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Odd-skipped genes specify the signaling center that triggers retinogenesis in *Drosophila*

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Although many of the factors responsible for conferring identity to the eye field in *Drosophila* have been identified, much less is known about how the expression of the retinal 'trigger', the signaling molecule Hedgehog, is controlled. Here, we show that the co-expression of the conserved odd-skipped family genes at the posterior margin of the eye field is required to activate *hedgehog* expression and thereby the onset of retinogenesis. The fly Wnt1 homologue *wingless* represses the odd-skipped genes *drm* and *odd* along the anterior margin and, in this manner, spatially restricts the extent of retinal differentiation within the eye field.

KEY WORDS: Retinal differentiation, Eye, *Drosophila*, *odd* genes, Hedgehog, *wingless*

INTRODUCTION

In *Drosophila*, the eye primordium is specified as a subdomain of the *Pax6*-expressing cells in the center of the eye disc, by the co-expression of a set of retinal determination genes (Bonini et al., 1993; Cheyette et al., 1994; Dominguez and Casares, 2005; Halder et al., 1998; Mardon et al., 1994; Pappu and Mardon, 2004). Then, retinogenesis is triggered by the *hedgehog* (*hh*) and the *hh* target *decapentaplegic* (Dpp/Bmp4) signals that are produced by the surrounding posterior margin cells (Fig. 1A), at the so-called 'firing point' (Treisman and Heberlein, 1998). These margin cells abut the eye primordium and give rise to part of the adult head capsule surrounding the eye (Haynie and Bryant, 1986). Once initiated, retinal differentiation propagates in a posterior-to-anterior wave (Fig. 1B,C), with the differentiation wavefront marked by an epithelial indentation: the morphogenetic furrow (MF) (Treisman and Heberlein, 1998). The gene(s) responsible for this specialization of the posterior margin are unknown.

MATERIALS AND METHODS

Drosophila strains

odd^{5}, *drm*^{6}, *bowl*^{1}, *wg*$^{1\text{-}16}$ (*wg*^{CX3}), *odd*^{rK111} (*oddZ*), *hhP30* (*hhZ*), *dppBS3.0* (*dppZ*), *P{en1}wgen11* (*wgZ*), *P{GAL4}hhGal4* (*hh*-GAL4) are described in FlyBase. Df(2L)*drmP2* (Green et al., 2002; Hao et al., 2003) deletes from *tim* to *odd*, and uncovers ~30 predicted genes, including *drm*, *sob* and *odd*. UAS strains were UAS-*odd(A)* and UAS-*sob(6)* (Hao et al., 2003), UAS-*bowl(1.1)* (de Celis Ibeas and Bray, 2003), UAS-*drm* (on the III) and UAS-*lines* (Green et al., 2002; Hatini et al., 2000), and UAS-*Src-GFP* (Kaltschmidt et al., 2000). *odd*-GAL4 faithfully reproduces *odd* expression (a gift from G. Morata and M. Calleja, CMB, Spain). *drm*^{6} was recombined onto a *FRT40A* chromosome.

Loss-of-function clones:

odd^{5}, *drm*^{6} and *bowl*^{1} mitotic clones were induced between 24 and 48 hours after egg laying (AEL) by a 45 minute 37°C heat-shock in larvae from the crosses of *odd* FRT 40A*/balancer males to *yw* hsFLP 122; *Ubi-GFP FRT40A* females (*odd** represents each of the alleles used). Df*drm*P2 cells do not survive unless given a growth advantage, for which we used the 'Minute technique' (Morata and Ripoll, 1975). Clones were induced between 24 and 72 hours AEL by a 20 minute 37°C heat-shock in larvae from the crosses of *odd* FRT40A* males to *yw*, *hsFLP122*; *M* armZ FRT40A females. In some experiments, we used *yw ey-FLP* as flipase source (Newsome et al., 2000) to maximize the amount of mutant tissue in eye discs. Mutant cells were identified by the absence of β-galactosidase (armZ).

