Regulators of the Morphogenetic Furrow

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Unlike other imaginal discs, the *Drosophila* eye disc has a progressive pattern of differentiation. Photoreceptor clusters begin to form at the posterior margin of the eye disc in the third larval instar, and more anterior rows of clusters then differentiate in succession (Ready et al., 1976). Just prior to their differentiation, cells undergo an apical constriction and apical-basal contraction that produces an indentation in the disc known as the morphogenetic furrow (MF; Ready et al., 1976). Cells anterior to the MF divide actively and appear unpatterned. Just posterior to the MF, cells assemble into evenly spaced rosettes; slightly more posteriorly these transform into arcs, and the arcs then close to produce five-cell preclusters (Wolff and Ready, 1991). Concurrently, these cells initiate a program of gene expression resulting in the appearance of neural-specific markers in a defined sequence in the cells of each cluster (Tomlinson and Ready, 1987). Cells in the MF are arrested in the G1 phase of the cell cycle (Thomas et al., 1994); posterior to the MF, cells excluded from the preclusters undergo one more round of division, the second mitotic wave, to generate the remaining cells of each ommatidium (Ready et al., 1976; Wolff and Ready, 1991). This orderly and sequential pattern of differentiation, proliferation and morphogenesis is organized by a set of signaling molecules that also direct many other developmental processes. The expression patterns and interactions of these molecules in the developing eye disc are shown in Figure 1.

Notch activation defines the initiation point

Photoreceptor differentiation initiates at the intersection of the dorsal-ventral (D-V) midline of the disc with the posterior margin. Determination of the D-V midline, or equator, is thus critical to delimit the initiation site. The dorsal side of the eye disc is defined during embryogenesis by its expression of the GATA transcription factor encoded by *pannier* (*pnr* ; Heitzler et al., 1996; Maurel-Zaffran and Treisman, 2000; Ramain et al., 1993). *pnr* is required for the expression of *wingless* (*wg*), a member of the *Wnt* gene family (Cadigan and Nusse, 1997), at the dorsal margin of the eye disc (Maurel-Zaffran and Treisman, 2000). Together with the secreted protein Hedgehog (Hh), Wg then activates the expression of the three homeobox genes of the *Iroquois* complex (*Iro-C*), *mirror* (*mirr*), *araucan* (*ara*), and *caupolican* (*caup*; Cavodeassi et al., 1999; Gomez-

Skarmeta et al., 1996; Heberlein et al., 1998; Maurel-Zaffran and Treisman, 2000; McNeill et al., 1997). Expression of the *Iro-C* genes fills the dorsal compartment of the disc but ends sharply at the D-V midline; the mechanism by which this sharp expression boundary is established is not understood. The JAK/STAT pathway ligand Unpaired, which is present at the center of the posterior margin, appears to contribute to the ventral repression of these genes (Zeidler et al., 1999). This repression is maintained by chromatin-mediated mechanisms requiring the *Polycomb* group of genes (Netter et al., 1998).

The dorsally expressed Iro-C proteins repress the expression of *fringe* (*fng*), limiting it to the ventral compartment of the eye disc (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; Yang et al., 1999). Fng is a glycosyltransferase that modifies the transmembrane receptor Notch (N) by adding N-acetylglucosamine to O-linked fucose residues (Bruckner et al., 2000; Moloney et al., 2000). This modification increases the affinity of N for its ligand Delta (Dl; Bruckner et al., 2000); in vivo, *fng*-expressing cells also appear less sensitive to an alternative ligand, Serrate (Ser; Panin et al., 1997). *Dl* expression is restricted to the dorsal side of the early eye disc and *Ser* to the ventral side (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The presence of Fng in the ventral compartment thus limits N activation to the midline, where modified ventral N is exposed to dorsal Dl and unmodified dorsal N is exposed to ventral Ser. The initial domains of *Dl* and *Ser* expression may be controlled by Hh and Wg signaling from peripodial membrane cells (Cho et al., 2000).

