2007; Silver et al., 2003). How spatial and temporal information is integrated into the promoter to differentially recruit these differing activation and repression complexes is unclear but is also likely to be important for shaping gene regulatory networks.

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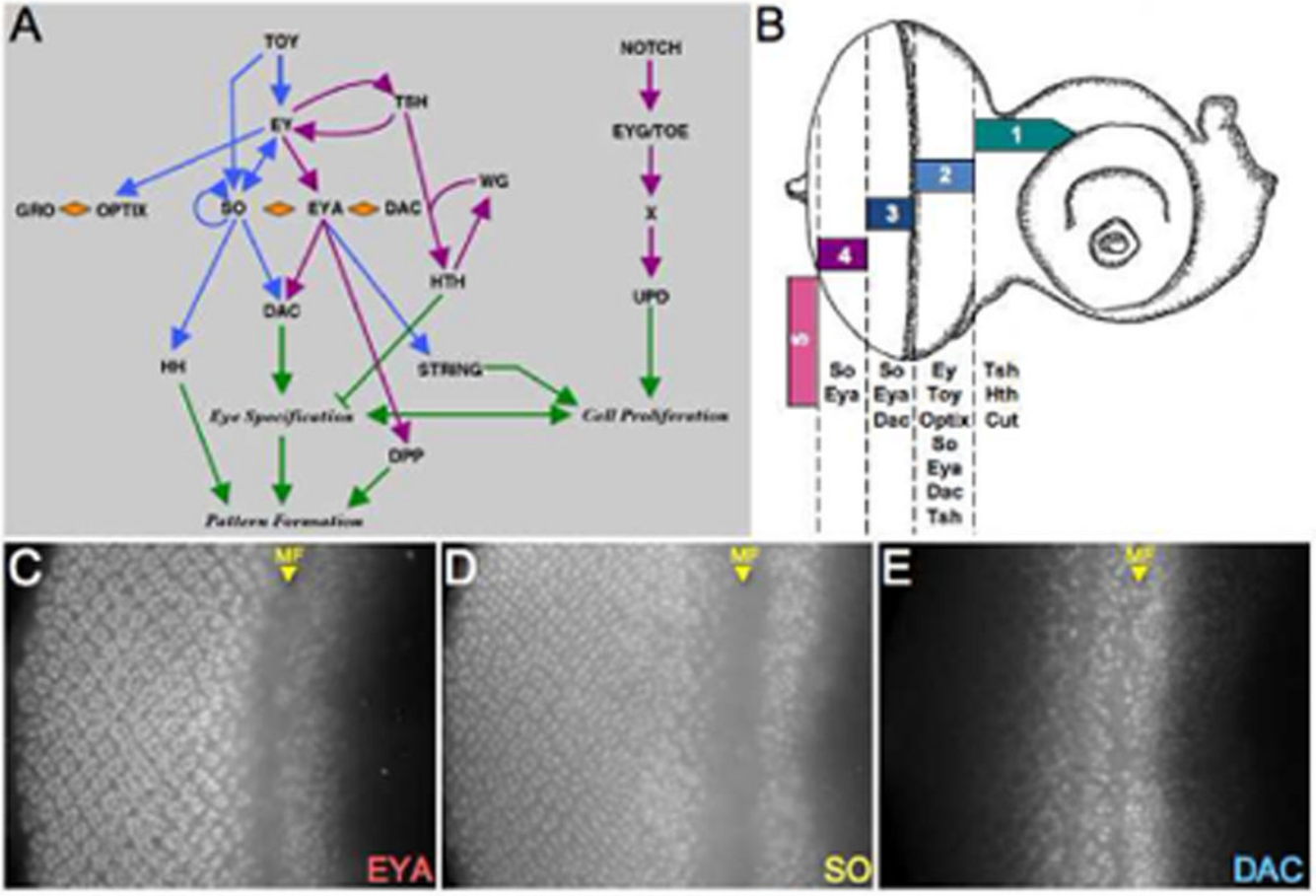

Figure 1. Spatial and Temporal Expression of the RD Network in the Fly Eye

(A) Schematic diagram of the retinal determination network. (B) Schematic drawing of a third instar eye-antennal imaginal disc. The expression zones are modeled after the data presented in Bessa et al., 2002. (C–E) Immunofluorescence images of wild type eye discs. Immunostained proteins are at listed at the bottom right of each panel. MF = morphogenetic furrow. Anterior is to the right.

