Early Retinal Development in *Drosophila*

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Like other insects, the fruit fly *Drosophila melanogaster* has a compound eye, made up of approximately 800 individual eyes or ommatidia. The eye arises from the eye imaginal disc, which is part of the compound eye-antennal disc. The cells that will form this disc invaginate from the ectoderm during late embryogenesis (Jurgens and Hartenstein, 1993); the disc primordium then grows inside the larva, where it consists of an epithelial bilayer. One columnar layer will give rise to all the cell types of the retina; it is covered by a squamous epithelial sheet called the peripodial membrane, which later contributes to the surface of the head (Haynie and Bryant, 1986). Differentiation of the retina begins in the third of the three larval stages, known as instars, and continues during the first few days of pupal development (Ready et al., 1976). In spite of the many morphological and developmental differences between fly and vertebrate eyes, many of the molecules involved appear to be conserved.

Specification of the eye primordium

Six genes have been implicated in the specification of the eye field because they are necessary for the formation of the eye and/or sufficient to cause ectopic eye development in other imaginal discs. These genes all encode nuclear proteins that are likely to function in a transcriptional regulatory hierarchy. The first of them to be expressed, in a broad domain in the anterior of the embryo, is *twin of eyeless* (*toy*), a homologue of the paired box/homeobox gene *Pax-6* (Halder et al., 1998). Its expression is then refined to the eye-antennal anlagen, where it is coexpressed with another *Pax-6* homologue, *eyeless* (*ey*) (Halder et al., 1998; Quiring et al., 1994). As its name suggests, mutations in *ey* result in the absence of the eye, due to a failure in photoreceptor differentiation in the eye disc (Halder et al., 1998). Both *ey* and *toy* have a striking ability to induce the formation of eye tissue when either is misexpressed in other imaginal discs (Halder et al., 1995; Czerny, pers. comm.). In this case *toy* activates the ectopic expression of *ey* but the reverse is not true, suggesting that *toy* functions upstream of *ey* (Czerny, pers. comm.); however, the phenotype of mutations in *toy* is not known. The placement of these genes very early in the process of eye development is intriguing in light of the phenotypes of mouse (*Small eye*) and human (*Aniridia*) mutations in *Pax-6*, both of which cause defects in eye development (Hill et al., 1991; Ton et al., 1991). *Pax-6* expression has subsequently been found in photoreceptive organs in a variety of evolutionarily

distant and developmentally diverse species (Glardon et al., 1997; Glardon et al., 1998; Loosli et al., 1996; Tomarev et al., 1997), suggesting that it was associated with visual or at least anterior sensory structures in a common ancestor.

ey is required for the expression in the second instar eye disc of *sine oculis* (*so*) (Halder et al., 1998), which encodes a homeodomain protein (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), and *eyes absent* (*eya*)(Bonini et al., 1997; Halder et al., 1998), which encodes a novel nuclear protein (Bonini et al., 1993). Again, mutations in either *so* or *eya* result in the complete absence of the eyes (Bonini et al., 1993; Cheyette et al., 1994). However, these genes are less efficient in inducing ectopic eye development than *ey*; ectopic eyes are only observed at a high frequency when *so* and *eya* are expressed in combination, and they are predominantly found in the antennal disc (Bonini et al., 1997; Pignoni et al., 1997). This ectopic eye development is associated with the activation of *ey* transcription, suggesting a positive feedback of *so* and *eya* on the earlier gene (Bonini et al., 1997; Pignoni et al., 1997). The reason for their combinatorial activity appears to be the association of the two proteins into a complex transcription factor with the DNA-binding domain of so and the transcriptional activation domain of eya (Pignoni et al., 1997). The proteins may be unstable outside this complex, as *eya* and *so* are each required for the normal level of expression of the other in the eye disc (Halder et al., 1998). However, *so* has an additional early role in the formation of the optic lobes of the brain that appears to be independent of *eya* (Cheyette et al., 1994; Serikaku and O'Tousa, 1994).