A boundary of Fng expression is essential to trigger N activation and define a central initiation point; either loss of *fng* function or ubiquitous *fng* expression causes a loss of the eye that can be rescued by expressing an activated form of N (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). Similarly, ubiquitous expression of *caup* or *mirr* can abolish the eye (Cho and Choi, 1998; Dominguez and de Celis, 1998), while removal of all three *Iro-C* genes from clones of dorsal cells induces ectopic eyes composed of both mutant and wildtype cells (Cavodeassi et al., 1999; Pichaud and Casares, 2000). Because *fng* is misexpressed in the mutant cells, N becomes activated at the clonal boundary and may promote the initiation of an ectopic

MF. Removal of the upstream gene *pnr* from clones of cells also leads to ectopic dorsal eye development, and ubiquitous expression of an activated form of *pnr* prevents MF initiation (Maurel-Zaffran and Treisman, 2000). These results show that activation of N at a line source is both necessary and sufficient, in combination with signals present at the disc margin, to initiate differentiation.

Hedgehog is essential for morphogenetic furrow movement

The *hh* gene is critical for initiation and progression of the MF. *hh* encodes a secreted protein related to vertebrate proteins of the Sonic hedgehog family (Fietz et al., 1994). Although *hh* has a very dynamic pattern of expression in the eye disc, its upstream regulators are unknown. During the second instar *hh* is expressed predominantly in the peripodial membrane, where it shifts from a ventral to a dorsal domain (Cho et al., 2000) that has been implicated in the regulation of D-V polarity (see above). In early third instar eye discs, *hh* is expressed at the center of the posterior margin, where it is required for the onset of photoreceptor differentiation (Borod and Heberlein, 1998; Dominguez and Hafen, 1997; Royet and Finkelstein, 1997). Using a temperature-sensitive allele, Borod and Heberlein (1998) determined that *hh* function was required for differentiation at the time of MF initiation, but not earlier.

During MF progression, *hh* is strongly expressed in the newly differentiating R2 and R5 photoreceptors, and more weakly in the other cells of the forming precluster (Heberlein et al., 1993; Ma et al., 1993). Hh secreted by these cells is essential to promote the differentiation of more anterior cells. Removal of *hh* function from the entire disc using eye-specific or temperature-sensitive alleles results in an arrest of MF progression that can be visualized by the presence of a full complement of photoreceptors in the most anterior row of ommatidia (Heberlein et al., 1993; Ma et al., 1993). When misexpressed in the anterior domain of the eye disc, Hh is sufficient to produce ectopic radially oriented morphogenetic furrows (Dominguez, 1999; Heberlein et al., 1995), and clones of cells lacking the negative regulators of Hh signaling *ptc* or *PKA* have the same effect (Chanut and Heberlein, 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995; Wehrli and Tomlinson, 1995). The range of Hh signaling in the eye is limited in part by the apical constriction of cells as they

enter the MF; cells that lack the actin binding protein Act up do not undergo apical constriction, allowing Hh to move further anteriorly and trigger precocious differentiation (Benlali et al., 2000). Despite a global requirement for Hh signaling during MF progression, clones of cells mutant for *smoothened* (*smo*), which encodes a cell autonomous receptor component, show only a delay in progression; this implies that Hh functions at least in part by activating a secondary signal (Dominguez, 1999; Greenwood and Struhl, 1999; Strutt and Mlodzik, 1997). This signal is probably the BMP family member encoded by *decapentaplegic (dpp;* see below).

Hh promotes differentiation by activating the expression of the proneural gene *atonal (ato* ; Borod and Heberlein, 1998; Greenwood and Struhl, 1999). *ato* encodes a basic helix-loop-helix (bHLH) protein that is required for the formation of the "founder" R8 photoreceptor (Jarman et al., 1994). A broad stripe of *ato* expression just anterior to the MF is later refined first to a proneural cluster of cells and then to individual R8 cells (Dokucu et al., 1996; Jarman et al., 1994, 1995). *ato* appears to be a direct target of Hh signaling, since it is autonomously lost in *smo* clones; however, *smo* is also required for the subsequent down-regulation of *ato* between the proneural clusters (Dominguez, 1999; Greenwood and Struhl, 1999). The repressive effect of Hh on *ato* may be mediated by the homeodomain protein Rough (Ro; Kimmel et al., 1990). *ro* and *ato* are expressed in complementary patterns, and Ro has been shown to repress the transcription of *ato* (Dokucu et al., 1996). Hh signaling is required for *ro* expression (Dominguez, 1999), and the gain of function mutation ro^{DOM} interferes with upregulation of *ato* by Hh (Chanut et al., 2000; Heberlein et al., 1993). Thus Hh regulates both the formation and the spacing of the R8 photoreceptors.