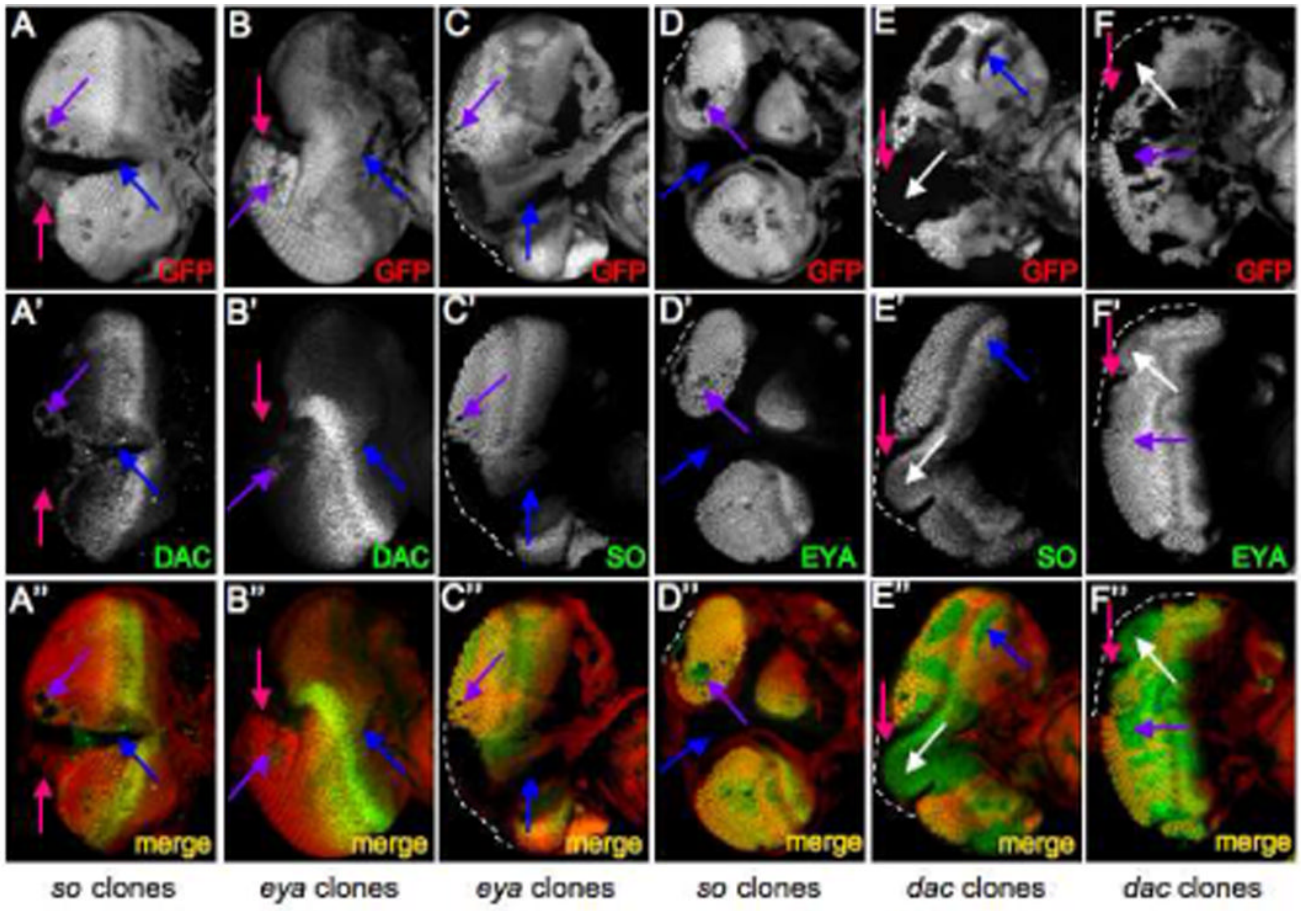

Figure 2. Position Dependency of the So-Eya-Dac Regulatory Relationship

(A–F) Immunofluorescence images of eye discs containing either so^3, eya^2 or dac^4 mutant clones. Immunostained proteins are at listed at the bottom right of each panel. GFP is labeled red and RD proteins are labeled green. Genotypes are listed at the bottom of each column. Anterior is to the right.

