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Wingless signaling in *Drosophila* eye development

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Abstract

The *Drosophila* eye disc gives rise to both the retina and the surrounding head capsule. The secreted morphogen Wingless (Wg) plays an important role in subdividing the disc between these two tissues; *wg* is expressed in the anterior lateral margins, regions that will give rise to the head capsule, and high levels of Wg signaling promote this fate. Wg is expressed earlier and more strongly in the dorsal than the ventral margin, and also contributes to specifying dorsal identity. In addition, Wg signaling can cause overgrowth of eye disc cells. Finally, at pupal stages, *wg* expression surrounds the eye and a gradient of Wg establishes several distinct peripheral cell fates. We describe how to generate clones of cells mutant for genes encoding components of the Wg signaling pathway in the eye disc and examine their effects on photoreceptor differentiation by antibody staining.

1. Wingless signaling in *Drosophila* eye development

1.1. Introduction

The *Drosophila* compound eye consists of a hexagonal array of approximately 800 light-sensing units called ommatidia. Each ommatidium contains 8 photoreceptor neurons, 4 lens-secreting cone cells, and 2 primary pigment cells; the ommatidia are surrounded by a lattice of secondary and tertiary pigment cells and mechanosensory bristles (1). The adult eye and head capsule develop from an epithelial bilayer known as the eye-antennal imaginal disc. This structure derives from a primordial group of about 20 cells determined early during embryogenesis by expression of the Pax6 transcription factors Twin of eyeless (Toy) and Eyeless (Ey) (2,3). The disc invaginates from the embryonic ectoderm and grows by asynchronous cell divisions until the third larval instar (1,4). The columnar epithelium of the disc is partitioned into separate eye and antennal primordia in the second larval instar (5). The squamous peripodial epithelium gives rise to head structures and may also communicate with the columnar epithelium (6,7).

Photoreceptor differentiation initiates at the posterior margin of the eye disc in the third larval instar and proceeds one row at a time towards the anterior. Differentiation is preceded by the morphogenetic furrow (MF), a transient groove in the epithelium formed by contraction of the cells in the apical/basal dimension (8). Cells in the MF are arrested in the G1 phase of the cell cycle (9). Immediately posterior to the MF, some of these cells are organized into a regularly spaced array of five-cell preclusters (10). The remaining cells undergo a final round of cell division, the second mitotic wave, before the remaining photoreceptors and cone cells are recruited to each cluster (10). The precise lattice of interommatidial cells is formed by differentiation and cell death during the pupal stages (11).

The *wingless* (*wg*) gene is the founding member of the Wnt gene family and encodes a secreted signaling protein that acts as a morphogen (12). In the eye-antennal disc, Wg acts primarily to

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promote head capsule differentiation and restrict eye development (13–16). In addition, Wg signaling contributes to the growth, patterning and differentiation of both the eye and head primordia. We will review these functions and also provide a detailed protocol to generate and stain clones of cells with high levels of Wg signaling within the developing eye disc.

1.2. Wg inhibits eye specification

Eye specification is controlled by the retinal determination genes, a set of transcription factors that function in a complex hierarchy. The two *Pax6* homologues *toy* and *ey* act at the top of the cascade, with *toy* upstream of *ey*; both are necessary and sufficient for eye development but are also required for formation of the entire head, reflecting their early expression in the antennal disc (2,3,17,18). Subdivision of the disc into separate eye and antennal primordia is first apparent in the early second instar, when Eyes absent (*Eya*), a target of *Ey*, is specifically expressed at the posterior margin of the eye disc in response to signaling by the secreted molecules Hedgehog (*Hh*) and Decapentaplegic (*Dpp*) (5,18,19). *Ey* also activates the expression of *Sine oculis* (*So*), a homeodomain protein that interacts with *Eya* and acts in conjunction with it to promote eye specification (18,20,21). Finally, the DNA-binding protein Dachshund (*Dac*) acts downstream of these genes but can also physically interact with *Eya* (18,22,23). Positive feedback regulation stabilizes the expression of these four genes to lock in the retinal fate (18,20,23–26).

