Chapter 12

Wingless Signaling in *Drosophila* Eye Development

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Abstract

The secreted morphogen Wingless (Wg) has a variety of functions throughout *Drosophila* eye development, controlling tissue specification, growth, and patterning. Wg plays a critical role in subdividing the eye imaginal disc into separate primordia that will give rise to the adult retina and the surrounding head capsule. During larval development, *wg* is expressed in the anterior lateral margins of the eye disc, regions that will give rise to head cuticle; Wg signaling promotes the head fate and prevents these marginal regions from initiating ectopic photoreceptor differentiation. Expression of *wg* at the dorsal margin is earlier and stronger than at the ventral margin, allowing Wg to contribute to specifying the dorsal domain of the eye disc. Finally, during the pupal stages, *wg* expression surrounds the entire eye and a concentric gradient of Wg establishes several distinct peripheral retinal cell fates. This chapter reviews these aspects of Wg function and describes 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 immunohistochemistry.

Key words: *Drosophila*, *wingless*, eye disc, photoreceptor, immunostaining, *dishevelled*, *axin*.

1. Introduction

The *Drosophila* compound eye consists of a hexagonal array of approximately 800 light-sensing units called ommatidia. Each ommatidium contains eight photoreceptor neurons, four lens-secreting cone cells, and two 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

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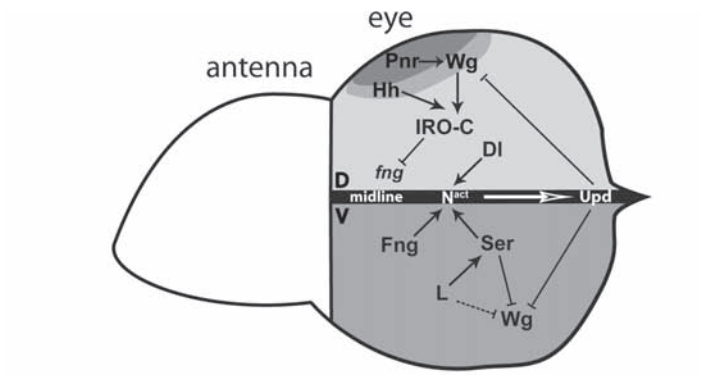
embryogenesis by expression of two Pax6 family transcription factors *(2–4)*. The disc invaginates from the embryonic ectoderm and grows by asynchronous cell divisions until the third larval instar *(1, 5)*. The retina arises from the columnar epithelial layer, which is partitioned into separate eye and antennal primordia during the second larval instar (**Fig. 12.1A**; ref. *6)*. The eye disc epithelium is further subdivided into a central field that gives rise to the eye, and marginal regions that instead form the head capsule *(7)*. The overlying squamous peripodial epithelial layer gives rise to additional head structures and may also communicate with the columnar epithelium *(8, 9)*.

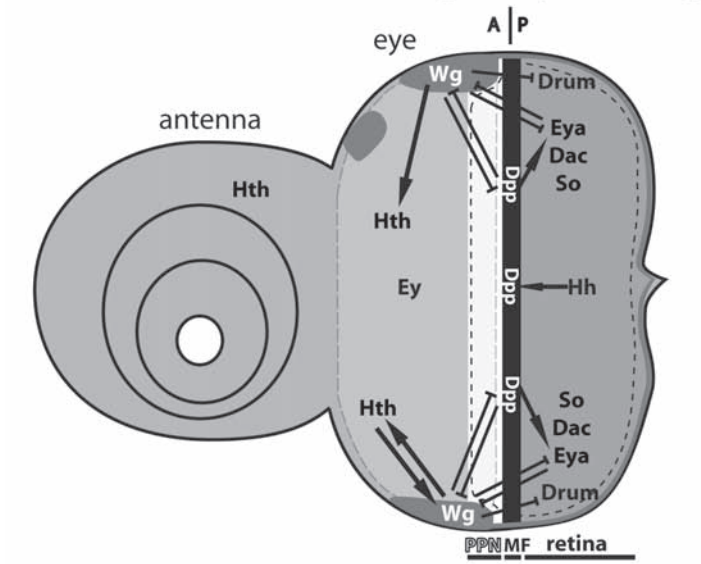
In the third larval instar, a wave of photoreceptor differentiation initiates at the posterior margin of the eye disc and progresses sequentially one row of ommatidia at a time toward the anterior. Differentiation is preceded by the morphogenetic furrow (MF), a transient vertical groove in the epithelium formed by contraction of the cells in the apical/basal dimension and constriction of their apical surfaces (**Fig. 12.1B**; ref. *10)*. In the MF, cells are arrested in the G1 phase of the cell cycle *(11)*. Cells immediately posterior to the MF become organized into a regularly spaced array of five-cell preclusters containing the precursors of photoreceptors R8, 2, 5, 3, and 4 *(12)*. The remaining cells undergo a final round of cell division, the second mitotic wave, before R1, 6, 7 and the four cone cells are recruited to each ommatidium *(12)*. The precise lattice of pigment cells and bristles surrounding the ommatidia is formed by differentiation and cell death of the interommatidial cells during the pupal stages *(13)*.