Both *eya* and *so* are required for the expression of *dachshund* (*dac*) (Chen et al., 1997; Pignoni et al., 1997), which encodes a novel nuclear protein (Mardon et al., 1994), in the early third instar eye disc. *dac* mutants lack eyes (Mardon et al., 1994), and *dac* expression can induce, although weakly, ectopic eye development (Shen and Mardon, 1997). Coexpression of *eya* with *dac* enhances the production of ectopic eyes, perhaps because the two proteins can physically interact (Chen et al., 1997). However, as neither protein contains a known DNA-binding domain and both contain domains capable of transcriptional activation (Chen et al., 1997; Pignoni et al., 1997), the basis of this synergy is less clear than for *so* and *eya*. Unlike *ey*, *so* and *eya*, *dac* is not required for normal growth of the early eye disc and thus seems to act at a slightly later stage of development (Mardon et al., 1994). Vertebrate homologs of *so*, *eya* and *dac* are all expressed in eye tissues (Hammond et al., 1998; Oliver et al., 1995; Seo et

al., 1998; Xu et al., 1997; Zimmerman et al., 1997), suggesting the possibility that these three genes define a conserved regulatory network downstream of *Pax-6* (Treisman, 1999). However, these genes are not limited to the eye in either flies or vertebrates; it is therefore not entirely clear how they determine the eye fate.

An additional gene required for formation of the eye is *eyegone* (*eyg*), which encodes a protein containing a Pax-6-like homeodomain but only the C-terminal half of the paired domain (Jang et al., 1999). *eyg* is expressed at the same stage as *ey* in the embryonic eye primordium, but neither gene requires the other for its expression; both might be targets of *toy* (Jang et al., 1999). *eyg* is not sufficient to induce eye development outside the eye disc, though it enhances the ability of *ey* to do so (Jang et al., 1999). A final gene that can induce eye development when misexpressed in the antennal disc, although it is not required for normal eye development, is *teashirt* (*tsh*), which encodes a zinc finger transcription factor (Pan and Rubin, 1998). It is possible that ectopic *tsh* results in the maintenance of *ey* expression present in the embryonic antennal primordium. A diagram outlining the known regulatory interactions between the eye specification genes *ey, eya, so,* and *dac* is shown in Figure 1.

Finally, two homeobox-containing genes, *homothorax* (*hth*) and *extradenticle* (*exd*) act to inhibit eye formation (Pai et al., 1998). Both genes are coexpressed along the anterior-lateral margins of the eye disc and the peripodial membrane, regions that will give rise to head cuticle. Clones of cells lacking either *hth* or *exd* cause ectopic eye formation near the ventral margin of the eye; conversely, ectopic expression of *hth* blocks retinal development (Pai et al., 1998). Hth protein acts by regulating nuclear localization of exd. How *hth* and *exd* interact with genes that promote (*ey*, *eya*, *so*, *eyg*, and *dac*) or inhibit (*wingless*, see below) eye development remains to be determined.

Dorsal-ventral patterning and growth of the eye disc

Growth of the eye primordium and its further development depends on asymmetry in the dorsal-ventral (DV) axis; signaling between the dorsal and ventral fields specifies the midline of the disc, which defines the site where differentiation will initiate. The outcome of these signals appears to be the activation of the Notch (N) cell-surface receptor at the DV midline. This activation is accomplished by two ligands, Delta (Dl) on the dorsal side of the midline, and Serrate (Ser) on the ventral side (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The asymmetric distribution of these

ligands is critically dependent on the restriction of the signaling molecule fringe (fng) to the ventral half of the disc (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). During both wing and eye development, fng has been shown to potentiate the response of N to Dl, leading to the activation of *Ser* expression; fng also inhibits the response of N to Ser, preventing it from activating *Dl* in ventral cells (Panin et al., 1997; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). A boundary of *fng* expression is thus essential for N activation; either loss of *fng* activity or misexpression of *fng* throughout the disc causes a failure of the eye disc to grow and differentiate (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). This failure can be rescued by a constitutively activated form of N, which leads to overgrowth of the eye field (Dominguez and de Celis, 1998).

