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Pattern formation in the *Drosophila* eye disc

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ABSTRACT Differentiation of the *Drosophila* compound eye from the eye imaginal disc is a progressive process: columns of cells successively differentiate in a posterior to anterior sequence, clusters of cells form at regularly spaced intervals within each column, and individual photoreceptors differentiate in a defined order within each cluster. The progression of differentiation across the eye disc is driven by a positive autoregulatory loop of expression of the secreted molecule Hedgehog, which is temporally delayed by the intercalation of a second signal, Spitz. Hedgehog refines the spatial position at which each column initiates its differentiation by inducing secondary signals that act over different ranges to control the expression of positive and negative regulators. The position of clusters within each column is controlled by secreted inhibitory signals from clusters in the preceding column, and a single founder neuron, R8, is singled out within each cluster by Notch-mediated lateral inhibition. R8 then sequentially recruits surrounding cells to differentiate by producing a short-range signal, Spitz, which induces a secondary short-range signal, Delta. Intrinsic transcription factors act in combination with these two signals to produce cell-type diversity within the ommatidium. The Hedgehog and Spitz signals are transported along the photoreceptor axons and reused within the brain as long-range and local cues to trigger the differentiation and assembly of target neurons.

KEY WORDS: *photoreceptor, autoregulatory loop, Hedgehog, Spitz, Delta*

Introduction

The *Drosophila* compound eye is a highly organized structure that constitutes an excellent developmental system in which to address the molecular and cellular mechanisms of pattern formation (Wolff and Ready, 1993). The retina is composed of 750-800 identical units called ommatidia, which are organized into a regular hexagonally packed array. Each ommatidial unit contains eight photoreceptors (R1-R8), four cone cells and two primary pigment cells arranged in a stereotypic pattern, and these units are surrounded by a lattice of secondary and tertiary pigment cells. The photoreceptors project axons into the optic lobes of the brain, where they form retinotopic projections in two separate ganglia, the lamina and the medulla (Clandinin and Zipursky, 2002).

The transformation leading from an unpatterned epithelial monolayer within the larval eye imaginal disc to the highly ordered adult eye has been extensively studied during the three decades since it was first introduced as an experimental system (Ready *et al.*, 1976). The eye disc is specified in the embryonic and early larval stages through the action of a network of transcription factors known as the retinal determination genes (Silver and Rebay, 2005). Pattern formation and ommatidial differentiation begin in the third larval instar with the appearance of a groove called the morphogenetic furrow (MF) at the posterior margin of the eye disc (Ready *et al.*, 1976). This indentation in the epithelium, which results from an apical constriction and apical-basal contraction of the cells, sweeps progressively across the eye disc from posterior to anterior over a 2-day period. Cells anterior to the MF are undifferentiated and proliferate asynchronously, whereas cells posterior to the MF are organized into columns of regularly spaced clusters within which photoreceptor differentiation occurs in a defined sequence (Tomlinson and Ready, 1987, Wolff and Ready, 1991). An average of 30 columns, each initiated every 90-120 minutes, will form the entire retinal field. Unlike other imaginal discs, which are patterned by organizers formed at stable bound-

Abbreviations used in this paper: Ato, atonal; Dl, delta; EGFR, epidermal growth factor receptor; Hh, hedgehog; LF, lamina furrow; LPC, lamina precursor cell; MF, morphogenetic furrow; PPN, preproneural domain; Spi, spitz.

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aries between cellular territories, the eye disc has a progressive pattern of differentiation controlled by signals that are constantly changing their spatial positions.

One critical signal driving the initiation and progression of the MF is the secreted protein Hedgehog (Hh) (Heberlein *et al.*, 1995, Heberlein *et al.*, 1993b, Ma *et al.*, 1993). Hh is expressed at the posterior margin of the eye disc prior to MF initiation, and induces differentiation of anterior cells; as they differentiate into photoreceptors, these cells also begin to express *hh* and can therefore act on cells anterior to them (Heberlein and Moses, 1995). One target of Hh signaling is the Bone Morphogenetic Protein (BMP) family member Decapentaplegic (Dpp), which functions redundantly with Hh in MF progression (Burke and Basler, 1996, Curtiss and Mlodzik, 2000, Greenwood and Struhl, 1999, Heberlein *et al.*, 1993b, Wiersdorff *et al.*, 1996). Another Hh target is the basic Helix-Loop-Helix (bHLH) transcription factor Atonal (Ato), which is required for the specification of R8 cells, the first photoreceptors to differentiate in each cluster. Ato is initially expressed in a broad stripe just anterior to the MF, and its expression is gradually refined to single R8 cells within the MF in a process requiring lateral inhibition mediated by the Notch receptor (Baker and Zitron, 1995, Dokucu *et al.*, 1996, Jarman *et al.*, 1994, Jarman *et al.*, 1995).

R8 orchestrates subsequent ommatidial development by recruiting surrounding uncommitted cells to differentiate into other photoreceptors, cone cells and pigment cells. Secretion of the Epidermal growth factor receptor (EGFR) ligand Spitz (Spi) from R8 and subsequently recruited cells promotes the sequential differentiation of photoreceptors R2/R5, R3/R4, R1/R6, R7, the cone cells and the primary pigment cells, as well as the survival of secondary and tertiary pigment cells (Freeman, 1996, Miller and Cagan, 1998). The Notch ligand Delta (Dl) acts as a critical secondary signal for several of the later differentiating cell types (Flores *et al.*, 2000, Nagaraj and Banerjee, 2007, Tomlinson and Struhl, 2001, Tsuda *et al.*, 2002). The founder role of R8 cells makes their specification and spacing critical for the organization of the adult eye. Both Hh and Spi are also transported along the photoreceptor axons and act on photoreceptor target cells in the brain (Huang and Kunes, 1996, Huang *et al.*, 1998), coordinating the development of the two tissues. We will describe the current understanding of the mechanistic basis of pattern formation in this relatively simple system.

