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My What Big Eyes You Have: How the *Drosophila* Retina Grows

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Summary

The compound eye of the fruit fly, *Drosophila melanogaster*, has for decades been used extensively to study a number of critical developmental processes including tissue development, pattern formation, cell fate specification and planar cell polarity. To a lesser degree it has been used to examine the cell cycle and tissue proliferation. Discovering the mechanisms that balance tissue growth and cell death in developing epithelia has traditionally been the realm of those using the wing disc. However, over the last decade a series of observations has demonstrated that the eye is a suitable and maybe even preferable tissue for studying tissue growth. This review will focus on how growth of the retina is controlled by the genes and pathways that govern the specification of tissue fate, the division of the epithelium into dorsal-ventral compartments, the initiation and progression of the morphogenetic furrow and the second mitotic wave.

Keywords

Drosophila; retina; cell proliferation; growth

1. Introduction

Growth of the *Drosophila* retina occurs in two waves during development. The first mitotic wave, as the initial burst of growth is called, is a continuous process that begins prior to the initiation of the morphogenetic furrow and continues in regions of the disc that lie anterior to the advancing differentiating front. By contributing to the number of cells within anterior regions of the eye, this first mitotic wave essentially sets the upper limit on how many ommatidia can be generated. Several developmental events such as tissue specification, midline formation and movement of the furrow itself will contribute to the overall impact that the first mitotic wave has on tissue growth. The second epoch of proliferation occurs posterior to the furrow and is called the second mitotic wave. This tight band of cell division is responsible for generating a subset of photoreceptor neurons as well as all accessory cone and pigment cells. The second mitotic wave gives birth to the majority of cells that comprise the eye and thus is key to setting the final overall size of the retina.

Studying tissue growth in the eye, particularly that of the first mitotic wave, presents a challenge since the cellular events that promote growth are not always cleanly separated in developmental time or geographical space. For example, cells within the first and second instar eye disc are simultaneously adopting a retinal fate while being sorted into dorsal and ventral compartments. As we will see later, both of these processes are important influences on the first mitotic wave. An equally important consideration is that the factors identified as promoting tissue identity are the very same ones establishing the midline. This can sometimes make it difficult to unambiguously attribute an increase or decrease in growth rates to either developmental process. Examinations of cell proliferation within the second mitotic wave presents similar challenges as several pathways that promote cell proliferation are also important for cell fate decisions in adjacent cells. How alterations in cell fates affect

cell proliferation in surrounding cells is a difficult question to answer with absolute confidence.

Interestingly though, the very issues that make studying cell proliferation in the retina a challenge are what make the developing fly eye an attractive system for studying tissue growth. We can take advantage of the integrative nature of eye development to uncover the mechanisms by which populations of cells simultaneously receive, sort, interpret and execute multiple growth signals. The asynchronous development of the eye also provides us with an opportunity to analyze the mechanisms by which a tissue recalibrates its rate of growth in real time. In this review I will attempt to document mechanisms by which retinal growth is promoted by the first and second mitotic waves.

2. Growth of Imaginal Discs: Unequal Beginnings

Adult compound eyes, like all other adult *Drosophila* structures, are derived from monolayer epithelia called imaginal discs. These epithelial sheets arise during embryogenesis when small numbers of cells are set aside from the rest of the developing embryo and begin developing asynchronously from the rest of the organism (Miall and Hammond, 1892; Kellog, 1902; Essa, 1953; Anderson, 1972a,b; Crick and Lawrence, 1975; Cohen, 1993; Held, 2002). Reflecting the differing sizes of adult appendages, each type of mature imaginal disc also contains distinct numbers of cells (Becker, 1957; Garcia-Bellido et al., 1970; Garcia-Bellido and Merriam, 1971; Morata and Garcia-Bellido, 1976; Steiner, 1975; Steiner, 1976; Martin, 1982). The different final organ sizes are achieved in part through three initial inequalities in growth control (Figure 1). First, distinct numbers of cells are set-aside during embryogenesis for each type of imaginal disc (Bryant and Schneiderman, 1969; Postlethwait and Schneiderman, 1971; Wieschaus and Gehring, 1976; Lawrence and Morata, 1977; Madhaven and Schneiderman, 1977). Second, the onset of the cell proliferative period differs for each type of disc (Madhaven and Schneiderman, 1977). And third, the cell-doubling rate is different for each kind of disc (Patterson, 1929; Bryant, 1970; Garcia-Bellido and Merriam, 1971; Postlethwait and Schneiderman, 1977; Haynie, 1975; Morata and Garcia-Bellido, 1976; Steiner, 1976; Madhaven and Schneiderman, 1977). Together, the inequality in starting cell numbers and the unique starting time and rate of proliferation allows for the eye disc to achieve a final size that is sufficient for the formation of approximately 800 ommatidia and accessory mechanosensory bristle complexes (Figure 1).

3. Retinal Determination Genes as Regulators of Growth

Tissue selector genes commit the small clusters of cells that are set-aside during embryogenesis to adopting distinct organ fates. Once the initial fate decisions are made, then growth control mechanisms take over and generate the appropriate numbers of cells. For example, during the final larval instar stage, the eye-antennal, wing, haltere and leg discs contain approximately 44,000, 49,000, 10,000 and 21,000 cells respectively (Becker, 1957; Garcia-Bellido et al., 1970; Garcia-Bellido and Merriam, 1971; Morata and Garcia-Bellido, 1976; Steiner, 1975; Steiner, 1976; Martin, 1982). Interestingly, mutations that alter the fate of the entire organ or tissue also reset cell proliferation targets. For example, gain-of-function Antp mutations and loss-of-function Ubx alleles are characterized by the conversion of antenna and halteres to legs and wings respectively (Lewis, 1978; Schneuwly et al., 1986; Schneuwly et al., 1987a,b). The ectopic organs are indistinguishable in size when compared to normal wings and legs. Thus, the initial act of committing tissues to defined fates sets in motion a series of developmental steps that ensure a degree of growth that is commensurate with the assigned tissue fate.

In the eye primordium, tissue specification is governed by a set of interlocking selector genes collectively termed the eye specification or retinal determination network (Figure 2A). Included within the network are the Pax6 genes *eyeless* (ey: Quiring et al., 1994) and *twin of eyeless* (toy: Czerny et al., 1999), the Pax6-like factors *eyegone* (eyg: Jang et al., 2003) and *twin of eyegone* (toe: Aldaz et al., 2003), the Six family members *sine oculis* (so: Cheyette et al., 1994; Serikaku and O'Tousa 1994) and *optix* (Seimiya and Gehring, 2000), the zinc finger transcription factors *teashirt* (tsh: Pan and Rubin, 1998) and *tiptop* (tio: Bessa et al., 2009; Datta et al., 2009), the pipsqueak DNA binding proteins *distal antenna* (dan: Curtiss et al., 2007) and *distal antenna related* (danr: Curtiss et al., 2007), the transcriptional co-activator and protein phosphatase *eyes absent* (eya: Bonini et al., 1993), a homolog of the Ski/Sno oncogenes *dachshund* (dac: Mardon et al., 1994), and the Nlk homolog *nemo* (Braid and Verheyen, 2008). A large body of experimental evidence has demonstrated that these genes regulate eye development by functioning through a labyrinth of complex genetic, molecular and biochemical interactions (Figure 2B).

These retinal determination genes share two unique and special characteristics. First, loss-of-function mutations often lead to a near complete loss of retinal tissue (Figure 2C; Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Serikaku and O'Tousa, 1994). The loss of retinal identity is accompanied by a severe inhibition of tissue growth and a dramatic increase in programmed cell death. (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). As a consequence, the larval disc is significantly smaller than wild type and the morphogenetic furrow fails to initiate thereby giving rise to an adult that is missing its compound eyes. As we will see below, the reduction in size is not a non-specific effect. Several retinal determination genes are tasked primarily with promoting cell proliferation within the eye disc (Singh et al., 2002; Chao et al., 2004; Dominguez et al., 2004; Singh et al., 2004; Lopes and Cesaras, 2009; Peng et al., 2009). A few are even involved in the direct regulation of cell cycle checkpoints (Jemc and Rebay, 2007).

The genes that comprise this network are also considered special because forced expression in non-retinal tissues is sufficient to coax populations of cells into forgoing their initial fate and into adopting an eye fate (Figure 2D; Halder et al., 1995; Bonini et al., 1997; Shen and Mardon 1999; Cznery et al., 1999, Seimiya and Gehring, 2000; Jang et al., 2003; Curtiss et al., 2007; Weasner et al., 2007; Braid and Verheyen, 2008; Yao et al., 2008; Bessa et al., 2009; Datta et al., 2009). Unlike homeotic genes, expression of retinal determination genes is not capable of transforming an entire tissue. Only small numbers of cell populations within imaginal discs have enough developmental plasticity to adopt an eye fate (Salzer et al., 2010). Thus, in these forced expression assays, the retinal determination genes can only influence growth locally.

While the network is organized into a loose hierarchy, expression of genes at the top of the network is often dependent upon factors that lie at lower levels (reviewed in Kumar, 2010). Thus, the small disc phenotype that is seen in loss-of-function mutants does not necessarily mean that every member of the retinal determination network promotes cell proliferation. *ey* is a case in point. It is one of only four network genes that are expressed during the early embryonic growth phase of the eye primordium. Loss of this expression leads to severe reductions in the size of the eye disc (Quiring et al., 1994). Forced expression of *ey* can also partially rescue the growth defects of *eyg* mutants (Jang et al., 2003). Together these observations initially suggested that *ey* promotes growth of the retina. However, an insightful paper by Dominguez and colleagues revealed that *eyg* and to a lesser degree its paralog *toe* are the true growth promoters in the retina (Dominguez et al., 2004). It had been previously established that the Notch pathway is a major inducer of cell proliferation in the retina (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998).

The authors demonstrated that only forced expression of *eyg*, *toe* and their mammalian homolog *Pax6(5a)* were capable of rescuing the growth defects of *Notch (N)* mutants, In contrast, expression of *ey* and its paralog *toy* failed to restore growth the mutant discs (Dominguez et al., 2004).