The ventral restriction of *fng* expression may be controlled by three related homeobox genes in the *Iroquois* complex, *araucan* (*ara*), *caupolican* (*caup*), and *mirror* (*mrr*) (Gomez-Skarmeta et al., 1996; McNeill et al, 1997). All three are expressed specifically in the dorsal half of the eye (Dominguez and de Celis, 1998; McNeill et al., 1997; Netter et al., 1998), and ectopic expression of either *caup* or *mrr* has been shown to repress *fng* expression (Cho and Choi, 1998; Dominguez and de Celis, 1998). The basis for their own dorsal restriction is less clear. Expression of *mrr* requires the function of the Wnt protein encoded by *wingless* (*wg*), and overexpression of *wg* can expand *mrr* expression, shifting the DV midline ventrally (Heberlein et al., 1998). However, the normal domain of *wg* present at the ventral margin does not induce *mrr*, indicating that other factors must be involved. Maintenance of *mrr* repression ventrally requires the *Polycomb* group of genes (Netter et al., 1998), implicating a repressive chromatin structure, though there is no evidence that these genes provide the spatial localization necessary to establish its initial repression. In addition to promoting growth of the eye disc and contributing to the selection of the site at which differentiation initiates, the DV boundary emits a signal defining ommatidial polarity (reviewed by Jarman, 1996).

Initiation of differentiation

In the third larval instar, photoreceptor clusters begin to differentiate in the eye disc, starting at the posterior margin and spreading anteriorly. Initiation of neuronal differentiation requires the function of the *hedgehog* (*hh*) gene, which

encodes a secreted protein (Dominguez and Hafen, 1997; Royet and Finkelstein, 1997; Borod and Heberlein, 1998). *hh* is present at the central posterior margin of the eye disc prior to the start of differentiation and may thus define the site of initiation (Dominguez and Hafen, 1997; Royet and Finkelstein, 1997; Borod and Heberlein, 1998). *decapentaplegic* (*dpp*), a BMP family member, is also required for initiation (Burke and Basler, 1996; Wiersdorff et al., 1996; Pignoni and Zipursky, 1997; Chanut and Heberlein, 1997). *dpp* is expressed around the posterior and lateral margins of the early eye disc (Masucci et al., 1990); its posterior expression is dependent on *hh* (Dominguez and Hafen, 1997; Royet and Finkelstein, 1997; Borod and Heberlein, 1998). Clones of cells mutant for downstream, cell-autonomous components of the *dpp* pathway, such as the receptors encoded by *thick veins* (*tkv*) and *punt* or the transcription factor encoded by *Mothers against dpp* (*Mad*), fail to initiate photoreceptor differentiation (Burke and Basler, 1996; Wiersdorff et al., 1996). Ectopic expression of *dpp* in the eye disc induces ectopic differentiation, but only initiating at the anterior margin of the disc (Pignoni and Zipursky, 1997; Chanut and Heberlein, 1997), suggesting that the margins express a factor required in conjunction with *dpp*. This ectopic initiation requires *hh*, the expression of which is activated at the anterior margin by *dpp*. This may reflect a normal feedback regulation of *hh* by *dpp*, which must be limited to the margins of the eye disc (Borod and Heberlein, 1998).

Localization of the site of initiation also requires negative regulation by *wg*, which is expressed at the dorsal and ventral margins of the eye disc. Its removal using a temperature-sensitive allele results in ectopic initiation, particularly from the dorsal margin (Ma and Moses, 1995; Treisman and Rubin, 1995), that is *hh*-dependent (Borod and Heberlein, 1998). Conversely, ectopic expression of *wg* at the posterior margin can inhibit initiation (Treisman and Rubin, 1995). *wg* is expressed throughout the second instar eye disc and is subsequently restricted to the anterior dorsal and ventral margins by *dpp* (Royet and Finkelstein, 1997). *wg* plays a role in promoting the differentiation of head cuticle by these regions of the eye disc, mediated in part by its activation of the expression of the homeobox gene *orthodenticle* (*otd*) (Royet and Finkelstein, 1997). Although ectopic *wg* is present in posterior margin cells that are unable to receive the dpp signal (Burke and Basler, 1996; Wiersdorff et al., 1996), it is not the only reason for their failure to differentiate as photoreceptors, as clones of cells doubly mutant for *Mad* and for *wg* still form no photoreceptors (Hazelett et

al., 1998). wg does not interfere with the expression or function of the dpp protein; it may act further downstream, preventing the epidermal growth factor (EGF) receptor pathway (see below) from triggering photoreceptor differentiation (Hazelett et al., 1998). Neuronal differentiation is severely impaired in eye discs lacking EGFR function (Kumar et al., 1998).