Ectopic-expression ('flip-out') clones of *odd*-family genes and *lines*

These clones were induced between 24 and 48 hours AEL (L1 stage) in larvae from the crosses between UAS-*odd** (where *odd** means *odd*, *drm*, *sob* or *bowl*) or UAS-*lines* males and *y*, hsFLP122, *actinP*>hsCD2>Gal4 females (Basler and Struhl, 1994). Clones were marked negatively by the absence of CD2 (CD2 was induced by a 45 minute 37°C heat-shock, followed by 45 minutes recovery at room temperature). The *hhZ*, *dppZ* or *oddZ* reporters were introduced in the genotypes of some experiments. The overexpression of *drm* in *bowl*$^{-}$ cells was achieved using the MARCM technique (Lee and Luo, 2001). *UAS-drm* was balanced over *TM6B, Tb*, so *drm*-expressing larvae were *Tb*$^{+}$. Clones were marked positively by expression of GFP.

Antibodies

We used rabbit anti-β-gal (Cappel), mouse anti-β-gal (Sigma), rabbit anti-GFP (Molecular Probes), mouse anti-CD2 (Serotec), guinea pig anti-Odd (Kosman et al., 1998) and mouse ant-Ptc (Nakano et al., 1989). Rat anti-Elav, mouse anti-Wg (4D4) and mouse anti-Eya are from the Iowa University Studies Hybridoma Bank. RNA probes for *odd*, *drm*, *sob* and *bowl* were as described previously (Hao et al., 2003). Phalloidin-FITC was used to mark filamentous actin. Appropriate fluorescent secondary antibodies were from Molecular Probes. Anti-mouse-HRP (Sigma) was used for immunoperoxidase staining.

RESULTS AND DISCUSSION

bowl, *odd*, *drm* and *sob* are expressed in the margin-peripodial cells in early eye discs, but their expression patterns differ later on in development

The eye disc is a flat epithelial sac. By early third larval stage (L3), columnar cells in the bottom (disc proper: Dp) layer are separated by a crease from the surrounding rim of cuboidal margin cells. Margin cells continue seamlessly into the upper (peripodial; Pe) layer of squamous cells (Fig. 1C-G). The Dp will differentiate into the eye, while the margin and Pe will form the head capsule (Haynie and Bryant, 1986). In addition, the posterior margin produces retinal-inducing signals (Treisman and Heberlein, 1998).

By examining gene reporters we found that the zinc-finger gene *odd-skipped* (*odd*) is expressed restricted to the posterior margin and Pe of L3 eye discs (Fig. 1). As the odd family members *drumstick*

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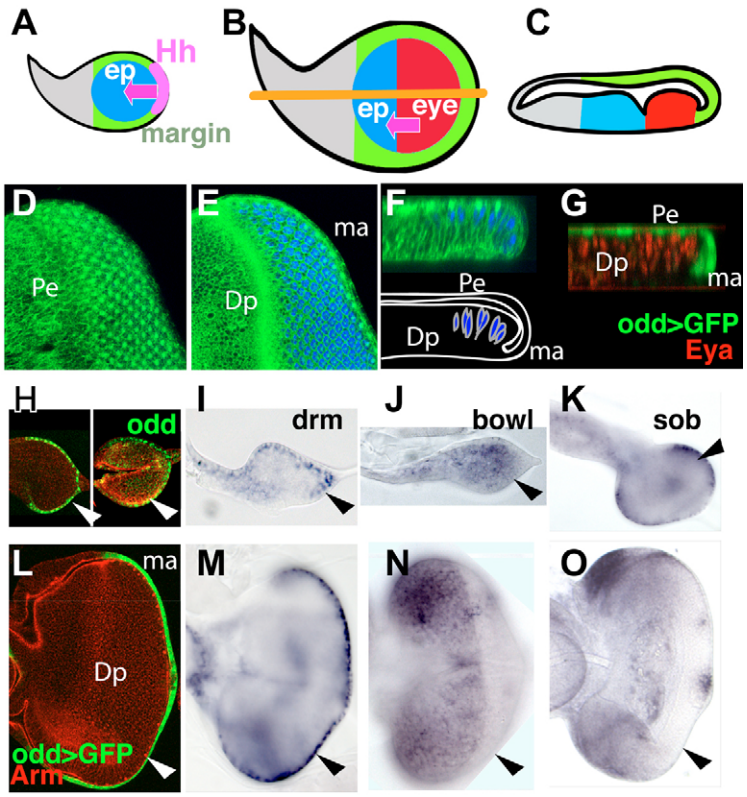