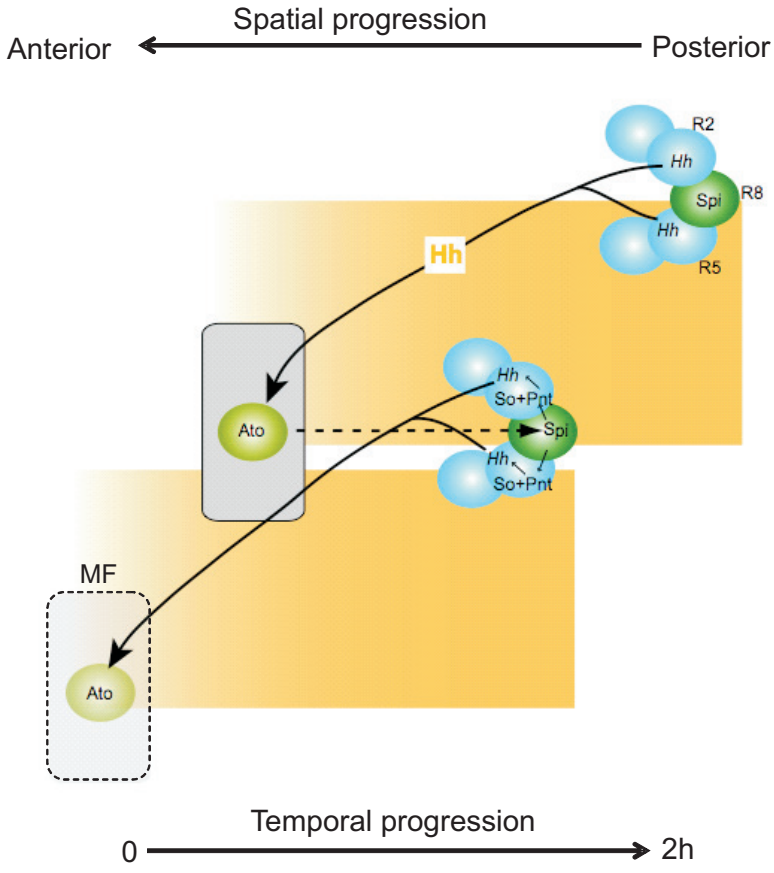

Fig. 1. A delayed autoregulatory loop of hedgehog (Hh) expression drives morphogenetic furrow (MF) progression. *Hh is secreted by differentiating photoreceptors, primarily R2 and R5, and diffuses anteriorly to activate* ato *expression in the MF. Ato then promotes R8 specification and expression of the proteases Rho and Ru, which cleave the Spi precursor to produce active Spi. Spi acts locally to enhance the activity of the transcription factor Pnt, which together with So activates an eye-specific enhancer of the* hh *gene. The resulting Hh expression reiterates the cycle, which repeats approximately every 2 h in a spatial progression from posterior to anterior.*

Progression of the morphogenetic furrow: a delayed autoregulatory loop

The MF initiates in the third larval instar at the dorso-ventral midpoint of the posterior margin of the eye disc. *hh* expression is activated at the center of the posterior margin in second instar eye discs (Cavodeassi *et al.*, 1999, Cho *et al.*, 2000) by members of the *odd-skipped* family of genes (Bras-Pereira *et al.*, 2006). Notch, which is activated at the dorsoventral midline by asymmetric distribution of its ligands and the glycosyltransferase Fringe, also contributes to MF initiation, as do Dpp, the JAK/STAT pathway ligand Unpaired, and the EGFR (Burke and Basler, 1996, Cavodeassi *et al.*, 1999, Cho and Choi, 1998, Dominguez and de Celis, 1998, Ekas *et al.*, 2006, Kumar and Moses, 2001, Papayannopoulos *et al.*, 1998, Tsai *et al.*, 2007, Wiersdorff *et al.*, 1996). The temporal control of initiation is not fully understood; it may be triggered by the hormone ecdysone (Niwa *et al.*, 2004), or by growth of the eye disc that brings the posterior margin out of the range of anterior inhibitory signals (Kenyon *et al.*, 2003, Ma and Moses, 1995, Treisman and Rubin, 1995).

While MF initiation is a unique developmental event, its progression occurs as a repeated sequence of events driven by an autoregulatory feedback loop. Cells that receive the Hh signal in and anterior to the MF are induced to differentiate as photoreceptors; as they differentiate posterior to the MF, these photoreceptors themselves begin to express *hh*, allowing them to drive the differentiation of more anterior cells (Dominguez and Hafen, 1997, Heberlein *et al.*, 1995, Heberlein *et al.*, 1993b, Ma *et al.*, 1993, Strutt and Mlodzik, 1995). This cyclical induction of Hh during MF progression depends on an enhancer element of the *hh* gene that reproduces *hh* expression specifically in photoreceptor cells; deletions of this element result in loss of *hh* expression in the

photoreceptors and arrest MF progression (Rogers *et al.*, 2005). This element is directly regulated by Pointed (Pnt), the transcription factor downstream of EGFR signaling (O'Neill *et al.*, 1994, Rogers *et al.*, 2005). Cells that receive the Hh signal from photoreceptors posterior to them turn on *atonal* expression, leading to the specification of R8 cells. R8 then secretes Spi, which acts through the EGFR to activate Pnt in the neighboring cells. Pnt promotes the differentiation of these cells into photoreceptors (O'Neill *et al.*, 1994), and simultaneously activates *hh* expression. This regulation of *hh* by Pnt, which is itself an indirect target of Hh signaling, creates a positive feedback loop that drives anterior propagation of *hh* expression and the MF (Fig. 1).