Over the years several retinal determination genes have been implicated in directly regulating growth. These include *so* (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), *eya* (Bonini et al., 1993), *hth* (Pai et al., 1997), *tsh* (Pan and Rubin, 1998) as well as *eyg* (Jun et al., 1998; Jang et al., 2003). The role of *eyg* in promoting growth appears to be related to midline formation and will be discussed below. The part that the remaining genes play in inducing cell proliferation appears tied to their roles in tissue specification and will be discussed here.

3.1 Teashirt and Homothorax: The RD Network Meets the Hippo Pathway

Several lines of evidence support roles for both *hth* and *tsh* in promoting growth in the developing eye. First, cells mutant for *hth* rarely survive in the anterior eye (Pichaud and Casares, 2000; Bessa et al., 2002) while cells over-expressing *tsh* overgrow (Singh et al., 2002). The genes are functionally dependent upon each other as expression of *tsh* leads to the induction of *hth* transcription (Singh et al., 2002) and as Hth and Tsh physically interact with each other (along with Ey) to repress the expression of downstream transcriptional targets (Bessa et al., 2002). Insight into potential mechanisms for *tsh* and *hth* in regulating growth came initially from an analysis of the expression patterns of each retinal determination network member (Bessa et al., 2002). Based on distinct transcriptional profiles the eye field can be divided into several regions (zone A–F) with the anterior portion of the eye occupying zones A–C (Figure 3; reviewed in Kumar, 2010).

Along the eye/antenna border (zone A) the only retinal determination gene that is expressed is *hth* along with its co-factor *extradenticle* (exd: Pai et al., 1997; Pichaud and Casares, 2000; Bessa et al., 2002). These cells are fated to adopt a head capsule fate and do not contribute to growth of the eye (Haynie and Bryant, 1986). Indeed, loss of either *hth* or *exd* within these cells leads to the formation of ectopic eyes with little effect on growth (Rauskolb et al., 1995; Pai et al., 1997; Gonzales-Crespo et al., 1995).

Just adjacent lies a narrow swathe of cells (Zone B) that are locked into a highly proliferative state. Several retinal determination genes including *ey/toy* (Quiring et al., 1994; Czerny et al., 1999), *eyg/toe* (Aldez et al., 2003; Jang et al., 2003), *hth* (Bessa et al., 2002), *tsh/tio* (Pan and Rubin 1998; Bessa et al., 2009; Datta et al., 2009) and *dan/danr* (Curtiss et al., 2007) are expressed within this tract of cells. One key to maintaining cells within this zone in a proliferative state is the repression of three retinal determination genes: *so*, *eya* and *dac*, a task that appears to be accomplished by the Tsh-Hth-Ey complex described above (Bessa et al., 2002). A consequence of this repression in Zone B that is relevant to tissue growth is down-regulation of *string* (*stg*), the fly homolog of yeast cdc25 and regulator of the G2/M transition. In zones A and B where *hth* is expressed *stg* expression is absent. In contrast, as *hth* expression is eliminated in cells just ahead of the furrow (zone C) *stg* expression is activated. A genetic relationship between these two genes exists as clones lacking *hth* show ectopic *stg* expression (Lopes and Casares, 2009). But *hth* does not appear to promote tissue growth through direct regulation of *stg*. As we will see below that appears to be the task of the *so* and *eya* genes. In cells closest to the morphogenetic furrow (Zone C) *hth* is lost and as a result the repression of *so*, *eya* and *dac* is relieved. The initiation of *so* and *eya* transcription will lead to the activation of *stg* (Jemc and Rebay, 2007) which is a key step in preparing cells for entrance into the furrow where they will undergo a genomic switch that will transition them to a state of terminal differentiation (Figure 4; Jasper et al., 2002).

In addition to the regulation of the cell cycle via *stg*, a recent study demonstrated that *tsh* and *hth* promote tissue growth through interactions with the Hippo tumor suppressor pathway (Peng et al., 2009). Hippo signaling is essential for correct growth control in both *Drosophila* and mammals (Dong et al., 2007; Pan, 2007; Halder and Johnson, 2011). The target of this pathway is *yorkie* (*yki*), a transcriptional co-activator (Huang et al., 2005; Dong et al., 2007, Oh and Irvine, 2008; Zhang et al., 2008). In its unphosphorylated state, Yki is localized to the nucleus and participates in the activation of growth promoting genes. One well-established binding partner is the TEAD/TEF transcription factor Scalloped (Sd: Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008). As loss of *sd* has little to no effect in the retina, the proliferative effects that Yki has in the eye must be mediated through other DNA binding proteins. The identity of that binding partner came to light when it was discovered that tissue overgrowths induced by simultaneous expression of *tsh* and *hth* are mediated by the Hippo pathway. Hth does indeed form a biochemical complex with Yki. The Hth-Yki transcription factor binds to an enhancer within the *bantam* (*ban*) microRNA locus thereby activating its expression (Peng et al., 2009). *ban* is known to block cell death by binding to the 3` UTR of *head involution defective* (*hid*) mRNA thereby inhibiting its translation (Brennecke et al., 2003). *ban* has been previously identified as a genetic target of the Hippo pathway in the wing (Nolo et al., 2006; Thompson and Cohen, 2006). It now is considered a molecular target of both Hippo and the RD network in the eye (Figure 4).

3.2 The So-Eya Complex: Talking to the Cell Cycle

The loss of either *so* or *eya*, the founding members of the Six and Eya protein families, in the *Drosophila* retina is characterized by a dramatic loss of the adult compound eyes. This loss in tissue identity is accompanied by a massive increase in programmed cell death thereby resulting in an eye disc that is only a fraction of normal size (Milani 1941; Milani 1951; Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O'Tousa, 1994). However, simple over-expression of *so* or its paralogs, *DSix4* and *optix*, does not lead to tumorigenesis in flies. This is in contrast to the dramatic effects on tissue growth that are seen when members of the Six or Eya families are mis-regulated in vertebrates. For example, over-expression of *Six3*, a vertebrate homolog of *so*, induces enlargement of the zebrafish rostral forebrain (Kobayashi et al., 1998). And inappropriate levels of several Six and Eya family members are observed in a multitude of human cancers (reviewed in Christensen et al., 2008). In total, these results suggest that Six and Eya family members function as cell survival factors as well as growth promoters.

So is a homeodomain-containing DNA binding protein (Cheyette et al., 1994; Serikaku and O'Tousa, 1994) while Eya is a transcriptional co-activator and protein tyrosine phosphatase (Pignoni et al., 1997; Xu et al., 1997; Ohto et al., 1999; Li et al., 2003; Silver et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Through direct physical interactions these proteins form a composite transcription factor (Pignoni et al., 1997) which initiates the expression of other retinal determination network members, genes that are involved in cell fate specification, as well as factors that control the movement of the morphogenetic furrow (Suzuki and Saigo, 2000; Yan et al., 2003; Pappu et al., 2005; Pauli et al., 2005; Zhang et al., 2006; Tanaka-Matakatsu and Du, 2008). Evidence from both flies and vertebrates indicates that the So-Eya complex influences cell proliferation by directly regulating several cell cycle components. In human cells, Six1 is known to regulate *c-myc*, *cyclin D1*, *cyclin A1* (Li et al., 2003; Coletta et al., 2004; Yu et al., 2006). In the fly retina the So-Eya complex has been shown to bind to an enhancer element within the *stg* locus (Edgar and O'Farrell, 1989; Jemc and Rebay, 2007). *stg* is expressed in a tight band just ahead of the morphogenetic furrow and is thought to shepherd the asynchronously dividing cells ahead of the furrow through G2 and into mitosis (Thomas et al., 1994). This is thought to be

important for synchronizing cells within the furrow where they are arrested in G1 (Ready et al., 1976; Horsfield et al., 1998; Escudero and Freeman, 2007; Firth et al., 2010). The loss of *stg* arrests cells in G2 and prevents them from entering mitosis and results in the over-recruitment of photoreceptor and cone cells (Mozer and Easwarachandran, 1999). Thus, one function of the So-Eya complex may be to synchronize cells at the G1 phase by activating and maintaining *cdc25/stg* expression ahead of the furrow (Figure 4).

4. The Midline: Growth from the Center

The division of a homogeneous tissue into broad compartmental domains is one of the first steps towards generating a system with multiple cell types. The *Drosophila* eye is initially borne with only a ventral address (Singh et al., 2003). This homogeneity is broken during the first larval instar when a dorsal fate is imposed on a subset of the eye field thereby creating two distinct retinal zip codes: ventral and dorsal (Maurel-Zaffran and Treisman, 2000; Singh et al., 2005). At the molecular level, the dorsal and ventral fates arise by the juxtaposition of selector genes (Iro-C and Slp1/2) whose expression patterns meet at but never cross the midline (Figure 5A, left panel; McNeill et al., 1997; Sato and Tomlinson, 2007). As a consequence, ventral cells express *fringe* (*fng*) while dorsal cells do not (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The confrontation of fng^{+} and fng^{-} cells is conserved although the orientation in the eye is inverted when compared to other tissues such as the wing (Irvine and Wieschaus, 1994). As a result, cells at the midline undergo Notch dependent tissue growth. Ubiquitous expression of *fng* throughout the eye, or the loss of Notch signaling blocks growth and the resulting eye discs are nearly indistinguishable from those that harbor mutations within the retinal determination network (Cagan and Ready, 1989; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). In contrast, activation of Notch signaling leads to tissue over-growth (de Celis et al., 1998; Go et al., 1998; Reynolds-Kenneally and Mlodzik, 2005).

4.1 Induction and Maintenance of Notch Signaling at the Midline

In *Drosophila*, the ligands for the N receptor are encoded by *Delta* (*Dl*) and *Serrate* (*Ser*: Dexter, 1914; Fehon et al., 1990; Fleming et al., 1990; Rebay et al., 1991; Thomas et al., 1991; Lieber et al., 1992; de Celis et al., 1993; Kooh et al., 1993). In the wing, where Notch signaling has been extensively studied, Dl and Ser are expressed in complementary patterns with the former restricted to the ventral compartment and the latter confined to dorsal territories (Doherty et al., 1996). In the eye the situation is reversed with *Dl* being present in the dorsal domain and *Ser* present in the ventral compartment (Cho and Choi, 1998; Cho et al., 2000). In both tissues the expression and activation of the N receptor is at its highest along the dorso-ventral boundary (Huppert et al., 1997). The juxtaposition of ligands on either side of the midline creates a positive-feedback loop that maintains Notch signaling across the midline and as a consequence promotes growth in the retina (Figure 5A, middle panel).