In addition to regulating genes that promote MF progression, *hh* lies upstream of another bHLH protein encoded by *hairy* (*h*; Heberlein et al., 1995; Ma et al., 1993; Pan and Rubin, 1995). H is present in a stripe anterior to the stripe of Ato, where it acts to inhibit differentiation in cooperation with the HLH protein Extramacrochaete (Brown et al., 1995). Hh signaling is required for *h* expression and is sufficient to ectopically activate *h* (Heberlein et al., 1995; Ma et al., 1993; Pan and Rubin, 1995); however, its effect on *h* is probably mediated by Dpp (see below).

Recent work has suggested that *hh* homologs may play a similar role in patterning the differentiation of neurons in the vertebrate retina (Jensen and Wallace, 1997; Levine et al., 1997; Neumann and Nüsslein-Volhard, 2000; Stenkamp et al., 2000). Retinal differentiation begins near the optic stalk and proceeds outward in a centrifugal pattern (Burrill and Easter, 1995; McCabe et al., 1999; Raymond et al., 1995; Schmitt and Dowling, 1996). The *hh* homologues *Sonic hedgehog (Shh)* and *Tiggywinkle hedgehog (twhh)* are expressed in the zebrafish ganglion cell layer at the time of ganglion cell differentiation and in the adjacent retinal pigmented epithelium at the time of photoreceptor differentiation (Neumann and Nüsslein-Volhard, 2000; Stenkamp et al., 2000). Reduction of Shh and Twhh production by antisense oligonucleotide injection, or using null mutations in the *shh* gene *sonic you (syu)*, reduces retinal ganglion cell and rod photoreceptor differentiation, while blocking all Hh family signaling with cyclopamine can prevent ganglion cell neurogenesis (Neumann and Nüsslein-Volhard, 2000; Stenkamp et al., 2000). In addition, the expression of a *shh* reporter is lost in *syu* mutants (Neumann and Nüsslein-Volhard, 2000), suggesting that, as in the fly retina, *hh* expression requires reception of the Hh signal.

Decapentaplegic promotes morphogenetic furrow movement

dpp, which encodes a homologue of the secreted Bone Morphogenetic Proteins (BMPs) 2 and 4 (Padgett et al., 1987), acts downstream of Hh to perform a subset of its functions in MF movement. *dpp* is first expressed at the ventral margin of the first instar eye disc, and its expression subsequently expands to include the dorsal and posterior margins (Cho et al., 2000). Following the initiation of differentiation, *dpp* expression becomes restricted to a stripe of cells within the MF (Masucci et al., 1990). At both early and late stages, *dpp* expression is dependent on *hh* (Borod and Heberlein, 1998; Heberlein et al., 1993; Ma et al., 1993; Royet and Finkelstein, 1997; Strutt and Mlodzik, 1997).

Dpp signaling is critical for the initiation of differentiation. Large clones of cells mutant for *dpp* can block the formation of posterior eye regions (Heberlein et al., 1993). An eye-specific enhancer mutation, $dpp^{d\text{-}blk}$, prevents initiation from occurring in the ventral region of the posterior margin (Blackman et al., 1991;

Chanut and Heberlein, 1997a; St. Johnston et al., 1990; Wiersdorff et al., 1996), and a similar effect can be seen using temperature-sensitive *dpp* alleles (Chanut and Heberlein, 1997b). In addition, clones of cells mutant for genes encoding components of the Dpp signaling pathway, such as the type I receptor Thickveins (Tkv), the type II receptor Punt, or the SMADs Mothers against Dpp (Mad) and Medea, fail to differentiate when they contact the posterior margin of the disc (Burke and Basler, 1996; Das et al., 1998; Wiersdorff et al., 1996). Consistent with a function specific to initiation, misexpression of Dpp can induce ectopic MF initiation from the anterior margin even at a distance, although it does not induce photoreceptor differentiation in internal regions of the disc (Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997).

In the absence of *dpp* or of the downstream component encoded by *Mad*, expression of the eye specification genes *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*; see previous chapter) is not induced despite the presence of the upstream Pax-6 homologues encoded by *eyeless* (*ey)* and *twin of eyeless* (Chen et al., 1999; Curtiss and Mlodzik, 2000; Quiring et al., 1994; Czerny et al., 1999). This may explain the requirement for *dpp* in initiation, as loss of *Mad* function at the posterior margin can be rescued by supplying *eya* (Curtiss and Mlodzik, 2000). Ectopic *dpp* induces expression of *eya*, *so* and *dac* at the anterior eye disc margin, and ectopic *ey* can induce expression of these genes in other imaginal discs only when *dpp* is also present, confirming a critical role for *dpp* at this stage (Chen et al., 1999; Halder et al., 1998; Pignoni and Zipursky, 1997). However, *eya* and *so* are necessary to maintain the expression of *dpp*, suggesting that these genes act in an autoregulatory loop (Hazelett et al., 1998; Pignoni et al., 1997). Another important function for Dpp signaling may be to repress the expression of the homeoprotein Homothorax (Hth), which is present in all cells of the early eye disc and then becomes restricted to the anterior margin, where it blocks MF initiation (Pai et al., 1998; Pichaud and Casares, 2000).