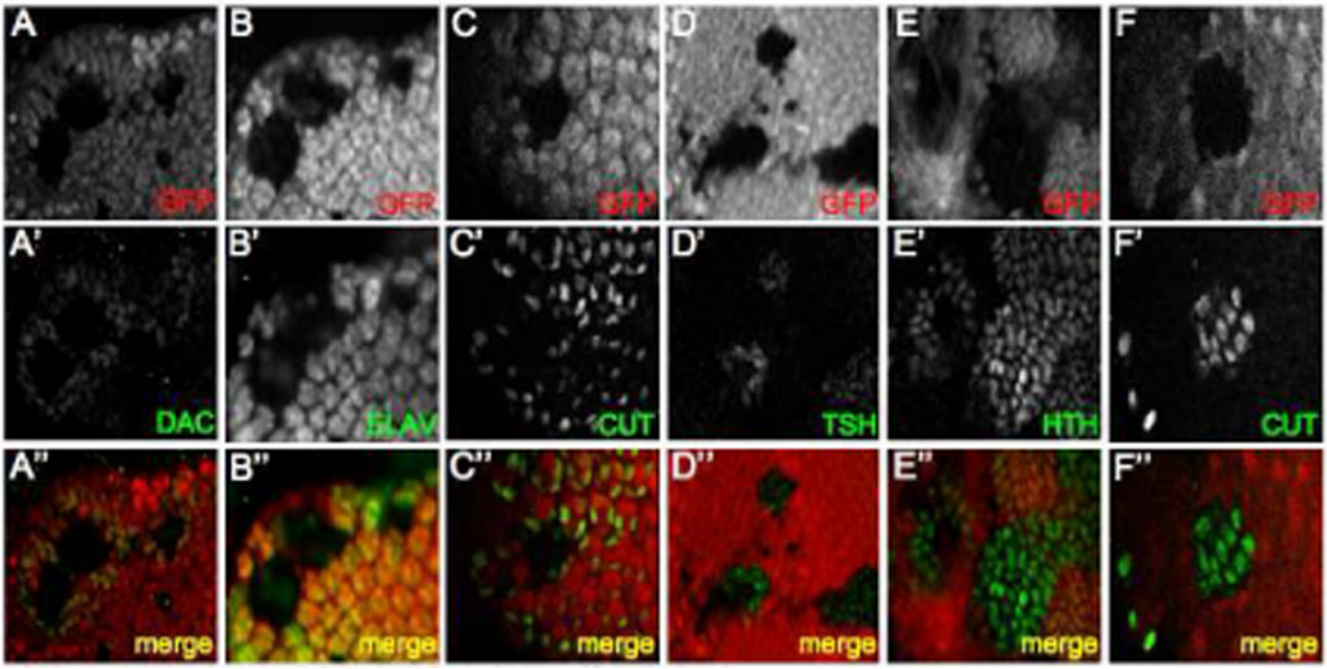

Figure 3. Molecular Signature of so Mutant Retinal Clones

(A–F) Immunofluorescence images of *so*^{3} mutant clones residing within zone 4. Immunostained proteins are at listed at the bottom right of each panel. GFP is labeled red and indicated RD proteins are labeled green. Anterior is to the right.