During development the patterns of *N*, *Dl* and *Ser* expression can vary considerably. At certain stages, *N* expression will be coincident with *Dl* and *Ser* while at other stages the receptor will be expressed in a complementary pattern to its ligands (Kooh et al., 1993). In the eye, cells along the dorsal side of the midline co-express *N* and *Dl* while their counterparts along the ventral side of the midline co-express *N* and *Ser*. Similar receptor-ligand relationships are also seen in the developing wing and are thought to mediate a positive-feedback loop between cells that straddle the D/V border (Diaz-Benjumea, 1995; Huppert et al., 1997). In the eye the model is that *Ser* expression in the ventral compartment signals across the midline to induce Notch-dependent activation of *Dl* transcription. *Dl*, in the dorsal compartment, in turn signals back across the midline to activate *Ser* expression

via *N*. As *Dl* and *Ser* are short range signaling molecules this reinforcing feedback mechanism has the effect of increasing *N* signaling just at the midline (Figure 5A, middle panel).

A role for the Ser ligand in the activation of N-dependent growth came from demonstrations that loss-of-function *Ser* mutants had small eyes (Speicher et al., 1994; Go et al., 1998). There was immediate interest in identifying the mechanisms that activated *Ser* expression. Its restriction to the ventral compartment suggested the upstream regulators would themselves either be expressed or function only within the ventral half of the eye. Earlier reports had proposed that, in the wing, *fng* could function as an upstream positive regulator of *Ser* (Diaz-Benjumea and Cohen, 1993; Kim et al., 1995). This also turned out to be case in the eye as loss of *fng* within the ventral compartment results in a down regulation of *Ser* expression (Cho and Choi, 1998). Another regulator of Ser expression turned out to be the product of the *Lobe* (*L*) gene. *L* mutants were first identified by the presence of a small eye (Morgan et al., 1925). And although *L* is expressed in both dorsal and ventral compartments the tissue that is lost is exclusively of ventral identity. In clones that lack *L*, *Ser* expression is also down regulated (Chern and Choi, 2002). Taken together, the evidence so far indicates that the selective induction of *Ser* in the ventral half of the eye is due to the activities of *fng* and *L* (Figure 5A. right panel).

4.2 Eyegone: At the Intersection of Growth Control and the RD Network

Pax genes encode transcription factors that are defined by the presence of two DNA binding domains: a Paired domain and a Homeodomain. The N-terminal (PAI) and C-terminal (RED) segments of the Paired domain can each interact with DNA when expressed separately but in the context of an intact Paired domain binding will occur pre-dominantly through the PAI domain. The RED domain becomes the predominant DNA binding domain only when the PAI domain is either removed or its function blocked (Czerny et al., 1993; Epstein et al., 1994; Kozmik et al., 1997). In flies, this situation manifests itself in the form of two Pax6-like proteins, Eyg and Toe, both of which contain only an intact RED domain (Jones et al., 1998; Jun et al., 1998; Jang et al., 2003; Aldaz et al., 2003). Similar situations exist in vertebrates as alternate splicing events in the *Pax3*, *Pax6* and *Pax8* genes generate protein isoforms that all contain a disrupted and inactive PAI domain (Cnerny et al., 1993; Epstein et al., 1994; Kozmik et al., 1997; Chi and Epstein, 2002).

Adult flies mutant for *eyg* display retinal phenotypes that range from having small to no compound eyes. This is reflected in the below average size of third instar larval eye disc (Jang et al., 2003). Forced over-expression of either *eyg* or *toe* induces tissue proliferation in areas of the surrounding head capsule that already expresses retinal determination factors but cannot induce ectopic eye formation in tissues such as the wing, leg and halteres (Jang et al., 2003; Yao et al., 2008). Taken together these results suggest that Eyg and Toe may play significant roles in promoting growth in the eye.

A link between *eyg* and Notch-dependent growth was first intimated by a comparison of expression patterns. At the midline *eyg* expression overlaps that of Notch signal activation (Jang et al., 2003). The same is true of *toe*, whose expression is identical to *eyg* (Yao et al., 2008). A functional connection between both Pax6-like proteins and Notch activation came from a pair of manuscripts showing that forced expression of either *eyg* or *toe* is sufficient to rescue the growth defects associated with loss of N. Similarly, loss of either Pax6-like gene could suppress the increased growth that results from hyper-activation of the Notch pathway (Chao et al., 2004; Dominguez et al., 2004). These reports also showed that Notch signaling induced expression of *eyg*. Of all the retinal determination genes, only *eyg* and *toe* mediate Notch-dependent induction of cell proliferation in the retina. Despite the small disc phenotype seen in *ey* mutants, forced expression of *ey* (and its paralog *toy*) fail to rescue the

growth defects of *N* mutants (Dominguez et al., 2004). As *ey* lies genetically and molecularly upstream of both *so*, *eya* and *dac* it is unlikely that any one of these factors would rescue *N* mutants. Thus the available data so far suggests that Notch signaling promotes growth in the retina via the transcriptional activity of the Pax6-like factors Eyg and Toe (Figure 5B, left panel).

4.3 From Notch to the JAK/STAT Pathway

The Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) signaling pathway is thought to be a global regulator of tissue growth. Eye discs devoid of the *unpaired* (*upd*) ligand have small eyes while forced expression leads to increased cell proliferation (Chen et al., 2002; Bach et al., 2003; Chen et al., 2003; Tsai and Sun, 2004). Like *N* and *eyg*, *upd* is expressed at the midline although its transcription is restricted to its intersection with the posterior margin (Tsai and Sun, 2004). This localized area is called "the firing point" because it is from here that the morphogenetic furrow initiates. One school of thought suggests that *N*/*eyg*-dependent growth is mediated by JAK/STAT signaling while another supports a model in which the JAK/STAT pathway sits at the top of the growth control circuit and promotes growth in the eye through Notch/eyg. Several lines of evidence support the first model. First, loss of either *N* or *eyg* leads to a reduction in *upd* transcription while forced expression of either component activates *upd* expression (Chao et al., 2004; Reynold-Kenneally and Mlodzik, 2005). Second, *N* and/or *eyg*-dependent growth can be blocked by the loss of *upd* and several other JAK/STAT components (Reynold-Kenneally and Mlodzik, 2005). Together, these data and those linking Notch signaling to *eyg* suggest a model in which localized activation of *N* at the midline initiates *eyg* expression which in turn leads to activation of the JAK/STAT pathway resulting in non-autonomous long-range growth of the entire eye disc (Figure 5B, left panel).

The second model, in which JAK/STAT signaling lies upstream of and promotes growth through *N*, has been recently proposed as an alternative (Figure 5B, middle panel: Gutierriz-Avino et al., 2009). In this paper the authors demonstrate that the growth defects associated with expression of *Protein Inhibitor of STAT* (*PIAS*) can be rescued by the activation of the Notch pathway. They also demonstrated that the tissue overgrowth phenotype which is observed when *upd* is over-expressed can be suppressed if the fng^{+}/fng^{-} border (and thus Notch activation) is disturbed (this was achieved by ubiquitous expression of *Iro-C* genes). At the moment it is not clear if these models are mutually exclusive of one another. There is room for both modes of growth control to exist if one were to incorporate a positive feedback loop between Notch signaling and the JAK/STAT pathway. This could act to continually promote localized Notch signaling at the midline while promoting long-range growth throughout the disc via JAK/STAT Figure 5B, right panel).

5. The Morphogenetic Furrow: How Point of Origin and Pace can Influence Organ Size

During the third and final instar a wave of morphogenesis initiates at the posterior margin of the disc and sweeps across the eye field until it reaches the eye/antennal border which lies at the far anterior edge of the eye disc (Weismann, 1864, Melamed and Trujillo-Cenoz, 1975; Ready et al., 1976). The leading edge of this wave front can be visualized by a dorso-ventral groove in the epithelium and is called the morphogenetic furrow. As it traverses the eye field it transforms a sea of undifferentiated cells into regularly spaced unit eyes or ommatidia (Krafka, 1924, Medvedev, 1935, Steinberg, 1943; Waddington and Perry, 1960; Ready et al., 1976; Tomlinson and Ready, 1987a,b; Wolff and Ready, 1991). Since the adult compound eye will ultimately contain approximately 800 such units, a continual re-adjustment of growth rates ahead of the furrow is predicted to accompany the addition of

each new ommatidial row behind the furrow (Figure 6). The overall size of the adult eye can be reduced by either altering the point along the retinal periphery at which the furrow initiates or by accelerating the pace at which the furrow races across the epithelium (Ma and Moses, 1995; Treisman and Rubin, 1995; Brown et al., 1995; Zelhof et al., 1997; Bhattacharaya and Baker, 2009). The reduction in tissue size is likely due to an imbalance between the rates of patterning and growth with d[P]/d[G] ratio being significantly greater than 1.

5.1 Wingless Signaling Inhibits the Initiation of Ectopic Furrows

Having a single firing center is important for maintaining a balance between patterning and growth rates. The creation of ectopic firing points directs the birth of new morphogenetic furrows (Ma and Moses, 1995, Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Kumar and Moses, 2001). In these situations cell proliferation rates ahead of the furrow do not increase therefore endogenous and ectopic furrows together quickly pattern all available cells before the number of cells needed to support an 800 unit display have been generated resulting in eyes that are small and disorganized.

Limiting the eye to a single firing center is a task that falls largely to the Wingless (Wg) signaling cascade. In late second/early third instar eye discs *wg* expression is found along the dorsal and ventral margins ahead of the advancing morphogenetic furrow. Its loss at the edges of the eye field leads to the birth of ectopic furrows (Figure 7, upper right panel; Ma and Moses, 1995). Together, the three furrows quickly overwhelm and pattern all available precursor cells before the full complement of retinal progenitors are produced. Wg signaling alone is sufficient to block pattern formation as ubiquitous *wg* expression throughout the eye field can inhibit the launching of the endogenous furrow from the firing center and its progression across the eye field (Figure 7, lower right panel; Treisman and Rubin, 1995).