Fig. 1. Expression of the odd-genes is associated to the margin-peripodial cells of the eye disc during development. (**A**,**B**) Schemes of late L2/early L3 (A) and late L3 (B) eye discs. (A) Posterior margin cells trigger retinogenesis in the adjacent eye primordium (ep) by producing Hh. (B) Once triggered, retinal differentiation progresses anteriorly (eye). (**C**) Cross-section through the line in B shows the peripodial and margin cells (green) overlaying the differentiating eye primordium. (**D**,**E**) Confocal images of the posterior region of a third larval stage (L3) disc through the peripodial (Pe, D) and disc proper (Dp, E) layers, stained with phalloidin-FITC and Elav (a photoreceptor marker used in this and following figures). The margin (ma) is a thin strip of cells adjacent to the posterior-most row of photoreceptors. (**F**) Confocal *z*-section through the same disc showing the three cell types (schematized below). (**G**) Confocal *z*-section through the posterior region of a L3 *odd*-GAL4>GFP disc, co-stained with Eya. *odd* is restricted to the Pe and margin. (**H-O**) Patterns of expression of the four odd genes in L2 (H-K) and L3 (L-O). Expression of *odd* is monitored by the *odd-GAL4* reporter (H, left; L) or with an anti-Odd antibody (H, right), and that of *drm* (I,M), *bowl* (J,N) and *sob* (K,O) by RNA in situ hybridization. The patterns of *drm* and *odd* seem identical. (H, left) Propidium iodide marks nuclei. (H, right) Rhodamine-phalloidin stains actin. (L) Arm expression marks cell membranes. Arrowheads indicate the margins. Discs are oriented with posterior towards the right and dorsal upwards.

(*drm*), *brother of odd with entrails limited* (*bowl*) and *sister of odd and bowl* (*sob*) are similarly expressed in leg discs (de Celis Ibeas and Bray, 2003; Hao et al., 2003), we examined them in eye discs. In L2, before retinogenesis has started, *odd* and *drm* are transcribed in the posterior Pe-margin (Fig. 1H,I), and this continues within the posterior margin after MF initiation (Fig. 1L,M). *bowl* is transcribed in all eye disc Pe-margin cells of L2 discs (Fig. 1J), but retracts anteriorly along the margins and Pe after the MF passes (Fig. 1N). In addition, *bowl* is expressed weakly in the Dp anterior to the furrow. *sob* expression in L2 and L3 is mostly seen along the lateral disc margins (Fig. 1K,O). Therefore *drm, odd* and *bowl* are co-expressed at the posterior margin prior to retinal differentiation initiation.

bowl is required for *hedgehog* expression in margin cells and for triggering retinal differentiation