A temporal delay is introduced into this loop by the indirect effect of Hh on Spi production. An inactive precursor form of Spi is produced in all cells, but secretion of the active form requires the activities of the chaperone protein Star and the proteases Rhomboid (Rho) or Roughoid/Rhomboid-3 (Ru) (Freeman *et al.*, 1992a, Shilo, 2003, Wasserman *et al.*, 2000). *rho* and *ru* are required in the R8 cell (Wasserman *et al.*, 2000), where their expression is regulated by Ato, a direct target of Hh signaling (Baonza *et al.*, 2001, Dominguez, 1999). Activation of the *hh* enhancer by Pnt thus requires reception and transduction of the Hh signal, transcription and translation of Ato, transcription and translation of Rho and Ru, processing and secretion of Spi, and reception and transduction of the Spi signal. An additional level of regulation ensures that this mechanism is specific to the eye disc. Pnt binding sites alone are not sufficient to drive *hh* expression in photoreceptors (Rogers *et al.*, 2005); the enhancer also contains essential binding sites for the retinal determination protein Sine oculis (So) (Pauli *et al.*, 2005). So is specifically expressed in the eye field (Cheyette *et al.*, 1994), restricting the control of *hh* expression by EGFR signaling to this developmental system.

Superimposed on this basic autoregulatory loop are a variety of other mechanisms that constrain the pace of MF progression. Several repressors of photoreceptor differentiation present in the region anterior to the MF are controlled by long-range and short-range ligands that are themselves targets of Hh signaling (Fig. 2). Dpp, which is expressed in a stripe of cells within the MF in a Hh-dependent manner (Heberlein *et al.*, 1993b, Masucci *et al.*, 1990), acts at a long range to repress the expression of Homothorax (Hth), a transcription factor that prevents retinal differentiation in the anterior of the eye disc (Bessa *et al.*, 2002). This repression of *hth* allows cells to enter a preproneural state in which they are able to respond to Hh. Dpp also activates the expression of *hairy* in a stripe anterior to the MF (Greenwood and Struhl, 1999); Hairy is a repressor of *ato* and acts in combination with another anteriorly expressed HLH protein, Extramacrochaetae, to prevent cells from initiating the differentiation process prematurely (Brown *et al.*, 1995). Since Hth has been shown to repress *hairy* (Bessa *et al.*, 2002), it is possible that the effect of Dpp on *hairy* is mediated indirectly through *hth* repression.

Down-regulation of *hairy* expression, which relieves the repression of *ato*, requires a short-range signal that is provided by Delta (Dl), a transmembrane ligand for the Notch receptor expressed in the MF under the redundant control of Hh and Dpp (Baonza and Freeman, 2001, Baonza and Freeman, 2005, Parks *et al.*, 1995). Misexpression of Dpp does not result in ectopic photoreceptor differentiation in most regions of the eye disc (Chanut and Heberlein, 1997, Pignoni and Zipursky, 1997); however, coexpression of Dpp with Dl can induce neural differentiation anywhere anterior to the MF, indicating that these signals are sufficient to induce a preproneural state that will progress to a proneural state (Baonza and Freeman, 2001). Through its activation of Dpp and Dl, Hh thus activates *hairy* expression at a long range and represses it at a shorter range, allowing precise spatial control of the initiation of *ato* expression. Hh also appears to be capable of repressing *hairy* by another mechanism in the absence of Notch signaling (Fu and Baker, 2003). The restriction of *ato* expression to single R8 photoreceptors is likewise Hh-dependent; Hh induces three negative regulators of *ato* expression, *rough, Bar*, and *daughterless*, in cells adjacent to R8 (Dokucu *et al.*, 1996, Dominguez, 1999, Lim and Choi, 2003, Lim and Choi, 2004, Lim *et al.*, 2008). The onset of expression of these genes is delayed relative to *ato* because like *hh* itself, they are indirectly induced by EGFR signaling (Dominguez *et al.*, 1998, Lim and Choi, 2004).

It is interesting that the mechanisms controlling Hh signaling in the eye disc differ from those used in the wing disc. The transcription factor Cubitus interruptus (Ci) is processed into its repressor form (Ci75) in anterior wing disc cells that fail to receive the Hh signal, and represses *hh* expression in these cells (Aza-Blanc *et al.*, 1997, Dominguez *et al.*, 1996, Methot and Basler, 1999). This suggests that positive autoregulation through inhibition of Ci75 production is intrinsic to the Hh pathway. This mechanism must be actively suppressed in the cells at the anterior-posterior boundary of the wing disc, which receive Hh signaling but do not express *hh* (Apidianakis *et al.*, 2001). However, Ci75 is not involved in *hh*