Prior to the initiation of the morphogenetic furrow *wg* expression and activity is down-regulated at the posterior margin of the eye disc by JAK/STAT and Decapentaplegic (Dpp) signaling (Figure 7, left panel; Heberlein et al., 1993; Wiersdorff et al., 1996; Chanut and Heberlein, 1997; Hazlett et al., 1998; Dominguez and Hafen, 1997; Ekas et al., 2006; Tsai et al., 2007). Loss of *dpp* expression is not accompanied by an increase in *wg* transcription suggesting that the functional antagonism between these two pathways is at the post-transcriptional level (Treisman and Rubin, 1995). In contrast, loss of JAK/STAT signaling leads to a cell autonomous increase in *wg* expression. However, it appears that JAK/STAT regulation of *wg* is indirect as STAT92E binding sites are not present within the STAT responsive *wg* 3` eye enhancer (Ekas et al., 2006; Tsai et al., 2007). As expected, forced activation of either pathway down-regulates *wg* transcription and leads to the formation of ectopic furrows from the lateral and anterior margins of the disc (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Ekas et al., 2006; Tsai et al., 2007). Together, these results suggest that the competition between Wg, Dpp and JAK/STAT signals limits the eye to one firing point at the intersection of the posterior margin and the D/V midline and that this is a key step in preserving proper growth of the eye field (Figure 7, left panel).

The competing activities of the of JAK/STAT, Dpp and Wg signaling cascades results in the activation of Hh signaling at the intersection of the posterior margin and the midline. It is from this point that the morphogenetic furrow initiates and begins its journey across the eye field (Ma et al., 1993; Dominguez and Hafen, 1997). The mechanism by which *hh* transcription was activated at the firing center remained elusive for many years. But recently, three zinc finger transcription factors, Odd skipped (Odd), Brother of odd with entrails limited (Bowl) and Drumstick (Drm) have been shown to be expressed along the posterior margin and are sufficient to initiate hh transcription and retinogenesis (Figure 7; Bras-Pereira et al., 2006). The results in this paper also provided a mechanistic link between

Wg and Hh signaling. The authors demonstrated that Wg inhibits *drm* transcription at the margins anterior to the furrow. Thus, it seems that Wg can prevent ectopic furrow formation by blocking the activation of *hh* by *odd/drm/bowl.* Similar linkages between Hh and Wg pathways using odd class genes are preserved in other imaginal discs as well as the insect gut and embryonic epidermis (Hatini et al., 2005).

5.2 Extramacrochaetae Regulates the Velocity of the Morphogenetic Furrow

An equally important factor in maintaining balance between patterning and growth rates is the speed at which the furrow navigates across the eye disc. Several indirect attempts to clock the furrow have been attempted over the years with contradicting results. An initial attempt registered a biphasic rate in which a new row was laid down once every 100 minutes in the posterior half of the eye and once every 70 minutes in the anterior half (Campos-Ortega and Hofbauer, 1977). A later measurement, one that is more widely accepted, suggests that the furrow creates a new ommatidial row every 120 minutes across the entire disc (Basler and Hafen, 1989). Whichever measurement is correct, it is imperative that the furrow does not move too rapidly across the eye field. If the furrow did move too rapidly then the pool of retinal precursors would be quickly transformed into photoreceptors and their capacity to generate the requisite number of cells needed for a fully formed adult eye would be exhausted.

One gene that appears to regulate the velocity of the furrow is *extramacrochaetae* (*emc*), which encodes a helix-loop-helix (HLH) protein (Botas et al., 1982). Emc and its vertebrate homologs, the Inhibitors of Differentiation (Id1-4) belong to a unique subclass of basic helix-loop-helix (bHLH) transcription factors. bHLH proteins form either homo or heterodimers through the HLH domain and can either activate or repress transcription of downstream target genes through binding of the basic domain dimer to the N or E box (Murre et al., 1989a,b; Bertrand et al., 2002). Emc works by forming inactive heterodimers with bHLH proteins. Since Emc lacks a basic domain (Ellis et al., 1990; Garrell and Modolell, 1990) the HLH-bHLH heterodimer is unable to bind nucleic acids. Thus Emc affects transcription through protein sequestration (van Doren et al., 1991). At the writing of this review very few binding partners have been identified *in vitro* and even fewer in the developing eye.

During eye development *emc* is expressed in a dorso-ventral stripe ahead of the advancing morphogenetic furrow (Brown et al., 1995). Simultaneous reductions in both *emc* and *hairy* (*h*) levels lead to an acceleration of the furrow and a dramatic decrease in the overall size of the eye disc (Brown et al., 1995; Bhattacharya and Baker, 2009). Initial reports put forth a model in which H, a bHLH transcription factor and Emc functioned together to slow the furrow (Brown et al., 1995), but more recent results have shown that the loss of *emc* by itself is all that is required for the furrow to accelerate across the disc (Bhattacharya et al., 2009). Reduction in the size of eyes that completely lack *emc* must be analyzed with some caution. Clones of *emc* null alleles do not survive unless they are generated in a genetic background that is heterozygous for dominant mutations within ribosomal proteins (referred to as Minute mutations). In this genetic background cells that lack *emc* have a competitive advantage over the surrounding tissue. Therefore the small eye phenotype may be due to a combination of phenotypes: the acceleration of the furrow across the eye field as well as potential regulation of the cell cycle. Indeed, several human cancers are associated with elevated levels of Id1-4 (Desprez et al., 1998; Parrinello et al., 2001; Dong et al., 2003; Itahana et al., 2003; Han et al., 2004; Kebebew et al., 2000; Kebebew et al., 2003; Ling et al., 2004; Chaudhary et al., 2005; Tsuchiya et al., 2005; Yuen et al., 2006; Tam et al., 2008; Zhao et al., 2008; Tang et al., 2009).

The mechanism by which Emc regulates the pace of furrow movement is yet to be resolved although several clear possibilities exist. Emc could potentially function to regulate the breadth and intensity of the Hedgehog signal, the transmission and execution of instructions from the Dpp pathway or the activity of the retinal determination network ahead of the advancing morphogenetic furrow. Equally likely is the potential for *emc* to simultaneously regulate multiple signaling events within and ahead of the furrow (Figure 8). Irrespective of which pathway is being regulated by Emc, a key to truly understanding the role of insect and vertebrate HLH proteins in growth control will require the identification of tissue specific binding partners and an understanding of how their sequestration affects regulation of the cell cycle.

6. The Second Mitotic Wave: Where Size Really Matters

As cells pass through the morphogenetic furrow a subset immediately exits the cell cycle and adopts a photoreceptor fate. These cells (R8, R2/5, R3/4) form the first five cells of the ommatidium and are collectively referred to as the 5-cell precluster (Ready et al., 1976). The remaining cells will undergo a final synchronized round of mitosis before exiting the cell cycle themselves. This second mitotic wave will give rise to three photoreceptor subtypes (R1, R6 and R7), the four cone cells, all thee pigment cell subtypes and the four cells that comprise the bristle complex (Ready et al., 1976; Tomlinson, 1985). Correct cell fate specification occurs in the absence of the second mitotic wave although not enough cells are generated to produce 800 complete ommatidia. Instead unit eyes within the anterior half of the retina contain the full balance of photoreceptor, cone, pigment and bristle cells while those within the posterior half are depleted for the later borne cells (de Nooij and Hariharan, 1995). This result indicates that the sole role of the second mitotic wave is to generate large quantities of cells. And since the majority of cells that comprise each ommatidium is generated in this round of cell division, the second mitotic wave can also be thought of as being the major determinant of the overall size of the adult retina (Figure 9).

6.1 A Passage to the Second Mitotic Wave: Notch Controls the G1/S Checkpoint

In order for cells, which are arrested in G1 within the morphogenetic furrow, to enter the second mitotic wave they must first negotiate the G1/S cell cycle checkpoint. This is accomplished through Notch signaling emanating from cells within the furrow (Figure 9). Cells within the furrow express and secrete the Dl ligand while non-precluster cells express the N receptor. Upon activation of the N receptor, cells enter the second mitotic wave by navigating the G1/S transition through modulations of RBF and E2F activity, two well-known regulators of G1 and S cell cycle phases (Baonza and Freeman, 2005; Firth and Baker, 2005). Other reports have extended these results by showing that Notch dependent entry into the second mitotic wave requires the CyclinE/Cdk2 complex, which is known to directly regulate Rb and E2F activity (Sukhanova and Du, 2008). Similarly, the CyclinD/Cdk4 complex also regulates both Rb and E2F activity (Mittnacht, 1998; Lundberg and Weinberg, 1998; Xin et al., 2002). Previous studies in vertebrate tissue culture systems had shown that expression of a mutant form of Rb lacking cdk phosphorylation sites can induce G1 arrest (Brown et al., 1999). However, at least within the second mitotic wave both CyclinE/Cdk2 and CyclinD/Cdk4 complexes may actually be required for the proper completion of S phase rather than regulating the initial entry into S phase. Despite forced expression of mutant Rb proteins in all cells behind the furrow, cells enter the second mitotic wave normally but do not exit S phase on time (Xin et al., 2002). The implication is that Notch signaling may actually regulate two steps: the initial entry into the second mitotic wave and the length of time that cells reside within S phase (Figure 9A).

6.2 The G2/M Checkpoint: EGF Receptor Signaling from the Pre-cluster

Entry into the second mitotic wave is not the only point at which non-autonomous signals from cells outside the wave are required. A second step, the transition into M phase from G2, also requires a long-range signal. In contrast to the G1/S transition, this step appears to be controlled by the EGF Receptor pathway and the signal emanates not from the furrow but rather from the five-cell pre-cluster (Figure 9). Initial insights into a potential role for EGF Receptor signaling in retinal growth control came from a study that demonstrated a requirement for this pathway in cell proliferation (Xu and Rubin, 1993). Other reports demonstrated that the ligand Spitz (Spi) and the two processing/presenting factors Star (S) and Rhomboid (Rho) are distributed within cells of the pre-cluster but not the furrow (Freeman et al., 1992; Heberlein et al., 1993; Tio and Moses, 1997). Similarly, loss of the EGF Receptor itself results in the loss of all photoreceptors that are normally borne in the second mitotic wave (Freeman, 1996; Kumar et al., 1998).