Cells in the eye disc initially express *Ey* together with two other transcription factors, Homothorax (*Hth*) and Teashirt (*Tsh*), and these factors are restricted to the most anterior region of the eye disc as differentiation proceeds (27). A proneuronal domain anterior to the MF is defined by the loss of *Hth* and the expression of *Eya*, *So* and *Dac* (27). Posterior to the MF, expression of *Ey*, *Tsh* and *Dac* is downregulated, but *Eya* and *So* are maintained and continuously required for photoreceptor differentiation (20,27,28). The early function of the interactive retinal specification and determination network thus ensures that cells anterior to the MF are already committed to become retinal tissue before they differentiate.

Many observations demonstrate that Wg signaling promotes head capsule formation at the expense of the retinal field (13–16). Reduction of Wg activity using a temperature-sensitive allele or removal of the downstream component *dishevelled* (*dsh*) results in expansion of the eyes into the lateral regions of the dorsal head (15,16,29). Conversely, removal of negative regulators of Wg signaling such as *shaggy* (*sgg*) or *axin* (*axn*) transforms eye tissue into head cuticle (13,29–31). Ectopic Wg activity also occasionally results in tube-like outgrowths that resemble legs or antennae more than head cuticle (30).

Wg protein is present throughout the entire second instar eye disc, where it blocks the induction of *Eya* expression by *Dpp* until disc growth brings posterior cells out of the range of Wg diffusion (5,30,32). Consistent with this model, the lack of eye development in mutants for *eyegone*, a target of Notch signaling that is required for disc growth (33), can be rescued by inhibiting Wg signaling in posterior cells (34). In the third instar eye disc, *wg* expression is restricted to the anterior lateral margins (6,13,15,29,35), where it continues to repress the expression of *Eya*, *So* and *Dac* in regions destined to form the dorsal head (30). Wg may also promote the head fate by enhancing expression of the anteriorly expressed transcription factor *Hth* (36) (Fig. 1B).

These expression patterns are maintained by negative feedback loops; *Eya* and *Dac* in turn repress expression of *wg* at the posterior margin of the eye disc (13,34), while *Hth* maintains Wg expression (36), contributing to the distinction between eye and head fates. Another regulator of *wg* expression is the JAK/STAT signaling pathway. The ligand Unpaired (*Upd*) is expressed at the posterior margin of the early eye disc and promotes the formation of the eye field through repression of *wg* transcription (37,38). The negative regulatory interactions

between anteriorly expressed Wg and posteriorly expressed signaling molecules including Hh, Dpp and Upd thus define the boundaries of the eye field.

Loss of *wg* activity transforms the head primordia into eye tissue by initiation of ectopic morphogenetic furrows, primarily from the dorsal margin of the eye disc which normally expresses *wg* more strongly, but also weakly from the ventral margin (13,15,35) (Fig. 3B, D). These furrows then progress inwards towards the center of the disc (13,15). Conversely, ectopic activation of the Wg pathway, using over-expression of *wg* or an activated form of *armadillo* (*arm*), or mutations in *sgg* or *axin*, prevents MF initiation and progression (13,14, 31). Although high levels of Wg signaling can inhibit expression of *dpp*, a positive regulator of MF movement (29), Wg also has an inhibitory effect downstream of Dpp receptor activation (34). This might be due to its ability to induce Hth, a negative regulator of photoreceptor differentiation that is repressed by Dpp signaling (27,36,39). Another target for Wg that is likely to be relevant for MF initiation is *drumstick* (*drum*), a member of the *odd-skipped* gene family, which normally contributes to MF initiation at the posterior margin and is repressed by Wg at the anterior lateral margins (40).

Taken together, these results suggest that division of the eye disc between an anterior head field and a posterior eye field may rely on the balance between antagonistic Wg signaling in the anterior and Dpp signaling in the posterior. The ranges of these signals initially overlap, but are separated by growth of the disc anlage, which is driven by Notch activity (5,33).