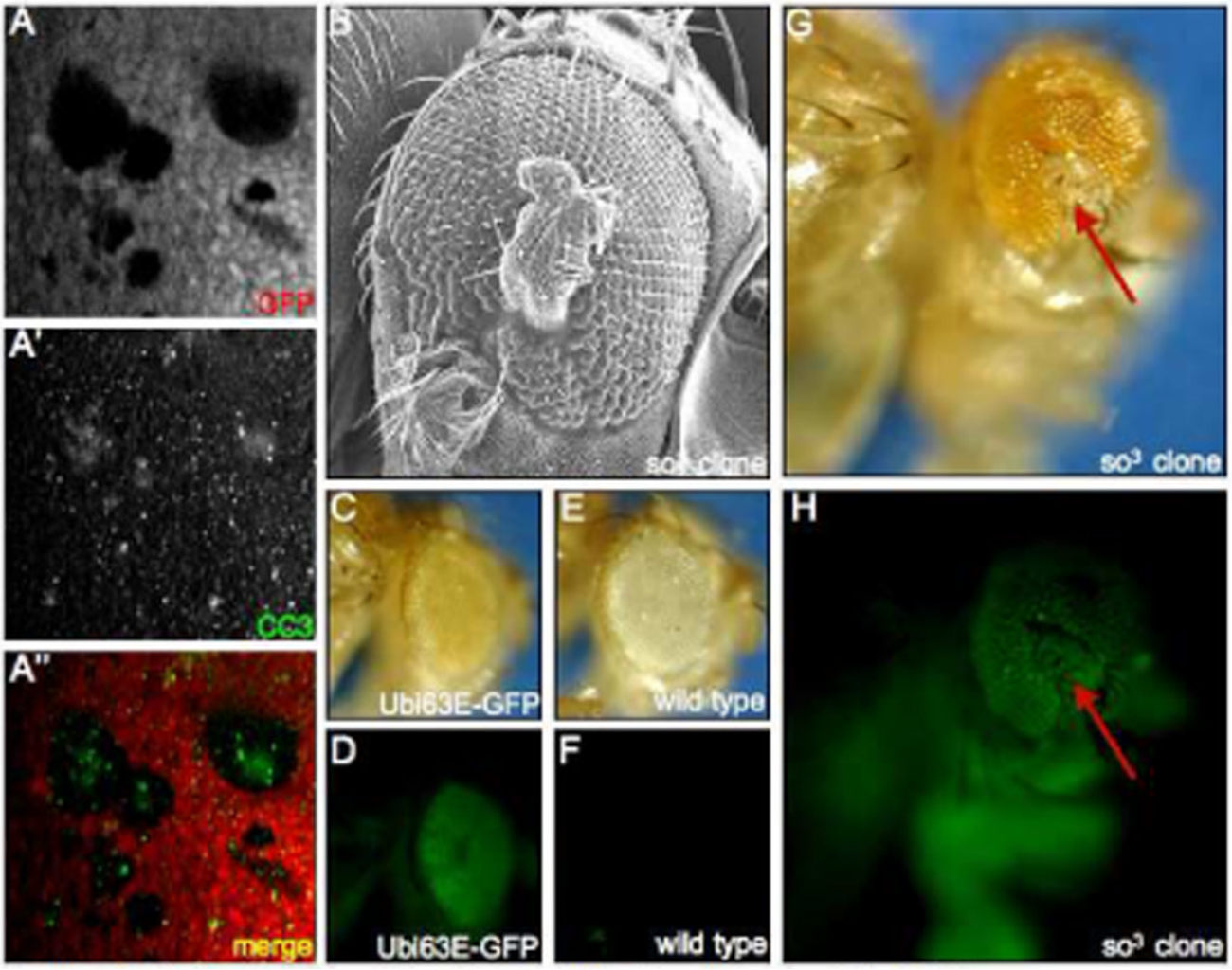

Figure 4. Fate of so and eya Mutant Tissue in the Developing and Adult Retina

(A) Immunofluorescence images of a so^3 mutant clone. Immunostained cleaved Caspase-3 is labeled green and GFP is labeled red. (B). Scanning electron micrograph of a cuticular outgrowth emanating from the compound eye. (C,D) Light and fluorescent images of an adult fly harboring a GFP reporter under the control of the Ubi63E promoter. Note that GFP is expressed in both the retina and the surrounding cuticle. (E,F) Light and fluorescent images of a fly lacking the Ubi63E-GFP transgene. Note that GFP expression is not observed. (G, H) Light and fluorescent images of an adult fly harboring *so*^{3} mutant clones. Red arrows point to cuticular outgrowth from the eye. Note the presence of GFP in the cuticular outgrowth. Genotypes are listed at the bottom right of each panel. Anterior is to the right.