The initiation of neuronal differentiation in the eye disc also requires the function of the proneural gene *atonal (ato)*. Flies carrying a loss-of-function *ato* mutation are nearly eyeless and the remaining narrow eyes contain no photoreceptor neurons (Jarman et al., 1994). The failure of photoreceptor differentiation in *ato* mutants is due to a defect in the specification of photoreceptor R8, the so-called ommatidial founder cell, which is the first photoreceptor cell to differentiate in each cluster. It is believed that in the absence of an R8 cell the remaining photoreceptors fail to be recruited and induced to differentiate as neurons. The expression of *hh*, *dpp*, and *wg* is normal in young third instar *ato* mutant eye discs. However, neuronal differentiation is absent and *dpp* expression decays (Jarman et al., 1994; Jarman et al., 1995; Borod and Heberlein, 1998). Ato protein is expressed along the posterior margin just prior to the start of differentiation and this expression requires *hh* function for its initiation and/or maintenance. This observation, together with the fact that *hh* expression at the margin is *ato*-independent, suggests that *ato* functions genetically downstream of *hh* in the pathway that leads to the initiation of differentiation in the eye disc (Borod and Heberlein, 1998; Dominguez and Hafen, 1997).

ato encodes a basic helix-loop-helix (bHLH) protein and is thus a potential transcriptional regulator. *In vitro*, ato protein forms heteromultimers with another bHLH protein encoded by the *daughterless (da)* gene; these ato-da multimers act as sequence-specific DNA-binding complexes (Jarman et al., 1993). Loss of *da* function in the retina leads to a failure of neuronal differentiation similar to that observed for *ato* (Brown et al., 1996). It is therefore likely that *ato* and *da* act as partners during proneural specification in the eye. A cartoon diagram summarizing our current understanding of the genetic hierarchy that controls initiation is shown in Figure 2.

How are specification and initiation related?

The connection between the early specification of the eye disc and its subsequent differentiation is not well understood. One important area of

investigation is the regulatory relationship between the eye specification genes *ey*, *eya*, *so*, *dac* and *eyg* on one hand, and the patterning genes *hh*, *dpp* and *wg* on the other. *eya* and *so* are necessary for *dpp* expression, which is only weakly initiated in eye discs mutant for either of these genes and is completely absent in *eya* or *so* mutant cells at the third instar (Hazelett et al., 1998; Pignoni et al., 1997). *eya* and *eyg* both play a role in the repression of *wg*, which is misexpressed at the posterior margin of *eyg* mutant discs and in third instar *eya* mutant clones (Hazelett et al., 1998; Jang et al., 1999). The lack of photoreceptor differentiation in *eyg* mutant discs can be rescued by inhibiting wg signaling at the posterior margin, indicating that repressing *wg* posteriorly is a critical function of *eyg* (Hazelett et al., 1998). This is not true for *eya* (Hazelett et al., 1998), consistent with a requirement for *eya* even at late stages of photoreceptor differentiation (Pignoni et al., 1997). *dpp* expression is normal in *dac* mutant eye discs (Mardon et al., 1994), though *wg* is ectopically expressed at the posterior of the disc (Treisman and Rubin, 1995); the role this ectopic *wg* plays in the *dac* mutant phenotype has not been tested. Potential regulatory interactions between the eye specification genes and *hh* have not yet been reported. However, both *eya* and *so* are required for normal *ato* expression (Jarman et al., 1995); whether *hh* acts as an intermediary in this process is unknown.