1.3. Wg patterns the dorsal-ventral axis in the eye disc

In addition to its effect on anterior-posterior patterning of the eye disc, Wg also contributes to distinguishing the dorsal and ventral domains. It is not clear when dorsal-ventral differences are first established, as transcripts encoding the GATA family transcription factor Pannier (*Pnr*) are present in the dorsalmost region of the embryonic eye disc (41), but a *pnr*-GAL4 line does not drive UAS-GFP expression in the dorsal eye disc until early second instar (42). *wg-lacZ* is expressed in the dorsal peripodial membrane and margin cells beginning in the first instar (6,35,43); at least at later stages, dorsal *wg* expression is dependent on *pnr* (41). *wg* expression in the dorsal peripodial epithelium has been traced to activation by Pnr of an eye-specific enhancer in the *wg* 3' cis-regulatory region (44).

Together with Hh, Wg activates the expression of the Iroquois complex (*Iro-C*) homeodomain genes, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*), in the dorsal half of the disc (31, 41,43,45). The *Iro-C* proteins repress the expression of *fringe* (*fng*), which encodes a glycosyltransferase that modulates the affinity of the Notch receptor for its ligands Delta (*Dl*) and Serrate (*Ser*) (46–48). The *Fng* expression border, in combination with the restriction of *Ser* to the ventral compartment and *Dl* to the dorsal compartment, results in activation of Notch precisely at the dorsal/ventral boundary of the disc, known as the equator (46–48) (Fig. 1A). Through its effect on the *Iro-C* genes, Wg thus contributes to the localized activation of Notch at the equator, which stimulates growth of the eye disc and determines the initiation point of the MF.

wg is not expressed ventrally in the early eye disc, but its expression appears at the ventral margin in the early third instar (35,43). Both *Ser* and its upstream activator *Lobe* are required for ventral eye development and growth (42). *Lobe* and *Ser* have been shown to repress ventral *wg* expression during the second instar; their absence results in ectopic Wg signaling that triggers cell death and loss of the ventral eye (49). Formation of a normally patterned eye is thus critically dependent on the timing of *wg* expression in this region. Later in development, Wg diffuses from both the dorsal and ventral margins to establish inverse gradients of *Dachsous* and *Four-jointed*, two molecules that set up planar polarity in the eye disc (50,51). This function of Wg is discussed in more detail in Chapter ... of this volume.

1.4. Wg controls growth of the eye disc

Both loss of function and gain of function studies suggest that Wg is a positive regulator of eye disc growth. Removal of *wg* early in larval development using a temperature-sensitive allele resulted in very small eye discs (13,15); however, this size reduction could be due to premature differentiation of cells that would otherwise have continued to proliferate. Activation of the Wg signaling pathway by overexpression of Wg or removal of the negative regulator Axin from clones of cells results in dramatic overgrowth, especially in clones that contact the posterior margin (13,30,31). Such clones do not respect the normal boundaries of the eye disc, but form large rounded projections beyond these boundaries (30,31) (Fig. 3E). A similar effect can be produced by misexpression of Tsh, which is not normally expressed in posterior margin cells, and this effect requires Wg signaling (27,52), perhaps because Tsh confers Wg responsiveness on these cells (53).

Final eye size must depend on the interplay between Wg and other growth regulators such as Dpp, Notch and its target Upd (6,46–48,54–56). Expression of Wg or activated Arm throughout the eye disc reduces eye size in addition to blocking differentiation (31,34,49), probably due to its effects on these other factors. The mitogenic effect of Wg may therefore depend on other signals received by the responding cells.

1.5. Wg surrounding the eye patterns the peripheral retina

As photoreceptor differentiation proceeds, *wg* is transcribed in a thin stripe of cells along the posterior margin of the eye disc, adjacent to the retinal field (15,29). When the MF reaches the anterior of the eye disc at the end of the first day of pupal development, *wg* expression remains in a ring of presumptive head cuticle cells, immediately adjacent to and surrounding the entire developing eye. This pattern is maintained throughout pupation and in the adult head. Wg diffusion from this peripheral source establishes a gradient that patterns the peripheral retina (29,57).