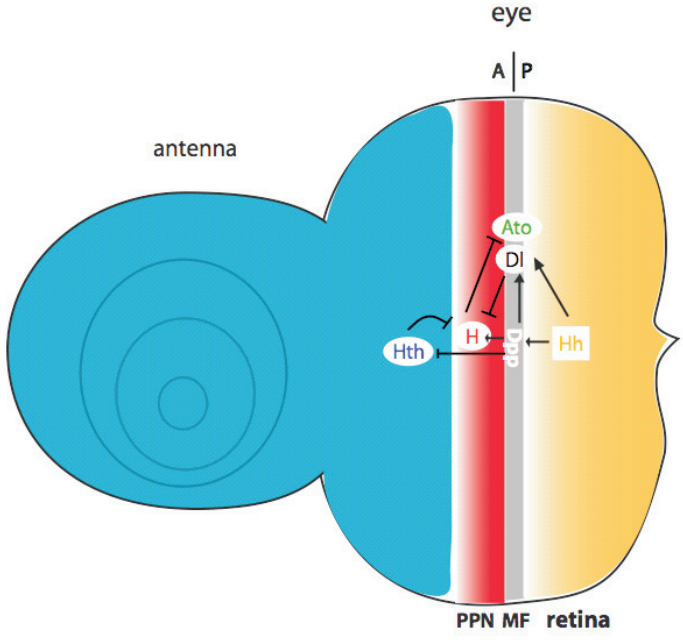

Fig. 2. Hedgehog (Hh) induces long-range and short-range secondary signals that control the precise position of the morphogenetic furrow (MF). *Hh acts over a short range to induce the expression of Dpp, which diffuses over a long range to turn off* hth *and turn on* hairy*, establishing a preproneural domain (PPN). Hh and Dpp also induce the expression of Delta, a transmembrane ligand that acts on adjacent cells to turn off* hairy *and allow* ato *expression, initiating photoreceptor differentiation.*

autoregulation in the eye disc; *ci* mutant clones do not result in premature photoreceptor differentiation (Fu and Baker, 2003, Pappu *et al.*, 2003), although Hh misexpression does (Heberlein *et al.*, 1995), and ectopic Ci75 cannot repress *hh* in the eye disc (Lee *et al.*, 2002). In the wing disc, Hh limits its own diffusion by upregulating the expression of Patched, a receptor that binds and sequesters Hh (Chen and Struhl, 1996). In the eye disc, Hh transport seems instead to be limited by apical constriction of cells in the MF, a shape change that is also Hh-dependent (Benlali *et al.*, 2000, Corrigall *et al.*, 2007, Escudero *et al.*, 2007, Heberlein *et al.*, 1995, Schlichting and Dahmann, 2008). Finally, Hh-expressing cells in the posterior compartment of the wing disc fail to respond to Hh signaling because the transcription factor Engrailed represses the expression of *ci* (Tabata *et al.*, 1995). In the eye disc, responsiveness to Hh is downregulated posterior to the MF by degradation of the Ci protein following its ubiquitination by an SCF complex containing Cullin 3 and Roadkill/Hh-induced MATH and BTB-containing protein, the same mechanism used in cells receiving high levels of Hh at the anterior-posterior boundary of the wing disc'(Kent *et al.*, 2006, Ou *et al.*, 2002, Ou *et al.*, 2007, Zhang *et al.*, 2006). The eye disc has integrated several secondary signals downstream of Hh to allow controlled movement of the boundary of Hh expression, which is stable throughout development in the wing disc.

Spacing of photoreceptor clusters is achieved through inhibitory mechanisms

To achieve a precisely ordered array of ommatidia in the adult eye, clusters within each column must initiate at regularly spaced intervals, and each column must also be offset from the preceding column. Each cluster initiates with the specification of its R8 cell, which depends on the proneural gene *ato* (Frankfort and Mardon, 2002). Tight control of *ato* expression is thus essential to guarantee the precision of the final lattice. *ato* is initially expressed in all cells in a stripe just ahead of the MF, and becomes restricted first to regularly spaced intermediate groups of about twenty cells, then to R8 equivalence groups of two to three cells, and finally to single cells that are the future R8 cells of each ommatidium (Fig. 3)(Baker *et al.*, 1996, Dokucu *et al.*, 1996, Jarman *et al.*, 1995).

Spacing of the intermediate groups is thought to involve an inhibitory signal from clusters in the preceding column that prevents new groups from forming directly anterior to existing clusters (Fig. 3A). The secreted glycoprotein Scabrous (Sca), which is expressed in a subset of cells in the intermediate groups and reaches its highest level in R8 (Baker *et al.*, 1990, Baker *et al.*, 1996, Baker and Zitron, 1995, Mlodzik *et al.*, 1990a), is a candidate for this signal. Sca secreted by R8 cells in posterior clusters is thought to diffuse anteriorly to repress *ato* expression between the forming intermediate groups; in *sca* mutants, R8 cells form too close together (Baker and Zitron, 1995, Lee *et al.*, 1996). The receptor for this activity of Sca is unknown. Although Sca interacts with Notch in other contexts (Baker and Zitron, 1995, Li *et al.*, 2003, Powell *et al.*, 2001), intermediate group spacing is unaffected by inactivation of Notch (Baker and Zitron, 1995, Lee *et al.*, 1996). The formation of intermediate groups coincides with organization of cells into clusters, in part because Ato activates the transcription of *DE-cadherin* (Baker and Yu, 1998, Brown *et al.*, 2006). Sca has been shown to alter DE-Cadherin localization and cell adhesion in