The *wingless* (*wg*) gene is the founding member of the *Wnt* gene family and encodes a secreted signaling protein that acts as a morphogen *(14)*. In the eye disc, Wg acts primarily to promote head capsule differentiation and restrict eye development *(15–18)*. In addition, Wg signaling patterns the eye primordium along the dorsal-ventral and central-peripheral axes, and contributes to its growth. The following reviews these functions and provides a detailed protocol to generate and stain clones of cells mutant for Wg pathway components within the developing eye disc.

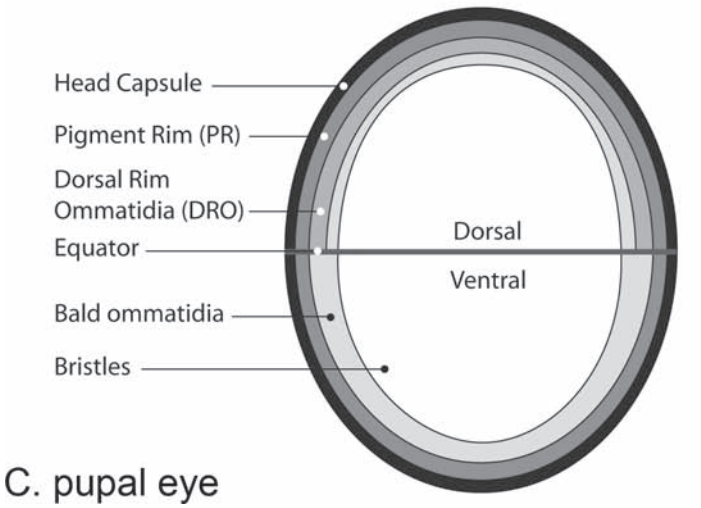
Fig. 12.1. Functions of Wg in eye development. Panels show schematic representations of second instar (**A**) and third instar (**B**) eye-antennal imaginal discs and a pupal eye (**C**). Dorsal is to the top and anterior is to the left. Wg–dependent genetic interactions governing D/V compartmentalization (**A**) and MF progression (**B**) are indicated. (**B**) shows *wg* expression in the third 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. MF (morphogenetic furrow), PPN (preproneural domain). (**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.