Odd family genes regulate diverse embryonic processes, as well as imaginal leg segmentation (de Celis Ibeas and Bray, 2003; Green et al., 2002; Hao et al., 2003; Hatini et al., 2005; Johansen et al., 2003). Bowl is required for all these processes (Green et al., 2002; Hao et al., 2003). In embryos, the product of the gene *lines* (Bokor and DiNardo, 1996) binds to Bowl and represses its activity, while Drm relieves this repression in *drm*-expressing cells (Hatini et al., 2005). As *drm/odd/bowl* expression coincides along the posterior margin around the time retinal induction is triggered, we asked whether they controlled this triggering. First, we removed *bowl* function in marked cell clones induced in L1. *bowl*$^{-}$ clones spanning the margin, but not those in the DP, cause either a delay in, or the inhibition of, retinal initiation (Fig. 2A,B) and the autonomous loss of *hh*-Z expression (Fig. 2C,E). Correspondingly, there is a reduction in expression of the *hh*-target *patched* (*ptc*) (Fig. 2D). These effects on *hh* and *ptc* are not due to the loss of margin cells, as *drm* is still expressed in the *bowl*$^{-}$ cells (not shown). The requirement of Bowl for *hh* expression is margin specific, as other *hh*-expressing domains within the disc (Royet and Finkelstein, 1997) are not affected by the loss of *bowl* (not shown). As expected from the *bowl*-repressing function of *lines* (Green et al., 2002; Hatini et al., 2005), the overexpression of *lines* along the margin phenocopies the loss of *bowl* (Fig. 2F). Nevertheless, the overexpression of *bowl* in other eye disc regions is not sufficient to induce *hh* (not shown). This suggests that, in regions other than the margin, either the levels of *lines* are too high to be overcome by *bowl* or *bowl* requires other factors to induce *hh*, or both.

drm and *odd* are required for and sufficient to initiate retinogenesis

drm and *odd* are expressed together along the posterior disc margin-Pe (Fig. 1), and *drm* (at least) is required for Bowl stabilization in leg discs (Hatini et al., 2005). Nevertheless, the removal of neither *drm* (Fig. 3A) nor *odd* (not shown) function alone results in retinal defects. *odd* and *drm* may act redundantly during leg segmentation (Hao et al., 2003) and this may also be the case in the eye margin. To test this, we induced clones of Df*drm*P2, a deficiency that deletes *drm*, *sob* and *odd*, plus other genes (Green et al., 2002). When Df*drm*P2 clones affect the margin, the adjacent retina fails to differentiate, suggesting that *drm* and *odd* (and perhaps *sob*, for which no single mutation is available) act redundantly to promote *bowl* activity at the margin (Fig. 3B,C) (although we cannot exclude that other genes uncovered by this deficiency also contribute to the phenotype). To test the function of each of these genes, we expressed *drm*, *odd* and *sob* in cell clones elsewhere in the eye disc. Only the overexpression of *drm* or *odd* induced ectopic retinogenesis (Fig. 3D and not shown), and this was restricted to the region immediately anterior to the MF, which is already eye

committed. Interestingly, *bowl* is also expressed in this region of L3 discs (Fig. 3E). The retina-inducing ability of *drm* requires *bowl,* because retinogenesis is no longer induced in *drm*-expressing clones that simultaneously lack *bowl* function (Fig. 3F). Therefore, it seems that in the eye, *drm* (and very likely also *odd*) also promotes *bowl* function.

The expression of *hh* (Heberlein et al., 1995) or activation of its pathway (Chanut and Heberlein, 1995; Dominguez and Hafen, 1997; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995; Wehrli and Tomlinson, 1995) anterior to the furrow is sufficient to generate ectopic retinal differentiation. As (1) *bowl* is required for *hh* expression at the margin, (2) this *hh* expression is