The outermost region of the eye is organized into a series of concentric rings with different morphological features. At the periphery of the eye, abutting the head capsule, the pigment rim (PR) is a thick layer of pigment cells, devoid of photoreceptors, that insulates ommatidia from extraneous light rays (57). On the dorsal side of the eye only, the ommatidia directly adjacent to the PR are specialized polarized light detectors called dorsal rim ommatidia (DRO) (57, 58). Finally, bristles are absent from the outermost ommatidial rings, but present in the remainder of the eye (Fig. 1C). A series of elegant gain and loss of function experiments demonstrated that a gradient of Wg signaling organizes the differentiation of these concentric features (57).

During the pupal phase, the most peripheral ring of ommatidia is eliminated by apoptosis (59–61), while the surrounding secondary and tertiary pigment cells survive and contribute to the PR (61). Wg signaling at mid-pupation is required for the death of these 80–100 ommatidia that often lack the full complement of cells (60–62). This programmed cell death also requires the Snail group transcription factors Worniu (Wor), Escargot (Esg) and Snail (Sna), which are targets of the Wg signaling pathway (62). Interestingly, *wg* overexpression or loss of the negative regulator of Arm activity Adenomatous polyposis coli 1 in the retinal lattice is sufficient to elicit *arm*-dependent apoptosis of all photoreceptors at mid-pupation (61,63,64).

While high levels of Wg activity result in photoreceptor death and pigment cell differentiation, lower levels of Wg can induce the differentiation of DRO. A critical target of Wg for this activity is the transcription factor Hth, which acts in combination with the Iro-C homeodomain proteins expressed specifically in the dorsal eye (57,58). Finally, even lower levels of Wg

signaling can prevent the formation of interommatidial bristles, at least in part through repression of the proneural gene *achaete* and its cofactor *daughterless* (57,65,66).

The multiple functions of Wg throughout eye development in part reflect its dynamic expression pattern, but probably also require spatial and temporal regulation of the responsiveness of surrounding cells. Combinatorial control of growth and differentiation by Wg and other secreted factors is also likely to play an important role in setting up the pattern of the eye disc. Elucidating the functions of Wg in eye development has been critically dependent on the ability to analyze mosaic eye discs in which random clones of cells are mutant for components of the Wg signaling pathway. This technique will be described in the following sections.

2. Materials

2.1. Useful tools to study Wg signaling in the *Drosophila* eye

Drosophila strains	Comment	References
<i>wg^{CX4}</i>	amorph	(67)
<i>wg^I</i>	viable hypomorph	(68)
<i>wg^{IN}</i>	not secreted	(69)
<i>wg^{IL114}</i>	temperature sensitive	(70)
<i>wg^{I-12}</i>	temperature sensitive	(71)
<i>wg^{en-11}</i>	enhancer trap, amorph	(72)
<i>arr^{I2-1}</i>	loss of function	(73)
<i>fz¹⁵</i>	amorph	(74)
<i>fz-2^{C1}</i>	loss of function	(75)
<i>dshV²⁶</i>	amorph	(76)
<i>axn^{E77}</i>	loss of function	(31)
<i>sgg^{D127}</i>	amorph	(77)
<i>dAPC^{Q8}</i>	loss of function	(78)
<i>arm^I</i>	amorph	(79)
<i>pan² / dTCF²</i>	amorph	(80)
<i>act5c>y⁺>wg</i>	FLP-out	(81)
<i>tub>w⁺>wg</i>	FLP-out	(50)
<i>GMR-wg</i>	expressed posterior to MF	(50)
<i>GMR-wg^{ts}</i>	temperature sensitive	(57)
<i>sev>w⁺>arm*</i>	Activated form	(50)
<i>UAS-wg</i>	GAL4-responsive promoter	(83)
<i>UAS-wg^{ts}</i>	temperature sensitive	(84)
<i>UAS-GFP-wg</i>	GFP-tagged	(85)
<i>UAS-Nrt-flu-wg</i>	Tethered, HA-tagged	(86)
<i>UAS-dTCF^{ΔN3} / UAS-dTCF^{DN}</i>	Dominant negative	(80)
<i>UAS-arm</i>		(78)
<i>UAS-arm^{K45}</i>	Activated form	(87)
<i>UAS-arm^{S10}</i>	Activated form	(88)
<i>UAS>CD2, y⁺>flu-Δarm</i>	N-terminal deletion, activated	(86)
<i>UAS-sgg</i>		(89)