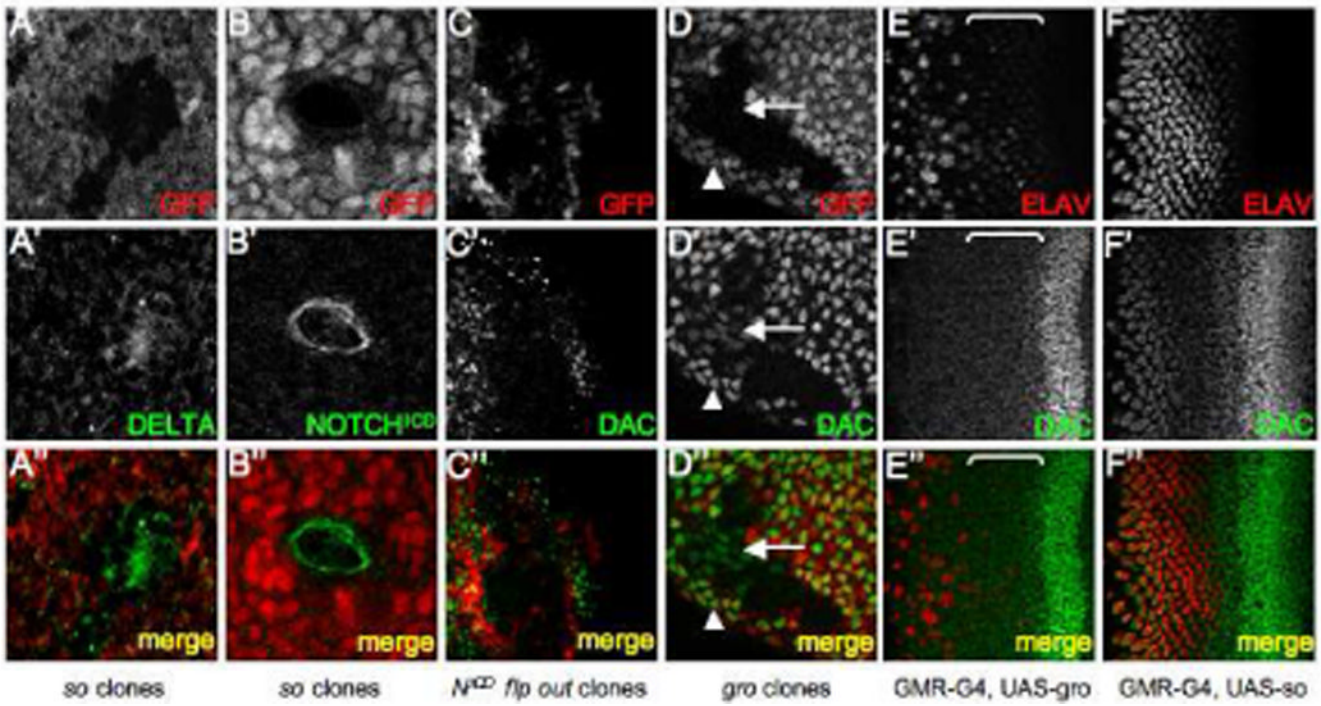

Figure 5. so Mutant Tissue Signals to Surrounding Cells via the Notch Pathway

(A–D) Immunofluorescence images of eye discs containing so^3 mutant, N^{ICD} flp-out, or *gro* mutant clones. All clones are positioned in zone 4. GFP is labeled in red. Immunostained proteins are in green. (E–F) Immunofluorescence images of eye discs in which GRO or SO is expressed behind the morphogenetic furrow via a GMR-GAL4 driver. ELAV is labeled red and DAC is green. The brackets in panels E indicate zone 3. Genotypes are listed at the bottom of each column. Anterior is to the right.