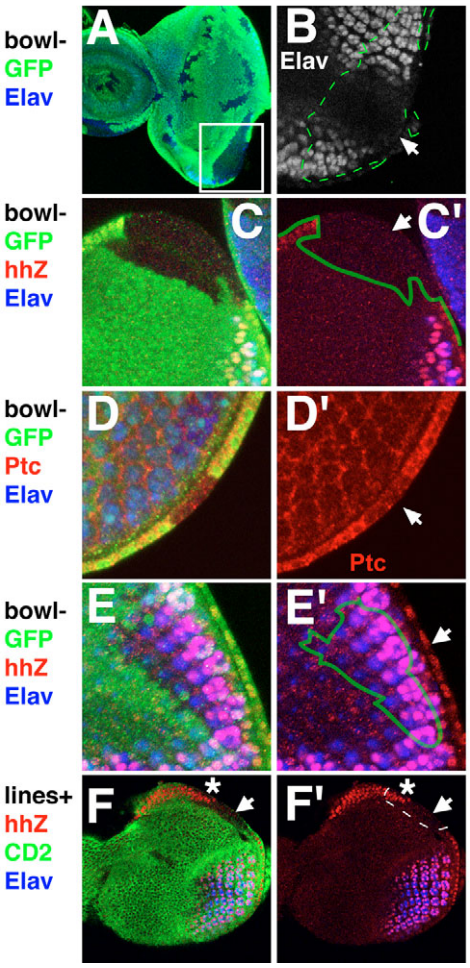

Fig. 2. *bowl* is required specifically at the margin for retinal triggering and *hh* expression. Clones are marked by the absence of GFP (A-E) or CD2 (F). (**A-E**) *bowl*$^{-}$ clones spanning the posterior margin. (A, inset in B) Defective retinal initiation is associated with *bowl*$^{-}$ mutant margin (arrow). Retinal initiation is partially rescued non-autonomously by neighboring tissue (clone outlined in B). (C,C′) *bowl*$^{-}$ clone spanning the margin loses *hh-Z* autonomously (arrow; clone outlined in C′). (D,D′) The expression of Ptc is also reduced in a *bowl*$^{-}$ clone (arrow). (E,E′) Internal *bowl*$^{-}$ clone abutting, but not including, the margin develops retina normally (clone outlined in E′). The *hh-Z* margin expression (arrow) is normal. (**F**,F′) *lines*-expressing clone at the margin resembles loss of *bowl,* causing loss of margin *hh-Z* and retinal failure (arrow). The *hh-Z* ocellar expression is not affected (asterisk). Discs are oriented with posterior towards the right and dorsal upwards.