<i>UAS-sgg^{S9A} / UAS-sgg^{act}</i>	Activated form	(34)
<i>UAS-axn^{A2}</i>		(90)
<i>wg2.11-lacZ</i>	reporter	(44)
<i>arm-lacZ</i>	reporter	(91)
<i>wg-GAL4</i>	GAL4 expressed in wg pattern	(92)
<i>arm-GAL4</i>		(93)
Antisera		
Anti-Wg		Developmental Studies Hybridoma Bank
Anti-dAPC		(82)
Anti-Arm		Developmental Studies Hybridoma Bank
Anti-Axn		(90)
Anti-Elav	recognizes photoreceptor nuclei	Developmental Studies Hybridoma Bank

2.2. Fly stocks

1. y,w, ey-FLP ; + ; FRT82B, arm-lacZ / TM6B
2. y,w ; + ; FRT82B, axn^{E77} / TM6B

2.3. Dissection materials

1. two pairs of fine forceps (A. Dumont & Fils, # 5)
2. a silicone dissection dish (Sylgard, Silicone Elastomer Kit)
3. a binocular microscope (Zeiss, Stemi SV 11)
4. a tungsten hook : ultra micro needle (Ted Pella, Inc.) bent into a hook
5. 30mm Petri dishes
6. polystyrene microwell mini trays, 60 wells (Nunc)
7. superfrost microscope slides, cover slips and nail polish

2.4. Solutions

- 1) 0.1 M phosphate buffer, pH 7.2: mix 1M Na₂HPO₄ and 1M NaH₂PO₄ in a 72:28 ratio, respectively, and add 9 volumes of water.
- 2) PEM: 0.1 M PIPES pH 7.0, 2mM MgSO₄, 1mM EGTA; it is usually kept as a 2X stock.
- 3a) 4% formaldehyde in PEM: for 10ml, mix 5ml PEM, 1ml H₂O and 4ml 10% methanol-free formaldehyde (Polysciences).
- 3b) or 2% formaldehyde in PLP: Prepare a phosphate buffer solution by mixing 75ml 0.1 M phosphate buffer pH 7.2, 2.5ml 1M Na₂HPO₄, and 122.5ml H₂O. To 15ml of this, add 1ml water and 0.27g lysine. Just before use, add 50mg sodium periodate and 4ml 10% formaldehyde (methanol-free).
- 4) 0.1 M phosphate buffer pH 7.2, 0.2 % Triton. For some antibodies other detergents such as saponin may be preferable.
- 5) normal donkey serum (Jackson Immunoresearch)

- 6) Rat anti-Elav (Developmental Studies Hybridoma Bank) and HRP-conjugated donkey anti-rat (Jackson ImmunoResearch)
- 7) diaminobenzidine (DAB) solution : for 250µl staining solution, add 225µl 0.1 M phosphate buffer, 0.2% Triton to 25µl 5mg/ml DAB; add 5µl 1% cobalt chloride for intensification and 2.5µl 0.3% H₂O₂ (freshly diluted from a 30% stock). For double staining with X-gal, include 5µl 1% nickel ammonium sulfate and 6µl 1% cobalt chloride in the DAB solution. DAB is toxic; handle with gloves and deactivate used tips etc. overnight in bleach before disposal.
- 8) X-gal staining buffer: For 50ml, mix 1.8ml 0.2M Na₂HPO₄, 0.7ml 0.2M NaH₂PO₄, 1.5ml 5M NaCl, 50µl 1M MgCl₂, 3.05ml 50mM K₃Fe(CN)₆, 3.05ml 50mM K₄Fe(CN)₆, H₂O to 50ml.
- 9) 8% X-gal solution in dimethylformamide (store in aliquots at -80°C)
- 10) 80% glycerol (Roche molecular biology grade) in PBS

3. Methods

3.1. Genetics

1. Cross 6–8 *y,w, ey-FLP ; FRT82B, arm-lacZ / TM6B* females with 4–6 *y,w ; FRT82B, axn^{E77} / TM6B* males in a freshly yeasted food vial. Allow the flies to lay eggs for 2–3 days. Select non-*Tubby* *y,w, ey-FLP / y,w or Y ; FRT82B, arm-lacZ / FRT82B, axn^{E77}* third instar larvae.