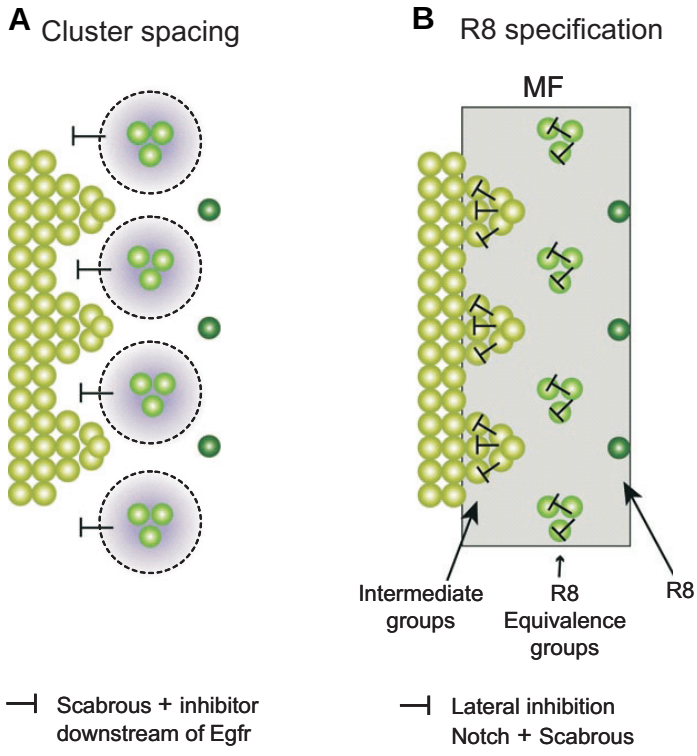

Fig. 3. Inhibitory signals control cluster spacing and R8 specification. **(A)** *Spacing of intermediate groups is controlled by inhibitory signals from clusters in the preceding column, including Sca and a second factor downstream of EGFR signaling.* **(B)** *The process of restriction of Ato expression within the MF from a continuous stripe of cells to intermediate groups, 3-cell R8 equivalence groups, and single R8 cells. R8 selection requires local lateral inhibition mediated by Notch and Sca.*

the notum (Renaud and Simpson, 2001), suggesting another possible mechanism for its effect on intermediate group spacing.

There may be another signal important for intermediate group spacing that is induced by EGFR signaling. Some studies have shown that *EGFR* mutant clones have a non-autonomous effect on spacing, suggesting that EGFR affects the secretion of a secondary factor involved in the restriction of *ato* expression (Chen and Chien, 1999, Spencer *et al.*, 1998). Since Ato activates *rho* expression (Baonza *et al.*, 2001), leading to secretion of Spi and activation of the EGFR pathway, this mechanism would function as a delayed feedback inhibitory loop that transmits spacing information from one column to the next, ensuring that successive columns are out of phase. The secreted signal involved has not been identified. One candidate is Argos (Aos), which is expressed in response to EGFR signaling and diffuses over a long range to bind the EGFR ligand Spi and antagonize its activity (Klein *et al.*, 2004, Schweitzer *et al.*, 1995, Spencer *et al.*, 1998). However, *aos* mutant clones show normal spacing unless the clones are very large (Baonza *et al.*, 2001, Yang and Baker, 2001), indicating that Aos does not transmit precise positional information about the location of each cluster. The molecule downstream of EGFR signaling must act in parallel to Sca in spacing intermediate groups, as *EGFR sca* double mutant clones have a more severe spacing defect than either single mutant (Baonza *et al.*, 2001). Since *sca* expression also depends on *ato* (Lee *et al.*, 1996), both signals

would operate through the same regulatory logic. However, the role of EGFR in the spacing process has been disputed (Rodrigues *et al.*, 2005).

Restriction of *ato* expression to a single R8 precursor cell within each intermediate group depends on lateral inhibition through the Notch receptor. The process of lateral inhibition has been well described during embryonic neurogenesis (Campos-Ortega and Jan, 1991). Slight differences between neighboring cells in the level of expression of the Notch ligand Dl are amplified because cells that receive a stronger Notch signal express less Dl. This process allows a single cell to be selected as the Dl-expressing neuron from a group of cells with equal potential (Bray, 1998). This mechanism appears to be conserved in the eye; when either *Notch* or *Dl* is inactivated using a temperature-sensitive allele, all cells in the intermediate groups continue to express *ato* and differentiate into extra R8 cells (Baker *et al.*, 1996, Baker and Yu, 1998, Baker and Zitron, 1995, Cagan and Ready, 1989a, Frankfort and Mardon, 2002) (Fig. 3B). This change from a positive effect of Notch and Dl on the early stripe of *ato* expression, described in section 2, to a negative effect on *ato* expression in the intermediate groups coincides with a switch in the enhancer element used to regulate *ato* (Baker *et al.*, 1996, Baker and Yu, 1997, Sun *et al.*, 1998). The 5' enhancer element used in the intermediate groups requires autoregulation by Ato, and Notch signaling appears to interfere with this autoregulatory process (Baker *et al.*, 1996, Sun *et al.*, 1998).

Precise spacing of R8 cells could not be achieved if R8 were randomly selected within the proneural cluster, suggesting that the choice must be biased in some way. However, examination of Dl expression did not reveal obvious differences between cells in the intermediate groups (Baker and Yu, 1998). One possible source of such a bias is Sca, which is secreted by the R8 precursor and can interact with Notch to sustain Notch signaling in surrounding cells (Baker and Zitron, 1995, Li *et al.*, 2003, Powell *et al.*, 2001). Sca appears to act within endosomes, perhaps preventing endocytic down-regulation of Notch (Li *et al.*, 2003). Another factor restricting the choice of R8 is Rough, a transcription factor that represses *ato* expression and is expressed in R2, 5, 3 and 4 under the control of EGFR signaling (Dokucu *et al.*, 1996, Dominguez *et al.*, 1998). Rough is excluded from the R8 precursor due to repression by Senseless, a transcription factor induced by Ato to lock in the R8 fate (Frankfort *et al.*, 2001). However, initial specification of the R8 precursor does not require Rough (Pepple *et al.*, 2008). Specification of R8 thus proceeds sequentially; the pattern of intermediate groups is imposed by signals from the preceding column, and a single R8 is selected within each group by biased lateral inhibition.