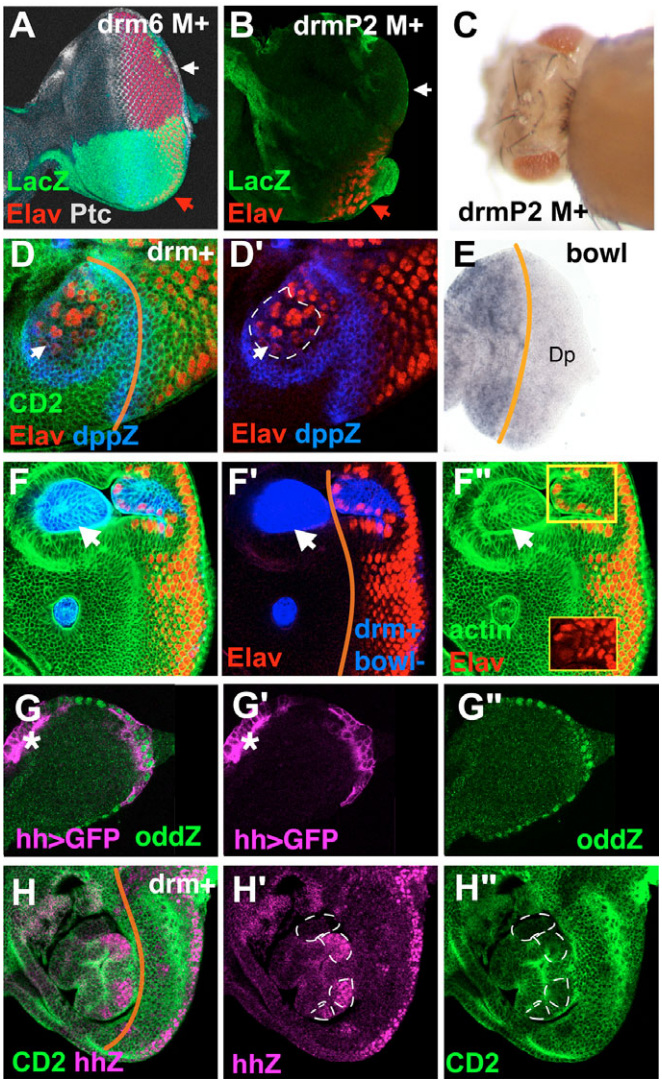

Fig. 3. *drm* and *odd* regulate *hh* expression, probably through enabling *bowl* function. (**A**,**B**) Eye discs containing *M+* clones mutant for (A) *drm*^{6} or (B) Df*drm*P2 (marked by absence of *lacZ*). (A) No effect on retinogenesis or Ptc expression is seen adjacent to *drm*-mutant margin. (Similar results were obtained for *odd*^{5}.) (B) Retinogenesis fails when the adjacent margin is mutant for Df*drm*P2. White and red arrows indicate mutant and wild-type margin, respectively. (**C**) Adult head from the Df*drm*P2, *M+* experiment showing severely reduced eyes. (**D**,D′) *drm*-expressing clone (absence of CD2, and outlined in D′) induces an ectopic furrow (marked by *dpp*-Z) and associated retinogenesis (detected by Elav). The line indicates the position of the endogenous furrow (D). (**E**) Disc proper (Dp) expression of *bowl* mRNA is detected anterior to the furrow (line) in late L3 discs. (**F**-F″) *drm*$^{+}$ *bowl*$^{-}$ clones (blue) do not induce ectopic retinal differentiation anterior to the morphogenetic furrow (arrow; line indicates the furrow). Phalloidin stains actin. A *drm*$^{+}$ *bowl*$^{-}$ clone located immediately after the furrow (boxed) shows Elav-positive neurons (inset). (**G**-G″) L2 eye disc from *oddZ*/UAS-GFP; *hh*-GAL4 larvae shows extensive overlap of *hh* and *odd* at the posterior margin. Asterisk indicates the *hh* ocellar domain, which, at this stage, does not express *odd-Z*. (**H**-H″) Most *drm*-expressing clones (absence of CD2, outlined in H′ and H″) induce *hh-Z* expression just anterior to the morphogenetic furrow (line). Discs are oriented with posterior towards the right and dorsal upwards.

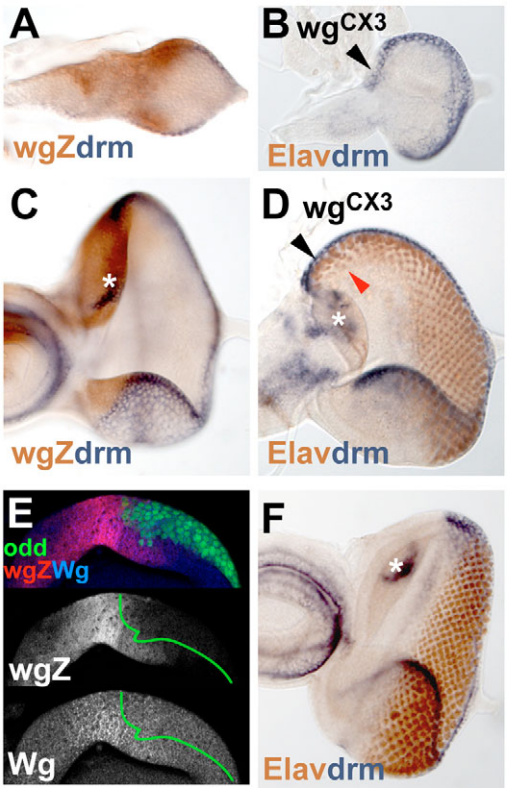

Fig. 4. *wingless* represses *drm* transcription in anterior eye disc margin. (**A**,**B**) Early and (**C-F**) late L3 discs. (A) In *wg*-Z discs (β-galactosidase, orange), prior to the initiation of retinal differentiation, *drm* and *wg* expressions are complementary. (C) In late L3 discs, this complementarity is maintained with the exception of the appearance of a dorsal head *drm*-expressing patch (asterisk in C,D,F). (B) In early L3 *wg*CX3 discs, *drm* transcription extends dorsally to reach the antenna (black arrowhead) before retinogenesis starts. (D) In older discs, ectopic retinogenesis (red arrowhead) can be seen progressing from the *drm*-expressing anterior margin (black arrowhead). (E) Dorsal margin of an *odd*-GAL4/*wg-Z*; UAS-GFP L3 disc. *odd* reporter expression (green and outlined in by the green line in the single channel panels) is complementary to both *wg* transcription (*wg-Z*) and protein expression (Wg). (F) A late wild-type L3 disc stained for *drm* and Elav is shown for comparison. Discs are oriented with posterior towards the right and dorsal upwards.