3.2. Dissection

- 1) Remove third instar larvae from the food vial with forceps. Select wandering larvae that have left the food but have not yet pupariated. Transfer the larvae to a puddle of 0.1 M phosphate buffer (pH 7.2) on a dissection plate to wash off excess food.
- 2) Select a larva and move it to a second puddle of 0.1 M phosphate buffer (pH 7.2) on the same plate. Under the dissecting microscope, hold it gently about halfway down the body with one pair of forceps. With a second pair of forceps, grasp the mouthparts and pull them out of the head (Fig. 2-1). Usually the eye-antennal discs will remain attached to the mouthhooks. Still holding the mouthhooks, remove other attached tissues such as the salivary glands and gut. Then hold the internal part of the mouthhooks and remove the external cuticle (Fig. 2-2). To remove the brain, hold the mouthhooks with one pair of forceps and pin the brain to the dissection dish between the two hemispheres with one point of a second pair of forceps. Gently pull on the mouthhooks to break the optic stalk that links the eye disc to the brain (Fig. 2-4). Leave the eye discs attached to the mouthhooks (see Note 1).
- 3) Using a tungsten hook, pick up the eye discs, placing the hook at the junction between the antennal imaginal disc and the mouthhooks (Fig. 2-5). Deposit the discs on the surface of the fix solution (usually 4% formaldehyde in PEM) in a 30mm Petri dish. Fix the discs for 25–35min on ice (see Note 2).
- 4) Using the hook, transfer the eye discs attached to the mouthhooks into a 30mm Petri dish filled with 0.1 M phosphate buffer, 0.2% Triton. Wash the discs in this solution for 15min on ice.
- 5) Transfer the discs to the antibody solution. A 60 well (10×6) microwell tray can be used for up to 6 different samples that will require 10 wells each (see Note 3). Place a folded wet Kimwipe in the tray to maintain its humidity. Incubate the discs in

primary antibody diluted in 0.1 M phosphate buffer, 0.2 % Triton, 10% serum, overnight at 4°C. Rat anti-Elav can be used at 1:100.

- 6) In the following steps, use the hook to transfer the discs to the next well at each step. Wash 3 × 5min in 0.1 M phosphate buffer, 0.2% Triton at room temperature. Incubate in secondary antibody diluted 1:200 in 0.1 M phosphate buffer, 0.2 % Triton, 10% serum, at least 2hrs at 4°C. Wash 3 × 5min in 0.1 M phosphate buffer, 0.2% Triton at room temperature.
- 9) Transfer the discs to freshly prepared diaminobenzidine (DAB) solution. Staining will occur rapidly, so transfer only a few discs at a time and be prepared to stop the reaction before the discs are overstained.
- 10) Stop the reaction by transferring the discs to 0.1 M phosphate buffer, 0.2% Triton.
- 11) Proceed with X-Gal staining if desired. Incubate in prewarmed (65°C) X-gal staining buffer with a 1:40 dilution of 8% X-gal (7.5µl in 300µl) at 37°C. Be careful not to let the staining buffer cool down; transfer discs directly to a tube at 37°C if possible. For *arm-lacZ* the staining time is usually 2–3 hrs, but staining should be checked under a dissecting microscope. Wash in PBS, 0.1% Triton, rocking for 1hr, to remove crystals of X-gal that may have become attached to the discs.
- 12) Mount discs in 80% glycerol in PBS. Deposit a 100 µl drop of glycerol solution on a microscope slide. Transfer the discs into the glycerol. Remove the mouthhooks and cover the discs with a cover slip on the slide. Seal the cover slip with nail polish (see Note 4).