Progression of the MF does not require Dpp signaling as strongly as initiation, as clones of cells mutant for *Mad, punt* or *tkv* in internal regions of the eye disc are able to differentiate almost normally (Burke and Basler, 1996; Wiersdorff et al., 1996) and to express *eya*, *so* and *dac* (Curtiss and Mlodzik, 2000). Because Dpp signaling is required for cell growth, large clones completely

lacking gene activity could not be analyzed; however, the alleles tested were able to completely block initiation while only delaying progression. Low levels of Dpp signaling may be required for MF progression, as complete removal of *dpp* function from the eye disc using a temperature-sensitive allele can arrest the MF (Chanut and Heberlein, 1997b). At this stage the functions of *dpp* and *hh* are partially redundant, as some photoreceptor differentiation is observed in clones mutant for the Hh receptor encoded by *smo* (Strutt and Mlodzik, 1997) but not in clones doubly mutant for *smo* and *Mad* or *tkv* (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999).

Despite this redundancy, loss of Dpp signaling alone causes more subtle phenotypes than loss of Hh signaling. Dpp signaling is required for cells in the MF to arrest in the G1 phase of the cell cycle (Horsfield et al., 1998; Penton et al., 1997). In addition, upregulation of the negative regulator H does not occur in *tkv* mutant clones anterior to the MF (Greenwood and Struhl, 1999). However, *smo* mutant clones show high levels of H, presumably due to Dpp diffusing in from neighboring wildtype cells (Greenwood and Struhl, 1999). Hh may thus control progression by inducing some target genes, such as *ato*, directly and others, such as *h*, indirectly through *dpp*. However, redundancy between the two pathways requires *ato* and other Hh target genes to be activated by Dpp in the absence of Hh. In *smo* mutant clones, the first broad stripe of *ato* expression is missing, but expression in single R8 cells is present, while *tkv* mutant clones also show a reduction in the initial broad *ato* expression (Dominguez, 1999; Greenwood and Struhl, 1999). The most likely explanation of these results is that separate enhancers of *ato* can respond to Hh and Dpp signaling.

Wingless inhibits morphogenetic furrow movement

wg is expressed in a dorsal domain of the margin and peripodial membrane of the early eye disc (Cavodeassi et al., 1999; Cho et al., 2000), although its secreted protein product is more broadly distributed (Cho et al., 2000; Royet and Finkelstein, 1997). Prior to MF initiation, *wg* expression becomes restricted to the anterior dorsal and ventral margins of the eye disc (Baker, 1988). The dorsal expression domain of *wg* is established in the embryo by *pnr* (Maurel-Zaffran and Treisman, 2000), while ventral *wg* expression is dependent on *hth*

(Pichaud and Casares, 2000). *dpp* is required to restrict *wg* expression to the anterior and to maintain its repression at the posterior margin (Royet and Finkelstein, 1997; Wiersdorff et al., 1996).

An important role of *wg* is to prevent ectopic photoreceptor differentiation from initiating at the lateral margins. When *wg* activity is removed during the larval stages using a temperature-sensitive allele, a MF initiates at the dorsal margin and progresses toward the center of the disc; the ventral margin is more weakly affected (Ma and Moses, 1995; Treisman and Rubin, 1995). Clones of cells mutant for *dishevelled* (*dsh*), a downstream component of the *wg* pathway, likewise form ectopic photoreceptors in the region that normally gives rise to dorsal head cuticle (Heslip et al., 1997). Conversely, ectopic expression of *wg* or activation of the Wg pathway can block both initiation and progression of the MF (Heslip et al., 1997; Treisman and Rubin, 1995).