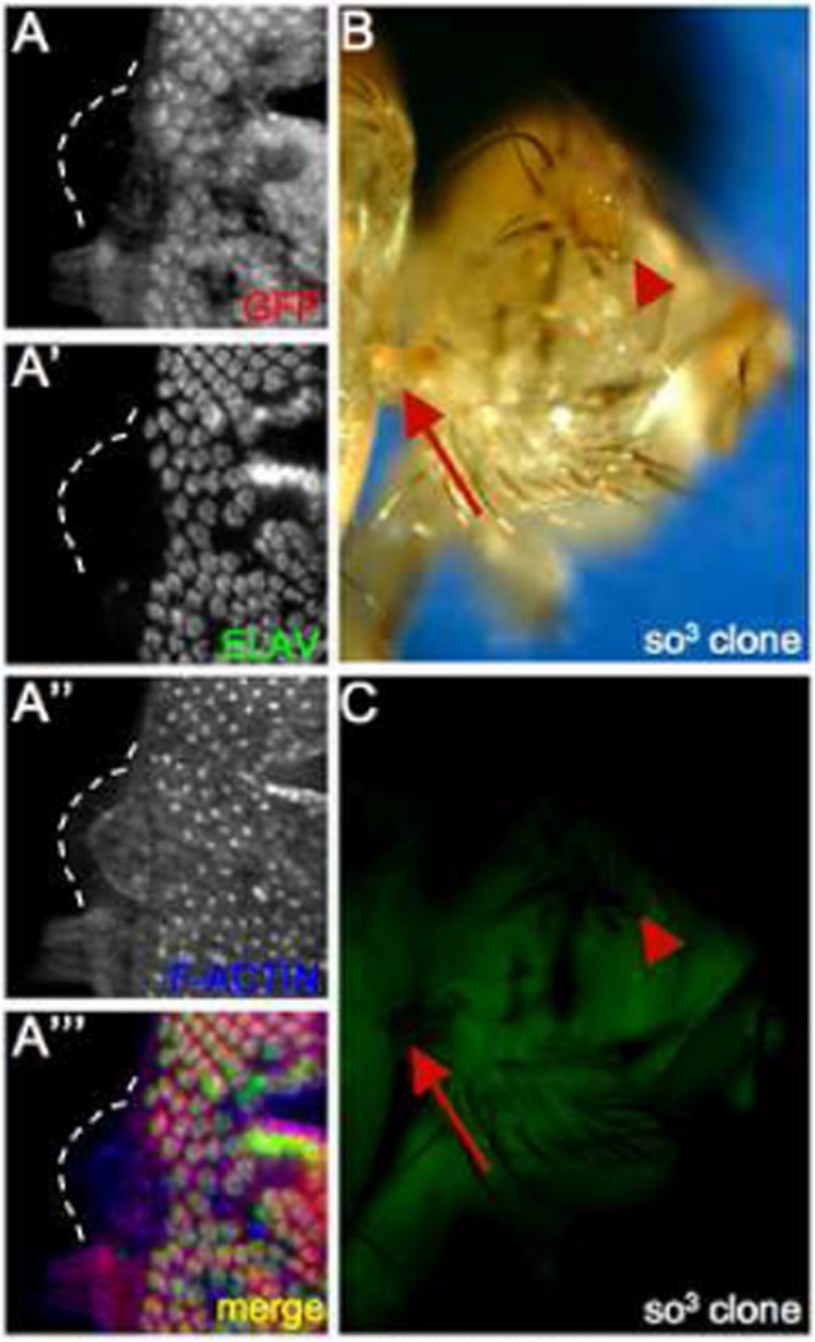

Figure 6. Autonomous growth in *so* clones on the margin

(A) Immunofluorescence images of a so^3 mutant clone. GFP is labeled red, immunostained ELAV is labeled green and F-actin is labeled blue. (B, C) Light and fluorescent images of an adult fly with so^3 mutant clones. Red arrows point to cuticular outgrowth from the head, red arrowheads point to the eye. Note the absence of GFP in the cuticular outgrowth. Genotypes are listed at the bottom right of each panel. Anterior is to the right.