Sequential differentiation within each cluster depends on signaling range

Spatial and temporal patterning of cluster formation is achieved by controlling the differentiation of a single photoreceptor in each cluster, R8. R8 then initiates the sequential recruitment of the other ommatidial cell types. First R2/R5 and then R3/R4 are recruited to complete the five-cell precluster; the surrounding undifferentiated cells undergo a final cell division, the second mitotic wave, before the remaining cells are recruited in the order R1/R6, R7, anterior and posterior cone cells, and equatorial and polar cone cells (Tomlinson and Ready, 1987, Wolff and Ready, 1993) (Fig. 4). During pupal development, two primary pigment cells are recruited to surround each ommatidium, and the lattice of secondary and tertiary pigment cells is formed by death of the surplus cells (Cagan and Ready, 1989b). All these cell fates except R8 are directly or indirectly dependent on Spi signaling through the EGFR. Mosaic clones lacking *spi* or *EGFR* function contain only R8 cells (Dominguez *et al.*, 1998, Freeman, 1994, Tio *et al.*, 1994), and activation of the pathway can induce additional recruitment of all cell types except R8 (Dominguez *et al.*, 1998, Freeman, 1996).

The dependence of all cell types on the same inductive signal raises the question of how their differentiation is ordered in a precise temporal sequence. This seems to be due in part to the short range of action of the ligand Spi. Spi is initially secreted by R8 due to Ato-dependent expression of the proteases Rho and Ru (Baonza *et al.*, 2001, Wasserman *et al.*, 2000). Spi then induces

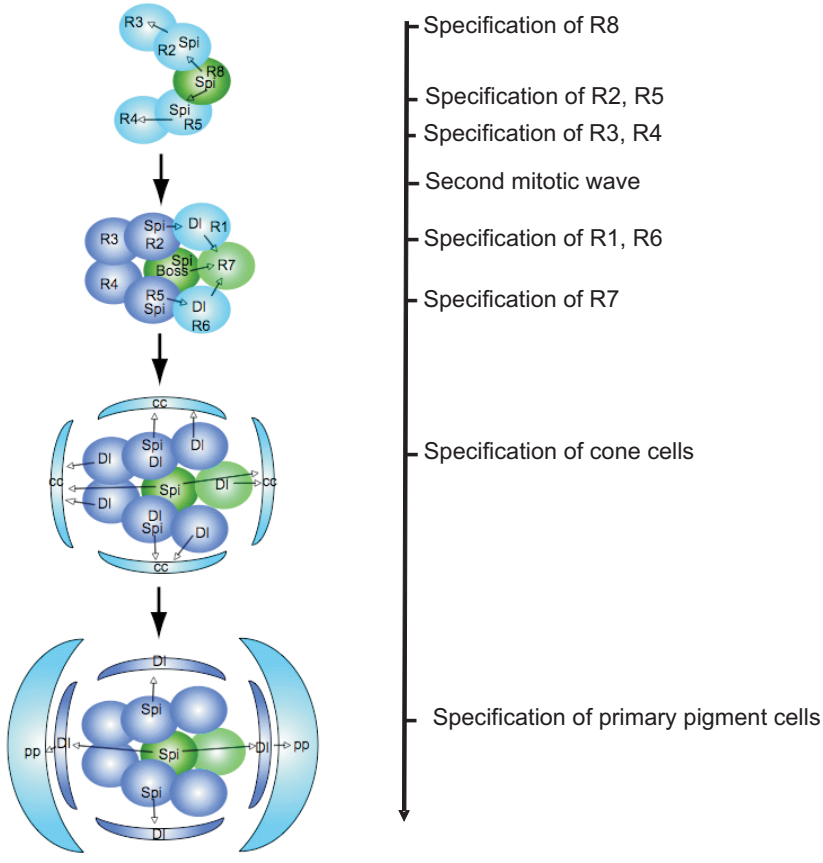

Fig. 4. Sequential recruitment of ommatidial cells is controlled by the short-range ligands Spi and Dl. *Spi promotes the differentiation of photoreceptors R2, 5, 3, 4, 1 and 6. It also induces the expression of Dl, which acts together with Spi and Boss to promote R7 differentiation, together with Spi to promote cone cell differentiation, and alone to promote primary pigment cell differentiation. Cells are added sequentially because Spi and Dl can only act over a short distance.*

the cells immediately neighboring R8 to differentiate into R2 and R5. These cells also express Rho, under the control of the transcription factor Rough (Freeman *et al.*, 1992a), as well as the chaperone protein Star (Heberlein *et al.*, 1993a), and can therefore produce Spi to induce the differentiation of their neighbors in the arc-shaped cluster into R3 and R4 (Freeman, 1997, Wolff and Ready, 1991). It has been proposed that an autoregulatory loop expands the expression of Rho and Ru to additional photoreceptors as they differentiate, allowing subsequent waves of recruitment (Freeman, 1997, Shilo, 2005). However, mosaic analysis has shown little evidence of a requirement for *rho*, *ru*, *Star* or *spi* in any photoreceptors other than R8, 2 and 5 (Freeman, 1994, Heberlein and Rubin, 1991, Tio *et al.*, 1994, Wasserman *et al.*, 2000). Indeed, R1, 6 and 7 differentiate adjacent to R8, 2 and 5 (Tomlinson and Ready, 1987), suggesting that they are responding to Spi produced by these cells. Spi secreted from other photoreceptors may nevertheless contribute to the recruitment of cone and pigment cells, which were not examined in the mosaic studies.