Removal of *spi* from the pre-cluster or of the EGF receptor from cells within the second mitotic wave does not affect the level of BrdU incorporation, suggesting that passage from the G1 to S phase does not require EGF Receptor signaling. In contrast, Cyclin B accumulation is higher than normal indicating that cells within the second mitotic wave are arrested in G2. In complementary experiments, hyper-activation of EGF Receptor signaling results in the elimination of Cyclin B and an increase in the number of cells undergoing mitosis. These experiments suggested that EGF Receptor signaling is necessary and sufficient for cells to transition through the G2/M checkpoint within the second mitotic wave (Baker and Yu, 2001). A subsequent paper demonstrated that the effect of the EGF Receptor pathway on the G2/M checkpoint was genetically dependent upon canonical Ras/MAPK signaling and the Ets transcription factor Pointed (Pnt), a downstream target of the cascade (Brunner et al., 1994; O'Neill et al., 1994; Yang and Baker, 2003).

A direct link between EGF Receptor signaling and the G2/M checkpoint was established by an analysis of several transcription factors that are known downstream components of the EGF Receptor pathway (Baonza et al., 2002). These include *pnt* (see above), *yan* (Rebay and Rubin, 1995) and *tramtrack* (*ttk*: Read, 1992; Lai et al., 1996; Tang et al., 1997). Removal of Pnt, a transcriptional activator, results in a severe reduction in the number of mitotic figures (Baonza et al., 2002; Yang and Baker, 2003). In contrast, an increase in the mitotic index is seen in cells that lack Ttk, a transcriptional repressor. (Baonza et al., 2002). Furthermore, both Pnt and one isoform of Ttk (Ttk69) were shown to directly bind to regulatory sequences within an eye specific enhancer element of stg/cdc25, the universal regulator of the G2/M checkpoint.

Is *stg* the only target of EGF Receptor signaling within the second mitotic wave? A recent paper suggests that the answer is no. The authors used a microarray-based approach to compare the expression profile of wild type eye discs to those that lack the second mitotic wave due to manipulations in EGF signaling. A number of new genes were identified and may play roles at the G2/M checkpoint (Firth and Baker, 2007)

7. Concluding Remarks

Despite the increased awareness of the *Drosophila* retina as a model system for studying growth control mechanisms, our understanding of how cell proliferation is regulated in the eye is still in its infancy. In fact, in terms of identifying the full complement of genes, pathways and networks that regulate the size of the adult eye, we have only scratched the surface. In this review, I have briefly discussed the contributions that the retinal determination network, the dorsal-ventral midline, the morphogenetic furrow and the second mitotic wave make to the growth of the *Drosophila* retina. These components are just a few

inputs into the overall growth control circuit. Additional inputs that are known to play important roles in tissue growth (but are not discussed here) include, but are not limited to, tumor suppressor pathways, apical-basal polarity regulators and programmed cell death cascades. How all of these influences are integrated into the central growth pacemaker is an open question and certainly one of fundamental importance. As increasing numbers of studies continue to use the eye to study growth control, future authors of similar reviews will be able to provide a significantly more complete picture of how cell proliferation levels are modulated during development to produce adult structures of the appropriate size and shape: not too big, not too small.

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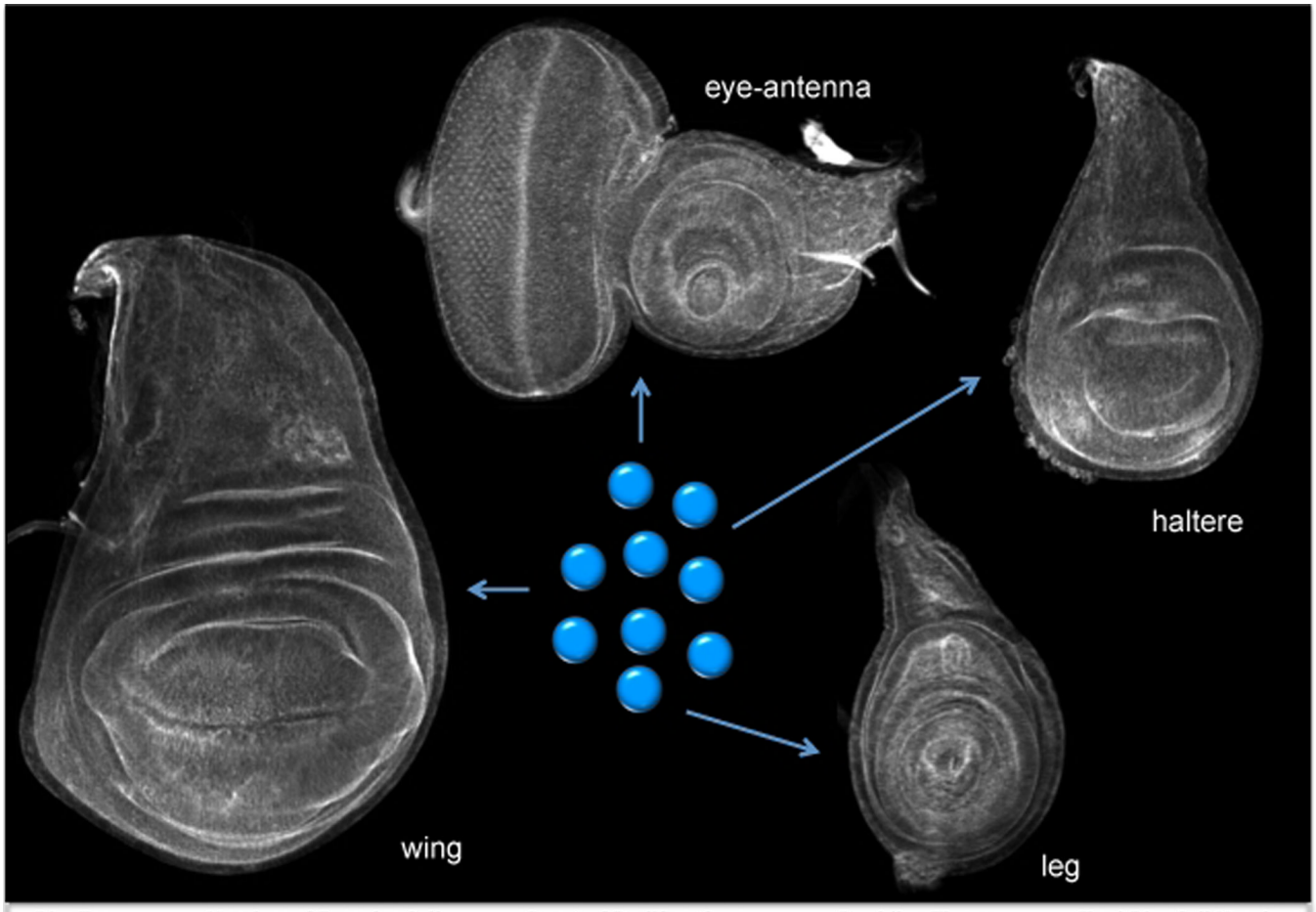

Disc Type	Number of Founder Cells	Onset of Proliferation	Doubling Time	Final Disc Cell Number
eye-antenna	~70	13-15 (eye), 24-26 (ant)	11.9 (eye), 11.5 (ant)	~44,000
wing	~10-38	15-17	8.5-10.6	~43,000-49,000
leg	~10-40	24-26	11.3	~17,000-21,000
haltere	~10-20	18-20	N.D	~7,500-10,000
		(after egg deposition)	(in hours)	

Figure 1. Tissue Growth in the Imaginal Discs of Drosophila

This figure includes confocal images of the eye-antennal, wing, haltere and leg discs of Drosophila. The discs have been stained with phalloidin, which marks F-actin, so that the outlines of each disc can be seen. Below are the starting and final number of cells of each disc as well as the doubling times and onset of cell proliferation times.

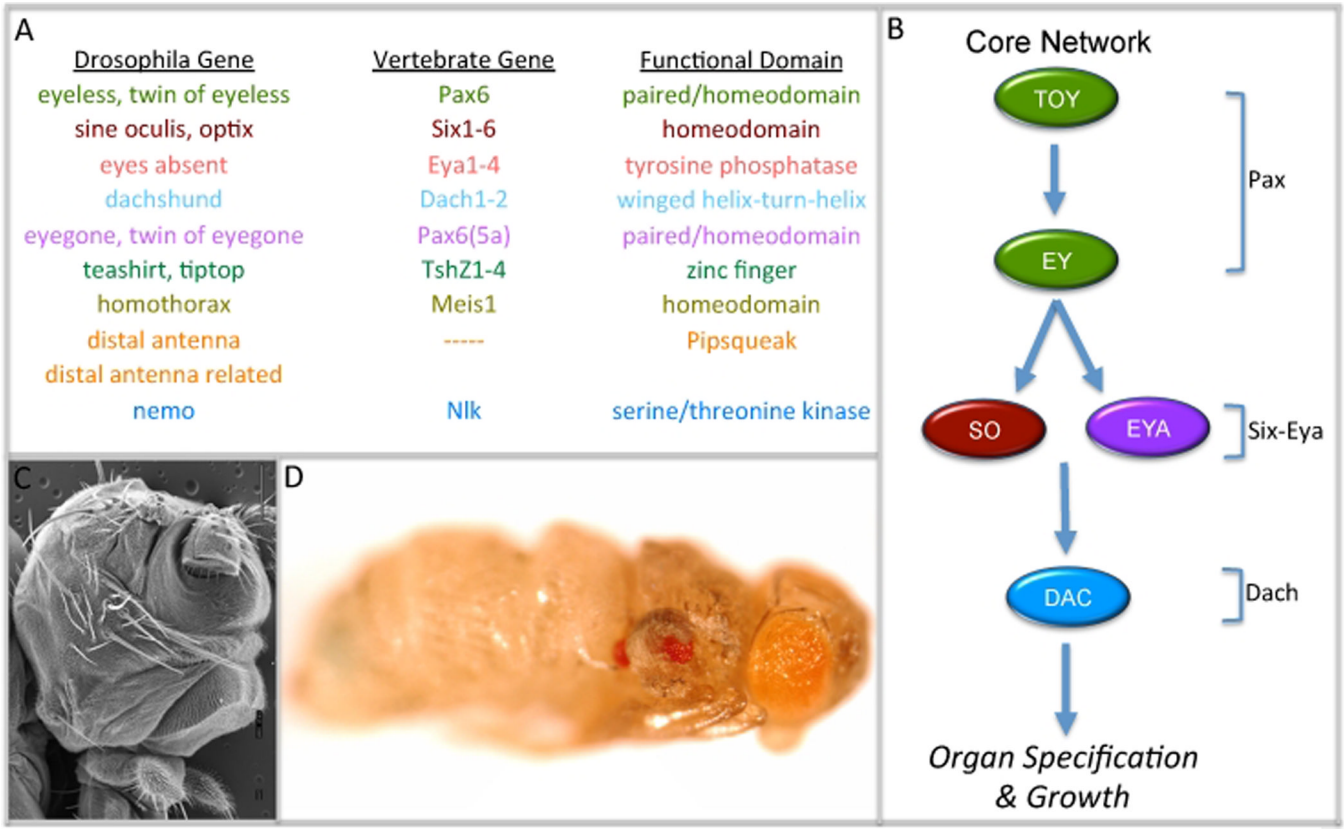

Figure 2. The Retinal Determination Network: Genes, Pathway, Loss and Gain-of-Function Phenotypes