There is also evidence that *dpp, hh* and *wg* may act upstream of or in conjunction with the specification transcription factors. Although *ey* is still present in *dpp* mutant eye discs, *eya* and *so* are only weakly expressed (Chen et al., 1999). Also, widespread misexpression of *ey* in other imaginal discs results in photoreceptor formation only in regions of the discs where *dpp* and *hh* are present and *wg* is absent (Halder et al., 1998). Coexpression of *dpp* with *ey* expands the domain in which it can induce eye development considerably, though it is still restricted to the posterior of the discs, where *hh* is expressed (Chen et al., 1999). *dpp* also enhances the efficiency of ectopic eye induction by *ey*, *eya* or *so* (Chen et al., 1999). *hh* and *dpp* also act to pattern other imaginal discs and the embryo, so in themselves they have no specificity for eye development. Presumably the late requirement observed for *eya* and *so* reflects their function in interpreting *hh/dpp* as a signal for photoreceptor differentiation.

Progression of differentiation

Differentiation of the retina is asynchronous; once initiated at the posterior tip of the eye disc, it progresses across the epithelium one row at a time,

reaching the anterior margin approximately 2 days later. The anterior edge of this differentiation wave is marked by an indentation in the apical surface of the epithelium, the morphogenetic furrow (MF), which spans the disc along its dorsoventral axis (Ready et al., 1976). The MF is a consequence of localized and transient changes in cell shape (Wolff and Ready, 1991). The MF coincides temporally and spatially with several important events in retinal morphogenesis. Ahead of the furrow cells are unpatterned and undifferentiated, and they divide asynchronously. In the MF, cells become synchronized in the G1 phase of their cell cycle as they begin to associate into evenly spaced clusters that will develop into the individual ommatidia (Ready et al., 1976; Tomlinson and Ready, 1987; Wolff and Ready, 1991). The first five cells of the ommatidium exit the cell cycle and begin differentiating as neurons immediately posterior to the furrow, while the remaining cells undergo one more round of synchronous cell division prior to differentiation.

hh, expressed in and secreted by cells located posterior to the MF, plays a crucial role in furrow progression (Heberlein and Moses, 1995). Reduction or elimination of *hh* function in the eye disc leads to a halt in MF progression (Heberlein et al., 1993; Ma et al., 1993). Conversely, ectopic expression of *hh* or ectopic activation of the *hh* signal transduction pathway in undifferentiated cells located ahead of the MF causes their precocious differentiation (Heberlein et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Wehrli and Tomlinson, 1995). However, clones of cells mutant for the *smoothened* (*smo*) gene, which is required for the transduction of the *hh* signal, still allow neuronal differentiation and furrow progression, albeit at a reduced rate (Strutt and Mlodzik, 1997). This suggests that other signals, in addition to *hh*, may contribute to proper MF progression (see below).

How does *hh* regulate furrow progression? *hh* induces the expression of several genes in and around the MF, including *dpp* and *ato*. *dpp* is expressed in the furrow (Blackman et al., 1991), but its function in MF progression is not clear. Clones of cells mutant for the autonomous components of the *dpp* signal transduction pathway, such as *tkv*, *punt*, and *Mad*, display only minor irregularities when located in the interior regions of the eye disc (Wiersdorff et al., 1996; Penton et al., 1997; Burke and Basler, 1996). However, global reduction of *dpp* function, by means of a temperature-sensitive allelic combination, significantly slows the progression of differentiation (Chanut and Heberlein, 1997). It is possible that *dpp* plays a permissive role in furrow progression by

ensuring proper cell cycle control; indeed, it has been shown recently that *dpp* is required for G1 arrest in the furrow (Horsfield et al., 1998).

A more direct mechanism by which *hh* regulates furrow progression is through its induction of *ato* expression. Ato protein is expressed in a strip of cells immediately anterior to the MF. In the furrow, expression is restricted to evenly spaced groups of cells and shortly thereafter to single R8 cells (Jarman et al., 1994). Removal of *hh* function, by means of a temperature-sensitive mutation, leads to a loss of ato in the MF (Borod and Heberlein, 1998); ectopic expression of *hh* induces ectopic expression of ato (Heberlein et al., 1995). During the process of MF progression cells that receive the *hh* signal must in turn become cells that send the *hh* signal to their more anteriorly located neighbors. This transition from recipient to sender of the *hh* signal requires the receiving cell to differentiate as a photoreceptor neuron, a process to which *ato* and *da* function are central (for a review see Treisman and Heberlein, 1998). The mechanisms by which *hh*, *ato* and *da* regulate each other's expression is thus an important and unresolved issue.