One reason for the sequential, rather than simultaneous, recruitment of photoreceptors is likely to be the very short range of Spi action, which is limited to 1-2 cell diameters by several mechanisms (Miura *et al.*, 2006, Schlesinger *et al.*, 2004, Wasserman *et al.*, 2000). Palmitoylation of the cleaved extracellular domain of Spi tethers it to the plasma membrane, restricting its diffusion (Miura *et al.*, 2006). Small wing, a phospholipase Cγ, prevents the secretion of any Spi that is cleaved in the endoplasmic reticulum by Ru (Schlesinger *et al.*, 2004). Finally, Spi signaling induces the expression of a secreted feedback inhibitor, Aos (Golembo *et al.*, 1996), which binds to Spi and prevents it from binding to the EGFR (Klein *et al.*, 2004). *aos* mutant eyes contain extra photoreceptors, cone cells and pigment cells (Freeman *et al.*, 1992b). Aos may prevent Spi from reaching distant cells, restricting its activity to the region where its local concentration exceeds that of Aos (Freeman, 1997). Although mosaic studies suggest that Aos can act over a range of about 10 cell diameters (Freeman *et al.*, 1992b), mathematical modeling of Aos function in the embryo indicates that Aos could exert a long-range effect by acting as a ligand sink, without itself diffusing over a long distance (Reeves *et al.*, 2005). Interestingly, a second negative feedback loop involving the intracellular inhibitor Sprouty subsequently turns off *aos* expression in R8, 2 and 5; *aos* is maintained in other photoreceptors because *sprouty* expression is inhibited by the transcription factor Seven-up (Svp) expressed in these cells (Iwanami *et al.*, 2005). This reduction in Aos production may allow Spi levels to increase sufficiently to induce the later differentiating cell types.

The use of Spi signaling to recruit all ommatidial cell types raises the question of how diversity is generated among these cells. Cell types are not simply determined by the level of EGFR activity, since ectopic activation of the EGFR pathway induces different cell types at different stages of development (Freeman, 1996, Hayashi and Saigo, 2001). One possible explanation is that Spi signaling acts in combination with other developmental signals. For example, R7 differentiation requires the activity of a second receptor tyrosine kinase, Sevenless (Sev), as well as the EGFR (Zipursky and Rubin, 1994). Bride of sevenless (Boss), the ligand for Sev, is a transmembrane protein specifically expressed in R8, which acts on the adjacent undifferentiated cell to induce its differentiation into R7 (Zipursky and Rubin, 1994). The combination of Sev and EGFR signaling is thought to increase signal transduction through their common downstream pathway, resulting in high-level expression of target genes such as *prospero* (Freeman, 1996, Tio and Moses, 1997, Xu *et al.*, 2000).

Another signal that interacts with Spi to promote cell fate diversity is the Notch ligand Dl (Fig. 4). Notch and Dl are required for the differentiation of R7, cone cells and primary pigment cells (Flores *et al.*, 2000, Nagaraj and Banerjee, 2007, Tomlinson and Struhl, 2001, Tsuda *et al.*, 2002). Dl is itself a target of EGFR signaling, which induces *Dl* transcription by promoting nuclear export and degradation of a corepressor for the Suppressor of Hairless transcription factor (Tsuda *et al.*, 2002). This relationship between the two signals introduces another temporally delayed autoregulatory loop. Spi signaling induces R1-6 to differentiate and to express *Dl*; Dl subsequently acts in combination with Spi to induce differentiation of R7 and the cone cells (Flores *et al.*, 2000, Tomlinson and Struhl, 2001, Tsuda *et al.*, 2002). As EGFR activation in the cone cells increases during the pupal stages, they

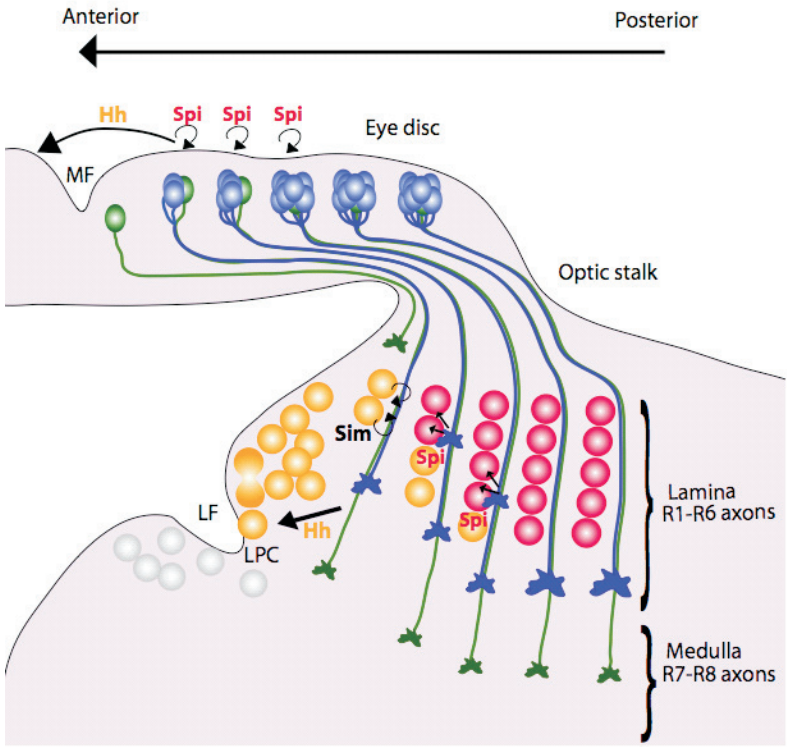

Fig. 5. Hh and Spi signals are transported down the photoreceptor axons to organize the target region. *Hedgehog (Hh) promotes the final division of lamina precursor cells (LPC) posterior to the lamina furrow (LF), and through Sim, their association with photoreceptor axons. Hh also induces the LPCs to express Dac, which activates expression of the EGFR, allowing them to respond to Spi. Cells that respond to Hh by expressing Dac and EGFR are colored yellow. Spi then promotes LPC differentiation adjacent to retinal axons. Cells that respond to Spi by expressing Elav are colored red. Release of the same signals from the cell body and the axons coordinates morphogenesis of the eye and the lamina.*