4. Notes

1. Keeping the mouthhooks attached to the eye-antennal discs provides a safe way to handle both discs from one larvae at the same time. One can then transfer the discs between wells with minimal damage or solution transfer by picking them up with a tungsten hook placed under the antennal discs, so that the mouthhooks fall to one side and the eye discs to the other.
2. The ventral and dorsal edges of the eye disc have a tendency to fold over, making it difficult to flatten the discs sufficiently when mounting them. When transferring the discs into the fix solution, pick them up by placing the tungsten hook under the antennal discs with the apical, convex, surface of the discs facing down. Gently deposit the discs apical side down on the surface of the fix, while moving the hook downward into the fix solution. Due to surface tension, the eye disc epithelium will unfold and flatten on the fix. PEM works well for antibodies to most nuclear and cytoplasmic proteins, but transmembrane proteins may be better preserved by fixing in PLP for 40–60 min on ice.
3. The use of a microwell mini tray allows each step of the staining protocol to take place in a 10–15 µl volume, conserving precious antibodies.
4. To improve the quality of the mounting, drag each disc toward one edge of the glycerol drop with its apical surface upward, until the disc immobilizes and flattens. Then gently place the cover slide on the drop beginning with the edge closest to the discs. The glycerol drop will enlarge but the flattened discs won't disperse or twist. Discs can be further flattened by gently pressing on the coverslip with forceps while observing the discs through the dissecting microscope to be sure the force is not too great.

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Figure 1.

Schematic representations of 2nd instar (A) and 3rd instar (B) eye-antennal imaginal discs and a pupal eye (C). Dorsal is to the top and anterior is to the left. Wg-dependent genetic interactions governing D/V compartmentalization (A) and retinogenesis (B) are indicated by arrows (positive regulation) or intersecting lines (negative regulation). (B) shows *wg* expression in the 3rd instar eye disc, which is restricted to small patches at the anterior dorsal and ventral margins and a thin stripe extending around the posterior margin. Expression of the retinal determination network proteins (*Eya*, *Dac* and *So*) is encircled by a dashed black line. Note that *Dac* expression does not extend as far posteriorly as *Eya* and *So*. Expression of *Ey* in the anterior part of the eye disc is encircled by a dashed gray line. (C) indicates the concentric

arrangement of head capsule, PR, DRO and ommatidia lacking or containing bristles, which is established at pupal stages by Wg expressed in a ring surrounding the eye.

Figure 2.

Cartoon of the dissection protocol for eye discs stainings. (1) Using fine tweezers, open the larva by pulling on the mouthhooks (black). (2–3) remove the larval cuticle and extract the eye-antennal discs attached to the mouthhooks. (4) pin down the brain between the two hemispheres and pull on the mouthhooks to separate the eye discs from the brain. (5) Pick up the discs carefully with a tungsten hook placed under the antennal discs, with the apical, convex side facing downward. (6) Deposit the discs on the surface of the fix, allowing the discs to flatten due to surface tension.

Figure 3.

Effects of genetic manipulation of the Wg pathway on photoreceptor differentiation. All pictures are 3rd instar eye discs stained with X-gal for β -galactosidase activity (grey) and with an antibody to the neuronal marker Elav (black). In each panel, anterior is to the left and dorsal to the top. (A) a wildtype disc expressing *dpp-lacZ*. Regularly spaced clusters of photoreceptors differentiate posterior to the MF, which is marked by *dpp-lacZ* activity. (B–E) show discs heterozygous for *dsh*^{V26} (B, D) or *axn*^{E77} (C, E) in which clones of homozygous mutant cells have been induced by the FLP/FRT technique and are visualized by the absence of *arm-lacZ* activity. (B, D) Removal of *dsh*, a positive intracellular effector of Wg signaling, in cells abutting the anterior dorsal margin (dotted line), prevents Wg signal transduction and allows

ectopic MF initiation as visualized by ectopic photoreceptor differentiation anterior to the normal MF (black arrow in D, compare with A). (C, E) Conversely, removal of *axn*, a negative regulator of Wg signaling, from clones of cells within the differentiating retina (dotted lines and asterisks), allows ectopic activation of Wg signaling posterior to the MF and eliminates photoreceptor differentiation in these clones. Mutant clones at the posterior margin of the eye disc also grow beyond the normal boundaries of the disc (arrow in E).

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