(A) List of retinal determination genes in flies and vertebrates. (B). Schematic of the core retinal determination pathway. (C) Scanning electron micrograph of an *eya*^{2} adult mutant head. (D) Light microscope image of an adult fly in which *eyeless* has been mis-expressed within the halteres and wings. Note the red ectopic eyes.

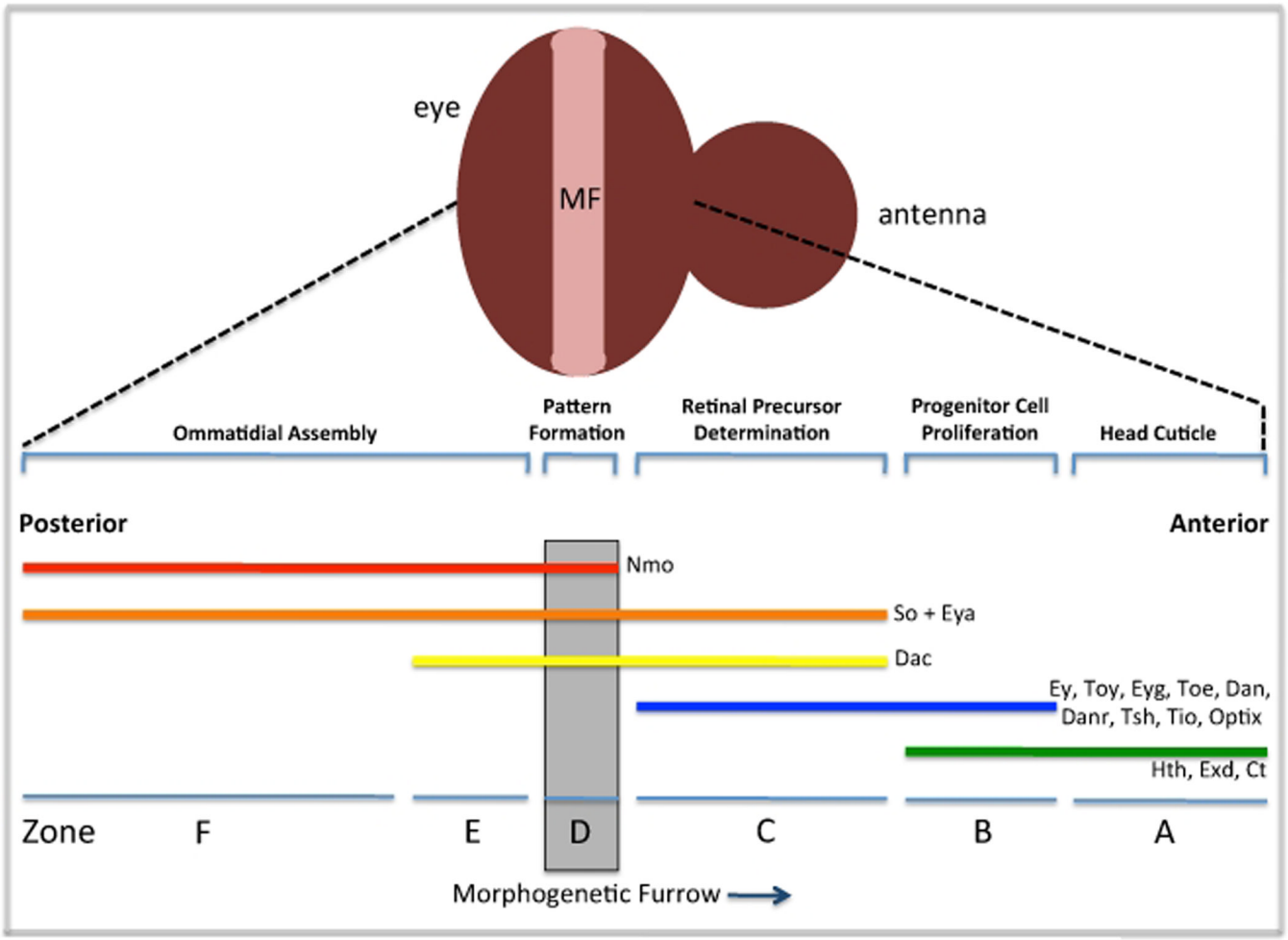

Figure 3. Expression Patterns of the Retinal Determination Genes in the Developing Fly Eye

Schematic diagram depicting the expression patterns of the retinal determination genes. Anterior is to the right.

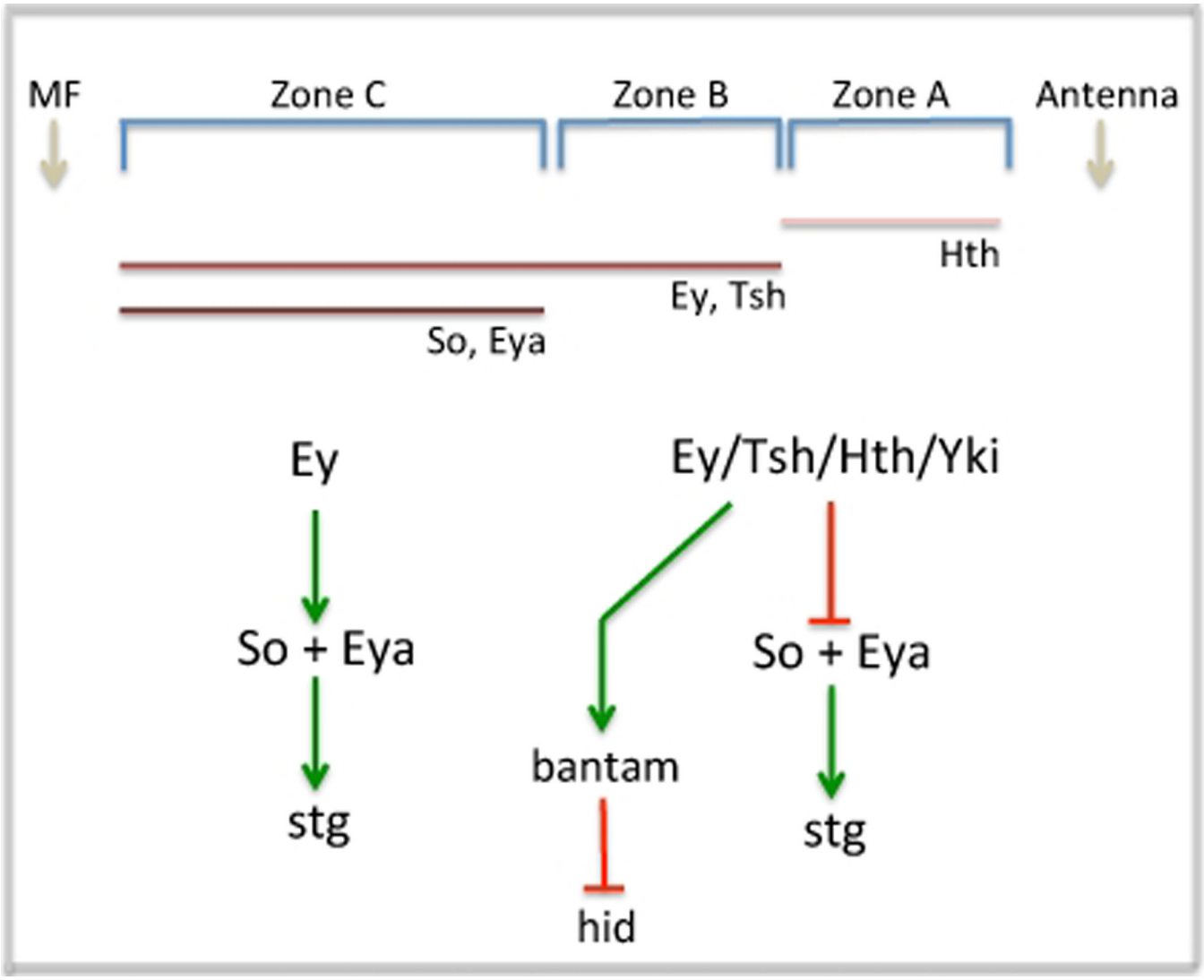

Figure 4. Model for RD Network Control of the Retinal Cell Cycle

In this model cell proliferation in the most anterior segments of the eye field is achieved by the activation of bantam, a microRNA that blocks translation of hid RNA, and transcriptional repression of both so and eya, two genes required for the activation of stg. In zone C cells, the So-Eya complex activates stg thereby moving cells through the cell cycle so that they can be arrested in G1 within the furrow.