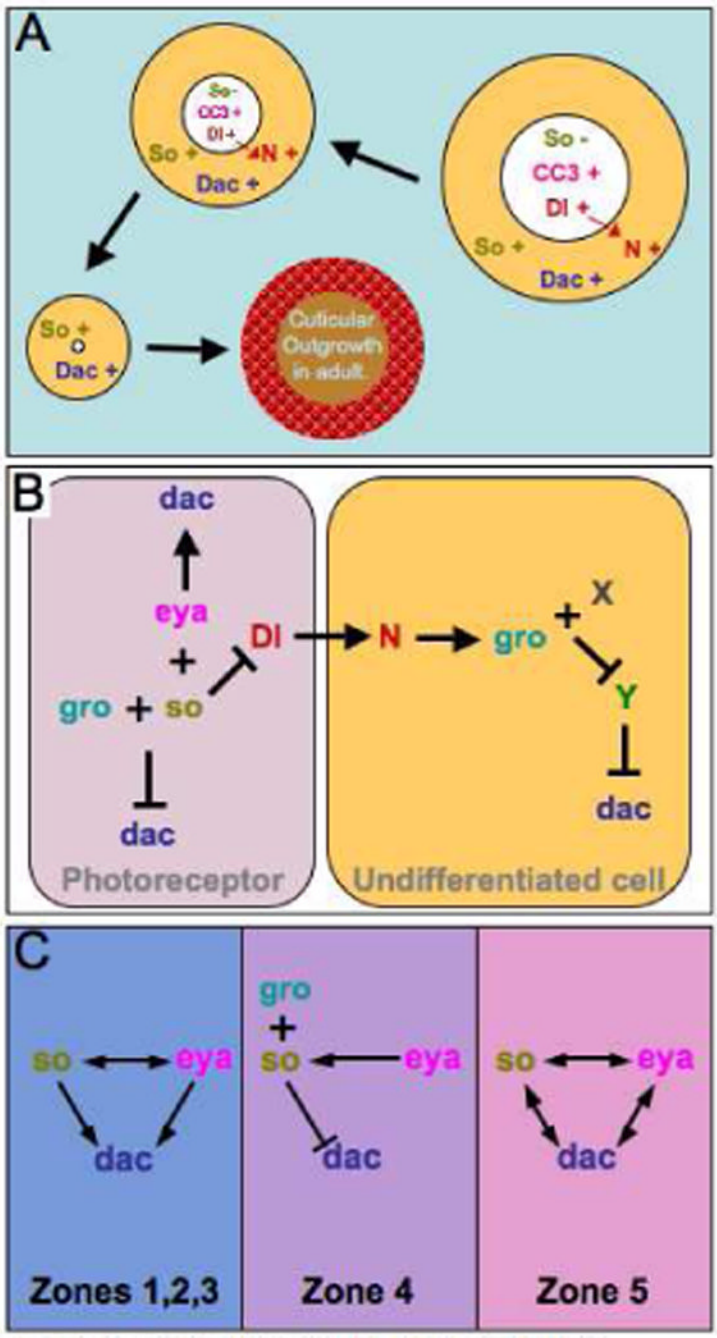

Figure 7. Models for So-Eya-Dac Regulation

(A). Schematic of the events observed in *so* or *eya* clones located in zone 4 of the developing eye disc. Note that the mutant tissue in the disc undergoes programmed cell death and is eliminated by the adult stage and is denoted by the shrinking size of the clone. The cuticular outgrowth in the adult is derived from the *dac* positive undifferentiated cells that surround the mutant clones in the eye disc. (B) A schematic depicting a potential mechanism for the repression of *dac* in photoreceptor neurons and undifferentiated cells in zone 4 of the retina. (C) A series of models describing the genetic interactions that we observe between so, eya and dac in the developing retina. Note that the relationships change depending upon the spatial orientation within the eye field.