Two additional genes of the HLH family negatively regulate furrow progression: *hairy* (*h*) and *extramacrochaete* (*emc*). *h* is expressed in a dorsoventral strip located immediately anterior to the domain of *ato* expression (Brown et al., 1991); *emc*, while fairly ubiquitous, is highest near the anterior margin (Brown et al., 1995). Clones of cells mutant for *h* or *emc* function in the eye have little effect on normal development. However, the MF and the front of differentiation are strikingly accelerated when crossing clones of cells doubly mutant for *h* and *emc* (Brown et al., 1995). It is likely that these two genes act normally by inhibiting the proneural functions of *ato*. Curiously, ectopic expression of *hh* or ectopic activation of the *hh* pathway ahead of the furrow leads to ectopic expression of *h* in nearby cells (Heberlein et al., 1995; Pan and Rubin, 1995). Thus, *hh* appears to induce not only an activator of neural differentiation (*ato*), but also an inhibitor (*h*). The correct balance of these two molecules may ensure the orderly patterning of the retina.

Another signaling pathway that has recently been shown to modulate *ato* expression in the MF is that activated by the EGFR: *ato* expression is reduced in clones of cells lacking the EGFR and induced in cells in which the EGFR pathway has been ectopically activated (Dominguez et al., 1998; Spencer et al., 1998). However, *ato* expression appears relatively normal in eye discs in which the EGFR has been inactivated by a temperature-sensitive mutation, perhaps

reflecting some residual function (Kumar et al., 1998). Thus, the EGFR pathway may not only induce the normal differentiation of the R1-6 photoreceptors (reviewed in Freeman, 1996), but also play a role in modulating furrow progression through the regulation of *ato* expression. This effect could, however, be mediated indirectly by EGFR induction of *hh*. Interestingly, ectopic activation of the EGFR pathway can induce neuronal differentiation in the eye disc even in the absence of *ato* function (Dominguez et al., 1998), showing that the normally obligate order of retinal differentiation can be bypassed by artificial activation of the EGFR. However, the resulting ommatidia are disorganized, underscoring the importance of a gradual and asynchronous differentiation process in proper patterning and, ultimately, proper vision.

A third signaling system that regulates *ato* expression and photoreceptor differentiation is the *Notch* (*N*) pathway. Clones of cells lacking the N receptor or its ligand Delta show reduced *ato* expression and impaired neuronal differentiation (Baker and Yu, 1997). It is not known whether *N* is required for cells to respond to *hh* or if it plays a more direct role in neuronal differentiation.

Finally, the steroid hormone ecdysone has recently been shown to control furrow progression (Brennan et al., 1998). Mutants that fail to produce or are impaired in the response to ecdysone show a disruption in furrow progression. Expression of *hh* and *ato* is lost in these mutants, providing a likely mechanism for their effect on the furrow. Curiously, loss of function of Ultraspiracle (Usp), the *Drosophila* RXR, in clones of cells leads to a slight acceleration of the furrow (Zelhof et al., 1997). Usp forms heterodimers with the Ecdysone receptor (EcR), and would therefore have been predicted to act as a positive modulator of furrow progression. It is possible that both USP and EcR have other dimerization partners that determine their specific roles in furrow progression.

In summary, the progression of the wave of neuronal differentiation is a highly regulated and complex process that involves the coordinate function of multiple signaling systems. An understanding of the interactions between the hedgehog, EGFR, Notch, and ecdysone signaling pathways will be crucial to our understanding of how the furrow moves across the eye disc leaving in its wake a beautifully precise developmental pattern. Vertebrate retinae also differentiate asynchronously, thus lessons learned from flies are likely to provide insights into their developmental mechanism.

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Figure legends:

Fig. 1. A cartoon depicting known and possible interactions in the genetic hierarchy that controls retinal specification in *Drosophila*. Adapted from Chen et al., 1999.

Fig. 2. A cartoon showing a complex series of genetic interactions that directly or indirectly regulate the initiation of photoreceptor differentiation in *Drosophila*. Arrows indicate positive interactions while intersecting lines depict negative interactions.