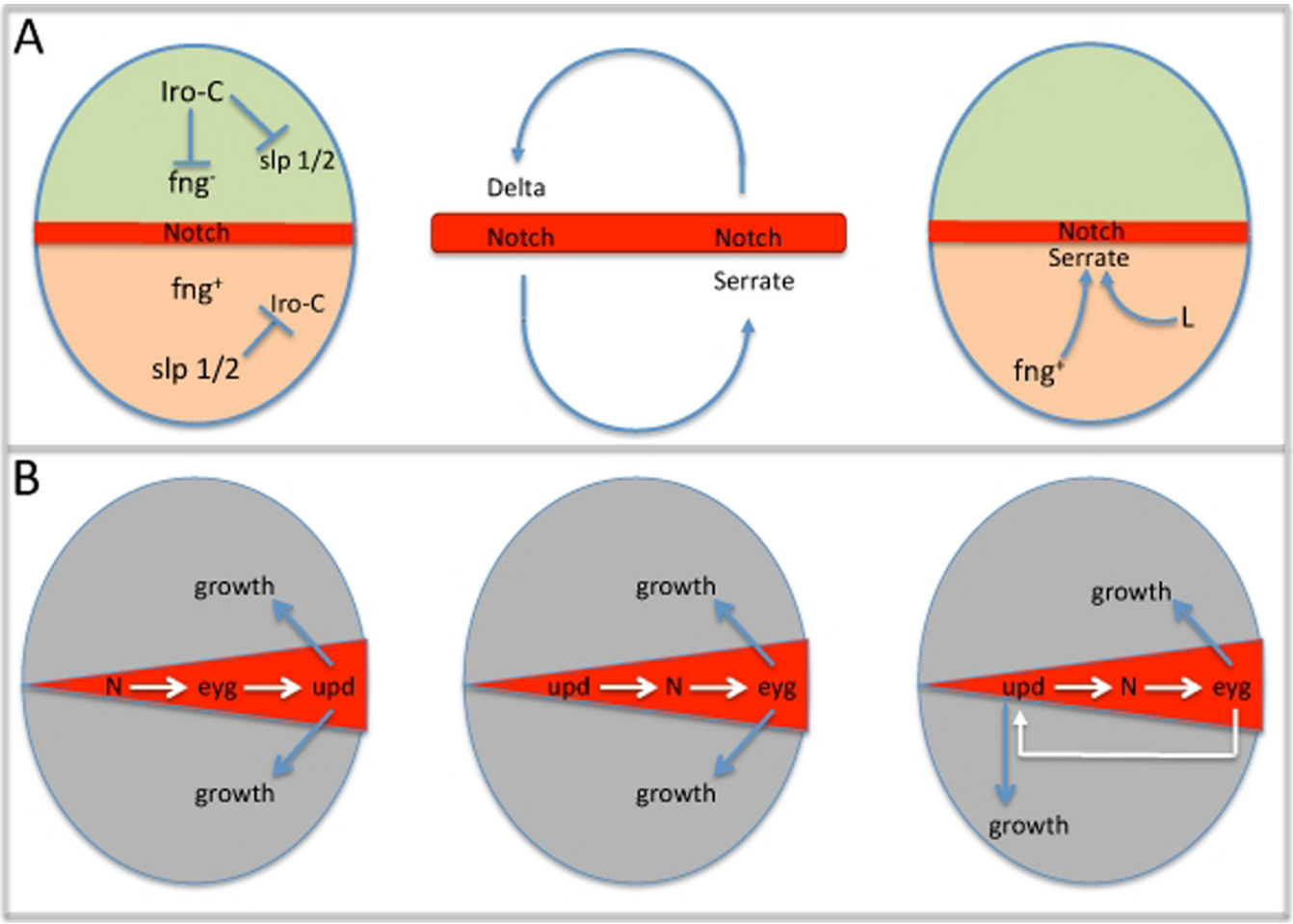

Figure 5. Notch Signaling at the Midline Promotes Growth

(A) Schematics depicting the activation of Notch at the midline, the amplification of the Notch signal and the activation of the Ser ligand within the ventral half of the eye. (B) Models depicting the genetic hierarchy of Notch mediated growth in the eye. These models include the activities of Notch, eyegone and JAK/STAT signaling.

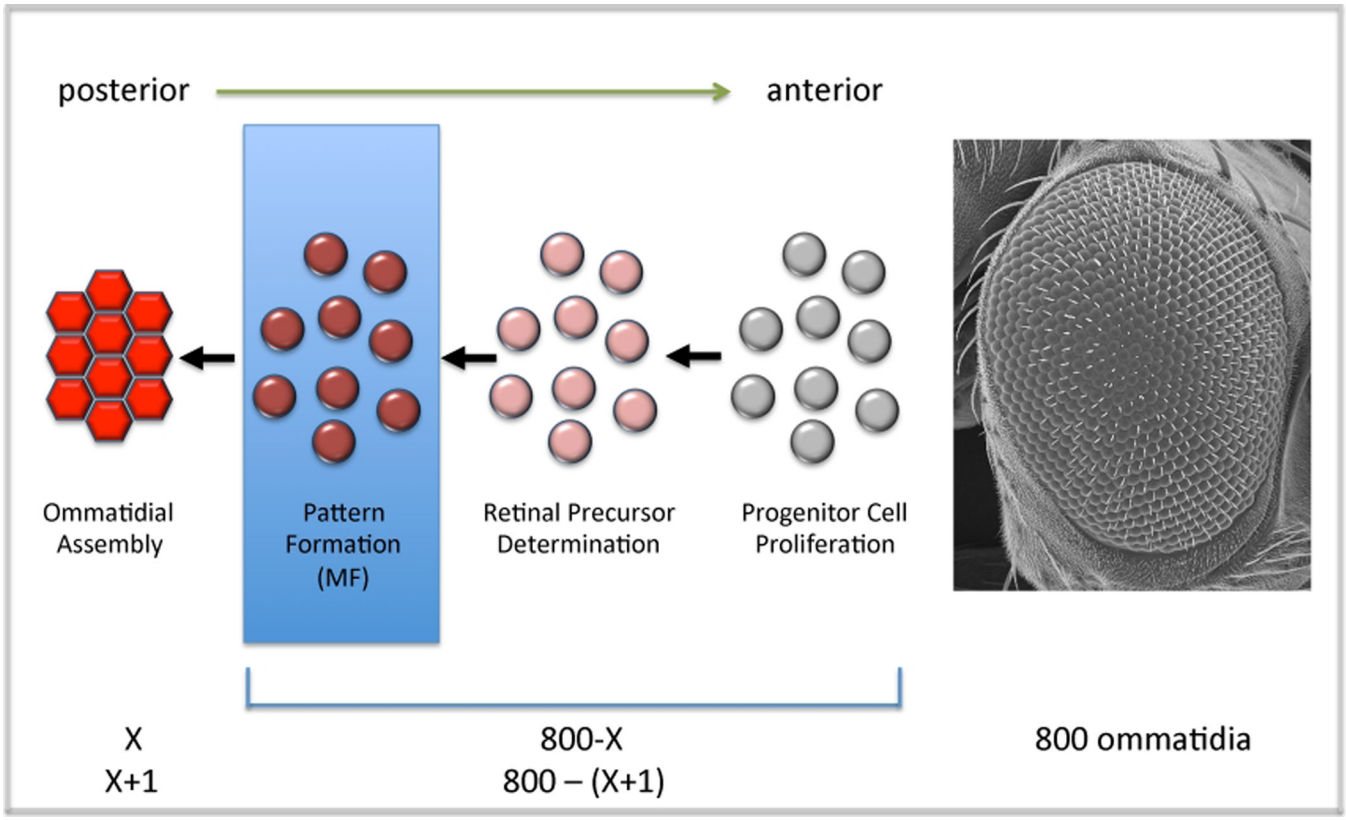

Figure 6. Progression of the Furrow and the Constant Recalculation of Growth Rates

This model describes the readjustment of growth rates that is likely to occur as new rows of ommatida are added to the developing eye. It also briefly describes the cellular steps that cells ahead of the furrow take on the way to ommatidial assembly.