too express *Dl*, which is sufficient to induce differentiation of adjacent cells into primary pigment cells (Daga *et al.*, 1996, Nagaraj and Banerjee, 2007). These cells require EGFR signaling only indirectly as an activator of *Dl* expression in cone cells (Nagaraj and Banerjee, 2007). Dl is a transmembrane molecule that can only signal to immediately adjacent cells (Artavanis-Tsakonas *et al.*, 1999); this limit on its range of action again contributes to the ordered sequence of cell recruitment.

A third contribution to cell fate diversity comes from intrinsic properties of the responding cells. The transcription factor Lozenge (Lz) is expressed in undifferentiated cells posterior to the MF and in cells that differentiate after the second mitotic wave: R1, R6, R7, and the cone and pigment cells (Flores *et al.*, 1998). Its misexpression in R3 and R4 transforms these cells into R7 cells, due to its positive effect on R7-specific genes such as *prospero* and its negative effect on genes such as *seven-up* that promote the identity of R3 and R4 (Daga *et al.*, 1996, Flores *et al.*, 1998, Xu *et al.*, 2000). Lz also positively regulates the *Bar* genes, which are expressed in R1 and R6 (Daga *et al.*, 1996, Higashijima *et al.*, 1992). Expression of *lz* itself requires the transcription factor Glass, which is expressed in all cells posterior to the MF, and the retinal determination protein So, but it is unknown how *lz* is excluded from R8, 2, 5, 3 and 4 (Moses and Rubin, 1991, Yan *et al.*, 2003). Other transcription factors expressed in different subsets of photoreceptors include Rough (R2, 5, 3, 4), Spalt (R3, 4), and Svp (R3, 4, 1, 6) (Barrio *et al.*, 1999, Kimmel *et al.*, 1990, Mlodzik *et al.*, 1990b). These specific expression patterns are likely to result from similar combinatorial control mechanisms as well as cross-regulatory interactions (Hayashi and Saigo, 2001, Heberlein *et al.*, 1991). In summary, differentiation of distinct cell types in an ordered sequence appears to be due to the combination of temporally delayed autoregulatory loops of the short-range ligands Spi and Dl with intrinsic prepatterning information in the form of transcription factor expression.

Photoreceptors coordinate the differentiation of their target cells in the lamina

As photoreceptor cells progressively differentiate in the eye disc, they extend axons into the brain, where R1-6 terminate in the lamina and R7 and R8 project through the lamina to terminate in the medulla (Clandinin and Zipursky, 2002). Within the lamina, the bundle of photoreceptor axons from each ommatidium associates with a cartridge composed of five lamina neurons, and these fascicles terminate in a retinotopic pattern, recreating a map of the visual field in the brain (Clandinin and Zipursky, 2002). Along the anterior-posterior axis, this organization arises because signals from the photoreceptor axons induce the formation of their target cells. Hh, which drives the propagation of the MF within the eye disc, is also transported along the photoreceptor axons and released to induce the final division of lamina precursor cells (LPCs) and their expression of the differentiation marker Dachshund (Dac) (Huang and Kunes, 1996) (Fig. 5). Interestingly, this axonal transport requires a targeting signal that lies within the C-terminal protease domain of Hh (Chu *et al.*, 2006), although LPCs respond to the N-terminal secreted domain through the canonical Hh signaling pathway (Huang and Kunes, 1998). Hh also induces the expression of the transcription factor Single-minded, which directs developing LPCs to associate with photoreceptor axons (Umetsu *et al.*, 2006). Each column of ommatidia in the eye disc thus induces the formation of a corresponding column of target cells, allowing progressive posterior to anterior differentiation to be coordinated between the eye and the brain.

In addition, Hh acts through Dac to induce LPCs to express the EGFR, making them responsive to a second signal transported down the axons (Chotard *et al.*, 2005, Huang *et al.*, 1998). Spi, the signaling molecule that recruits photoreceptors to each ommatidial cluster, is also essential to direct the assembly of each lamina cartridge (Huang *et al.*, 1998). Spi is necessary and sufficient for the neuronal differentiation, indicated by Elav expression, of the five lamina neurons associated with each ommatidial fascicle (Huang *et al.*, 1998). The very short-range activity of Spi presumably ensures that lamina neurons differentiate only in the immediate vicinity of retinal axons. Coordination of eye and brain development achieved by using the same signals in both tissues is likely to be important to establish the spatial precision necessary for accurate vision.

Conclusions

The progressive development of identical units within a monolayer epithelium makes the *Drosophila* eye disc an excellent system in which to address the regulatory mechanisms of pattern formation. Positive autoregulatory loops play an important role in driving the progression of differentiation, and a temporal delay can be introduced into such loops by requiring the production of a second signal dependent on the first. The range over which signals are distributed is also an important factor in controlling the sequence of differentiation. Although columns differentiate sequentially, they can also interact with each other through secreted signals that determine the placement of clusters. Finally, the long processes formed by neurons allow them to act on different target cell populations by releasing the same signals from their cell bodies and axon terminals. Several aspects of this differentiation process have now been described at a level at which mathematical modeling could be applied to test and extend our understanding of the mechanisms involved.

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