It was originally proposed that *wg* repression is the only role of *dpp* in MF initiation, as cells in which the Hh pathway is ectopically activated can become photoreceptors if they also lack both *dpp* and *wg* (Dominguez and Hafen, 1997; Wiersdorff et al., 1996). However, *Mad* mutant clones at the posterior margin fail to differentiate even if they also lack *wg*, indicating that *dpp* has additional functions in initiation (Hazelett et al., 1998). Conversely, although high levels of Wg signaling repress *dpp* (Heslip et al., 1997) a level of ectopic Wg that does not affect *dpp* expression can still block differentiation (Treisman and Rubin, 1995). Activating the Dpp pathway at the level of the receptor Tkv is not sufficient to restore photoreceptor differentiation in the presence of Wg (Hazelett et al., 1998). Interestingly, Wg can upregulate the expression of *hth*, and ectopic Hth similarly blocks differentiation downstream of *dpp* expression (Pai et al., 1998; Pichaud and Casares, 2000). Anterior/posterior patterning of the eye disc appears to depend on the balance between Wg and Dpp, as anteriorly expressed genes such as *ey* are activated by Wg signaling and repressed by Dpp signaling (Curtiss and Mlodzik, 2000; J.D.L. and J.E.T., submitted).

Some of the effects of *wg* on MF initiation may be due to its influence on D-V patterning. The early restriction of *wg* expression to the dorsal side of the eye disc allows it to contribute to the activation of the *Iro-C* genes, which act as dorsal determinants (see above). However, the *Iro-C* genes probably do not

mediate all the effects of *wg*. Although activation of the Wg pathway in the ventral domain is not sufficient to induce *Iro-C* gene expression (Cavodeassi et al., 1999), it does prevent MF initiation (Treisman and Rubin, 1995). Ectopic eyes produced by lack of *Iro-C* include wildtype tissue and an ectopic equator is induced at the clonal boundary (Cavodeassi et al., 1999; Pichaud and Casares, 2000), while ectopic eyes produced by *dsh* clones are entirely composed of mutant tissue (Heslip et al., 1997), and ectopic furrows in discs lacking *wg* function have no equator (Ma and Moses, 1995).

A final signal that appears to be important for MF progression is the steroid hormone ecdysone. Inactivation of the *ecdysoneless* gene, which is required for ecdysone production, leads to a block in MF progression characterized by the loss of *hh* and *ato* expression (Brennan et al., 1998). However, the mechanism by which this signal is transduced is unclear. The known ecdysone receptor EcR and another hormone receptor encoded by DHR78 are not required for normal photoreceptor differentiation (Brennan et al., 2001), while loss of the coreceptor Ultraspiracle leads to an acceleration of MF movement (Zelhof et al., 1997). A delay in MF progression does occur in clones of cells mutant for some components of the downstream ecdysone-regulated *Broad-complex* (Brennan et al., 1998, 2001).

Conclusions

The molecules that are used to establish spatial pattern in the eye disc are also used to provide positional information to other imaginal discs; however, the details of their interactions are tailored to fit the progressive nature of eye development. N activation defines the D-V boundary of the wing disc (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996), as it does in the eye, but the selector gene that determines the dorsal compartment of the wing is *apterous* rather than the *Iro-C* genes (Diaz-Benjumea and Cohen, 1993). Although *hh* in the posterior of the wing disc also activates *dpp* in more anterior cells (Basler and Struhl, 1994; Tabata et al., 1995; Zecca et al., 1995), the domain of *hh* expression is stable rather than progressive and depends on the selector gene *engrailed* (Tabata et al., 1992; Zecca et al., 1995), which is not required in the eye disc (Strutt and Mlodzik, 1996). The direct effects of *hh* on wing patterning are

restricted to the region near the compartment boundary (Mullor et al., 1997; Strigini and Cohen, 1997), while *dpp* organizes the long-range pattern (Lecuit et al., 1996; Nellen et al., 1996). In the eye, the progressive expansion of *hh* expression allows it to play a more significant direct role, taking over most of the functions of *dpp*. Eye development thus exhibits some interesting variations on the mechanisms known to specify spatial pattern.

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Figure legend

Figure 1: Expression and interactions of some of the molecules used to pattern the eye disc. Before MF initiation, *pnr* activates *wg* expression on the dorsal side of the eye disc, leading to the activation of the *Iro-C* genes in the dorsal compartment. The *Iro-C* genes repress *fng*, and the boundary of *fng* expression leads to N activation at the dorsoventral midline. The initiation point is specified by the combination of activated N with *hh* and *dpp*, which are expressed at the posterior margin. *wg* prevents ectopic initiation from the lateral margins. Later in development, Hh present in the differentiating photoreceptors activates *dpp* expression and differentiation in more anterior cells.