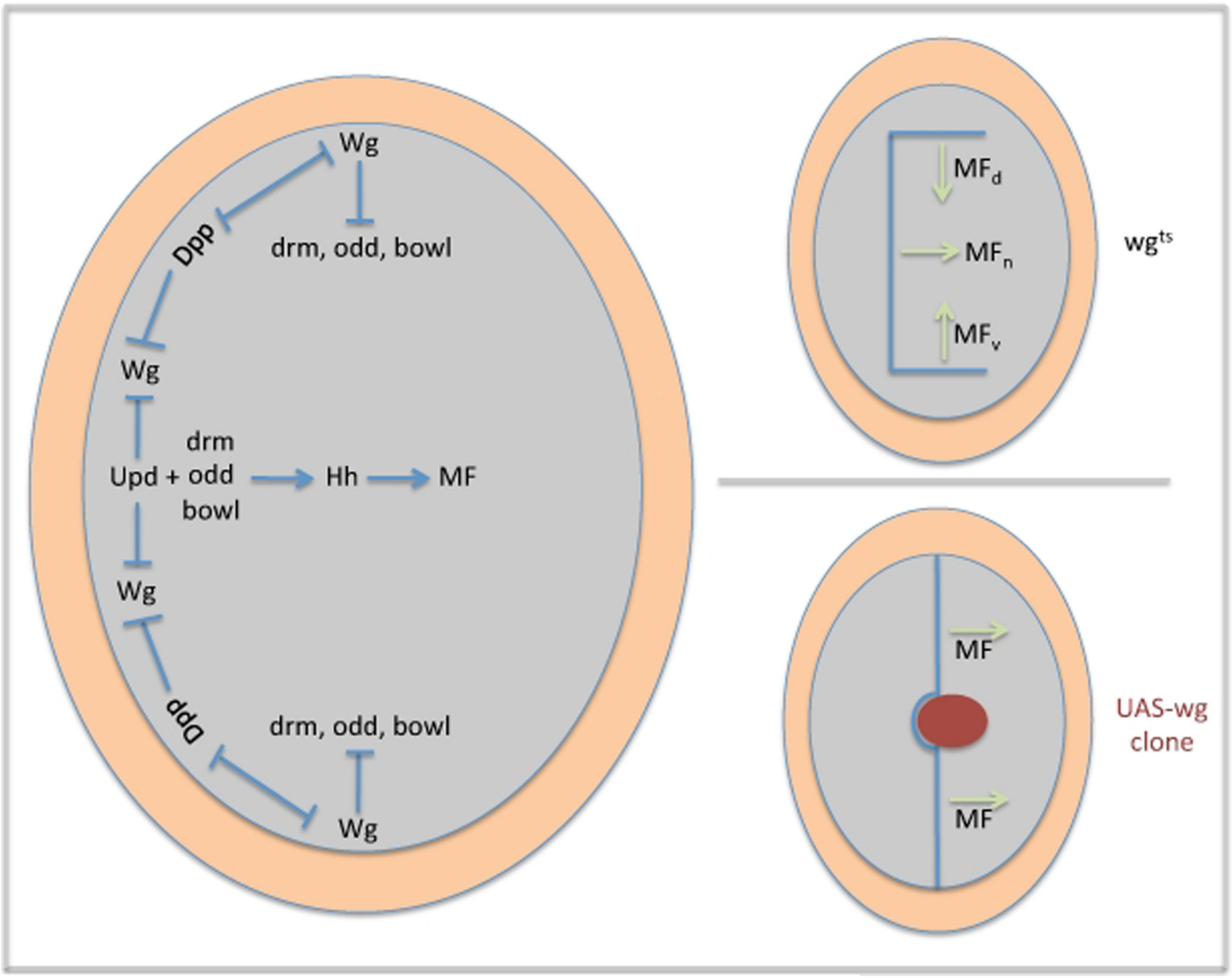

Figure 7. Initiation of the Morphogenetic Furrow and the Firing Center

The left panel describes the mechanisms by which Dpp, JAK/STAT and Wg restrict the firing center to the intersection of the posterior margin and the midline. The top right panel describes the initiation of ectopic furrows in response to the loss of *wg* expression. The bottom right panel describes a furrow stop phenotype that results from ectopic *wg* expression within the eye field.

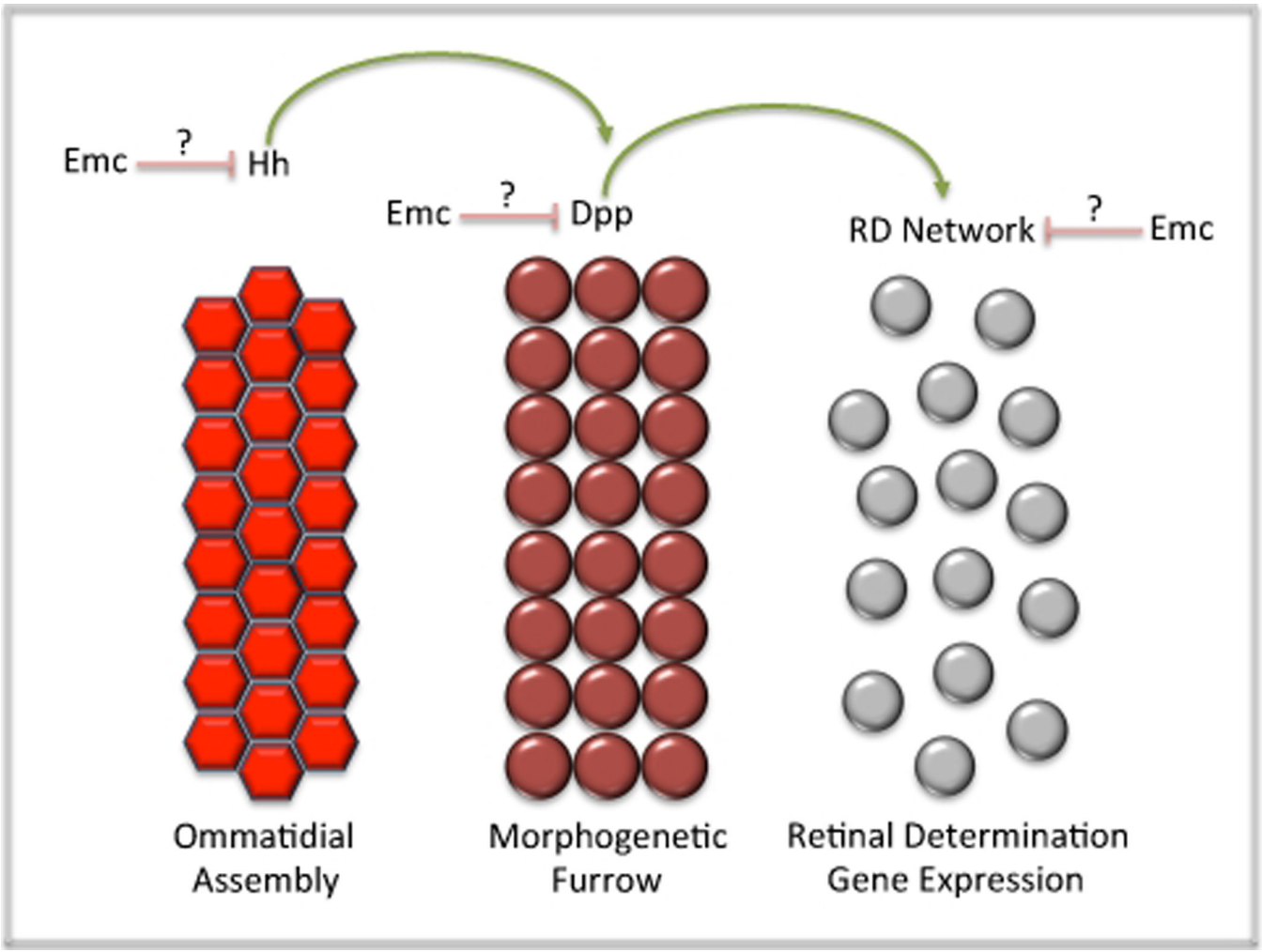

Figure 8. Regulation of Furrow Movement and Velocity by Extramacrochaetae

Hh and Dpp signaling as well as members of the RD network are known to regulate the progression of the morphogenetic furrow. Emc is known to slow the rate of furrow progression. This figure describes the points of potential regulation for Emc.

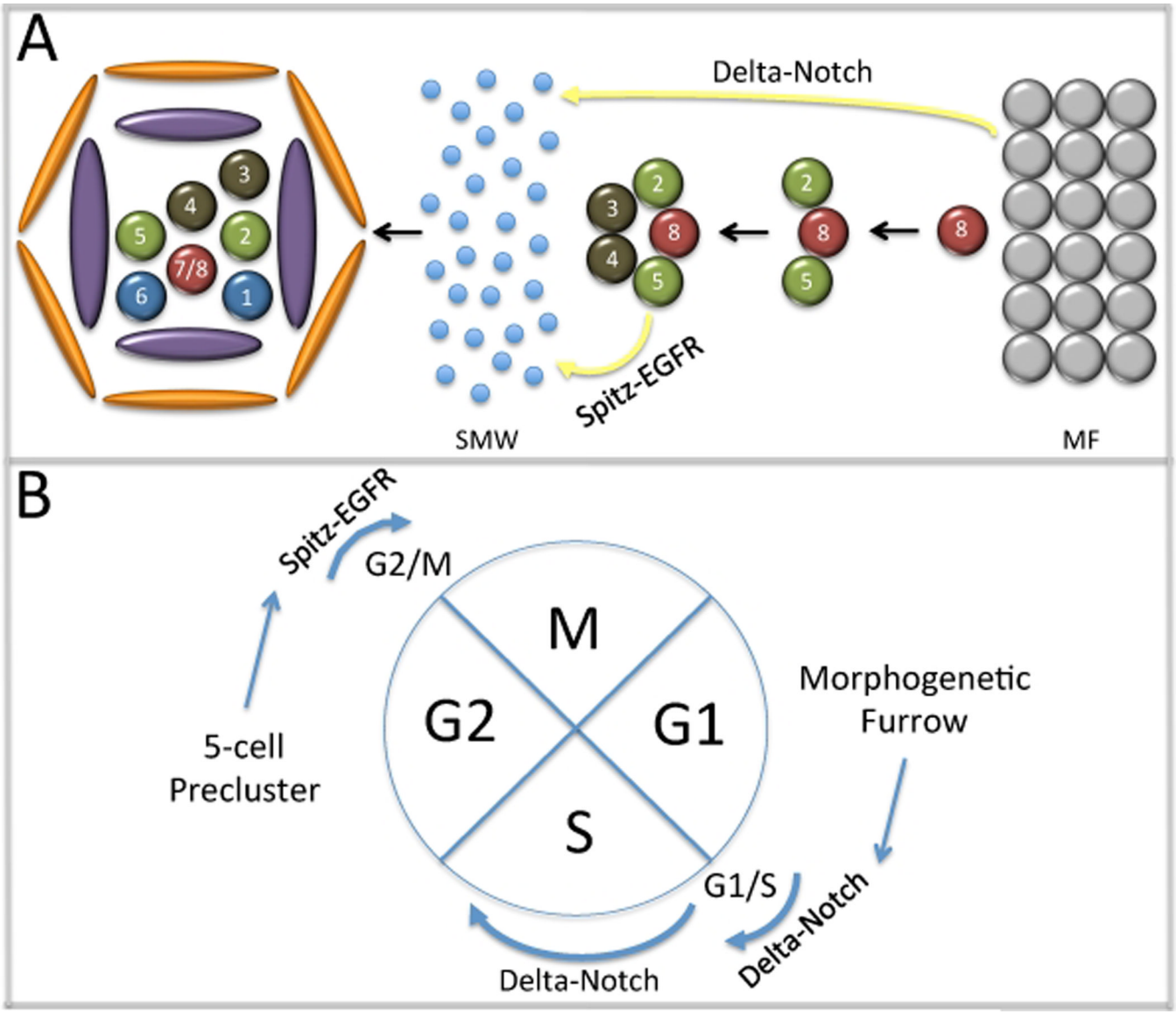

Figure 9. Notch and EGF Receptor Regulation of Second Mitotic Wave Checkpoints

(A) Schematic diagram of ommatidial assembly. Delta-Notch signaling from the furrow and Spitz-EGFR from the precluster regulate progression of cells through the second mitotic wave. (B) Notch signaling regulates entry into S phase and the length of time that cells remain in S phase. EGF Receptor signaling promotes the transition of cells into mitosis.