largely coincident with that of *odd* and *drm* (Fig. 3G), and (3) *drm* (and possibly *odd*) functionally interacts with *bowl*, we checked whether *drm*- and *odd*-expressing clones induced the expression of *hh*. In both types of clones *hh* expression is turned on autonomously, as detected with *hh-Z* (shown for *drm* in Fig. 3H), which would thus be responsible for the ectopic retinogenesis observed. That the normal *drm*/*odd*/*bowl*-expressing margin does not differentiate as eye could be explained if margin cells lack certain eye primordium-specific factors.

wingless represses *drm* transcription along the anterior dorsal eye disc margin

Our results indicate that the expression of *odd* and *drm* defines during L2 the region of the *bowl*-expressing margin that is competent to induce retinogenesis. How is their expression controlled? *wingless* (*wg*) is expressed in the anterior margin, where it prevents the start of retinal differentiation (Ma and Moses, 1995; Treisman and Rubin, 1995). *drm/odd* are complementary to *wg* (monitored by *wgZ*) during early L3, when retinal differentiation is about to start, and also during later stages (Fig. 4A,C,E). In addition, when *wg* expression is reduced during larval life in *wg*CX3 mutants, *drm* transcription is extended all the way anteriorly (Fig. 4B,D). This extension precedes and prefigures the ectopic retinal differentiation that, in these mutants, occurs along the dorsal margin (Fig. 4B,D,F). Therefore, *wg* could repress anterior retinal differentiation by blocking the expression of odd genes in the anterior disc margin, in addition to its known role in repressing *dpp* expression and signaling (Hazelett et al., 1998; Treisman and Rubin, 1995).

Interestingly, the onset of retinogenesis in L3 is delayed relative to the initiation of the expression of *drm/odd* (this work) and *hh* (Cavodeassi et al., 1999; Cho et al., 2000) in L1-2. This delay can be explained in three, not mutually exclusive, ways. First, the relevant margin factors (i.e. *drm/odd*, *hh*) might be in place early, but the eye primordium might become competent to respond to them later. In fact, *wg* expression domain has to retract anteriorly as the eye disc grows, under *Notch* signaling influence, to allow the expression of eye-competence factors (Kenyon et al., 2003). Second, building up a concentration of margin factors sufficient to trigger retinogenesis might require some time. In fact, the activity of the *Notch* pathway along the prospective dorsoventral border is required to reinforce *hh* transcription at the firing point (Cavodeassi et al., 1999). Third, other limiting factors might exist whose activity becomes available only during L3. Such a factor might be the EGF receptor pathway, which is involved in the triggering and reincarnation of the furrow along the margins during L3 (Kumar and Moses, 2001).

In addition to *hh*, other genes are required for retinal triggering, including *dpp* (Burke and Basler, 1996; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996), *eyes absent* (*eya*) (Bonini et al., 1993) and the target of *eya dachshund* (*dac*) (Mardon et al., 1994; Pignoni et al., 1997). These genes are expressed in both the posterior region of the eye primordium and the posterior margin. In addition to their role in eye specification, they might also specify the margin. Although the regulatory relationships between *hh* and *dpp*, or *dpp* and *eya* are obscured by cross-regulatory interactions (Borod and Heberlein, 1998; Chen et al., 1999; Curtiss and Mlodzik, 2000; Hazelett et al., 1998; Pignoni and Zipursky, 1997), recent functional data indicate that *dpp* and *eya* are functionally downstream of *hh* (Pappu et al., 2003). The possibility that the odd genes control the expression or function of *dpp* and *eya* at the margin remains to be tested.

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