A. 2nd instar disc illustrating D/V patterning



B. 3rd instar disc illustrating MF progression



C. pupal eye

1.1. Wg Inhibits Eye Specification

A critical role for Wg is to define anterior regions of the eye disc that will give rise to the head capsule rather than the eye. Eye specification is controlled by the retinal determination genes, a set of transcription factors that function in a complex hierarchy. The two *Pax6* homologues *twin of eyeless* (*toy*) and *eyeless* (*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 whole eye-antennal disc *(2–4, 19)*. Subdivision of the disc into separate eye and antennal primordia is first apparent in the early second instar, when Ey is downregulated in the anterior region that will give rise to the antenna *(6, 19, 20)*. Concomitantly with this change, the retinal determination protein Eyes absent (Eya) is specifically expressed at the posterior margin of the eye disc in response to signaling by the secreted molecules Hedgehog (Hh) and Decapentaplegic (Dpp) *(6, 19, 20)*. Ey is required for the expression of both Eya and Sine oculis (So), which interact with each other to form a compound transcription factor that promotes eye specification *(19, 21, 22)*. The final retinal determination protein, Dachshund (Dac), requires these proteins for its expression, but can also physically interact with Eya to promote eye development *(19, 23, 24)*. Positive feedback regulation stabilizes the expression of these four genes to lock in the retinal fate *(19, 21, 24–27)*.

Once the eye disc has been specified in the second instar, its cells express Ey together with two other transcription factors, Homothorax (Hth) and Teashirt (Tsh). When the MF initiates in the third instar, these factors become restricted to the most anterior region of the eye disc, where they promote proliferation *(28)*. A preproneural domain (PPN) anterior to the MF is defined by the loss of Hth, which is repressed by Dpp signaling, and the expression of Eya, So, and Dac *(28)*. Posterior to the MF, expression of Ey, Tsh and Dac is downregulated, but Eya and So are maintained and continuously required for photoreceptor differentiation (**Fig. 12.1B**; refs. *21*, *28*, and *29*). 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.

wg expression is limited to anterior marginal regions of the eye disc that will give rise to head cuticle (**Fig. 12.1**), and many observations demonstrate that Wg signaling promotes head capsule formation at the expense of the retinal field *(15–18, 30)*. Reduction of Wg activity using a temperature-sensitive *wg* allele or removal of the positively acting downstream component *dishevelled* (*dsh*) from clones of cells results in expansion of the adult eyes into the dorsal head *(17, 18, 31)*. Conversely, removal of negative regulators of Wg signaling such as *shaggy* (*sgg*) or *axin* (*axn*) maximally activates the Wg pathway and

transforms eye tissue into head cuticle or other cuticular structures *(15, 31–33)*.

Specification of the head primordium by Wg probably occurs early in eye disc development. Despite the limited domain of *wg* transcription, Wg protein diffuses throughout the entire early second instar eye disc, where it blocks Eya expression. Dpp signaling is only able to induce Eya once the disc has grown large enough that Wg can no longer reach the posterior cells *(6, 32, 34)*. Consistent with this model, the lack of eye development in mutants for *eyegone (eyg)*, a *Pax6*-like gene that is required for disc growth *(35)*, can be rescued by inhibiting Wg signaling in posterior cells *(36)*. In the third instar eye disc, *wg* expressed at the anterior dorsal and ventral margins *(8, 15, 17, 30, 31)* continues to repress the expression of the retinal determination genes *eya*, *so* and *dac* in regions destined to form the dorsal head *(32)*. Wg may also promote the head fate by enhancing expression of the anteriorly expressed transcription factor Hth (**Fig. 12.1B**; ref. *(37)*. These expression patterns are maintained by feedback loops; Eya and Dac repress expression of *wg* at the posterior margin of the eye disc *(15, 36)*, while Hth maintains ventral Wg expression *(37)*, 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 (**Fig. 12.1A**; refs. *38* and *39)*. 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 primordium into eye tissue by initiation of ectopic morphogenetic furrows. This occurs primarily at the dorsal margin of the eye disc, correlating with the strong expression of *wg* in this region, but can also occur at the ventral margin where *wg* is more weakly expressed (**Fig. 12.2B,D**; refs. *15*, *17*, and *30*). These furrows then progress inward toward the center of the disc *(15, 17)*. Conversely, ectopic activation of the Wg pathway, using overexpression of Wg or an activated form of Armadillo/β-catenin (Arm), or mutations in *sgg* or *axn*, prevents normal MF initiation and progression (**Fig. 12.2C,E**; refs. *15*, *16*, and *33*). Although high levels of Wg signaling can prevent the expression of *dpp*, a positive regulator of MF movement *(31)*, Wg overexpression also has an inhibitory effect downstream of Dpp receptor activation *(36)*. This might be due to its ability to induce expression of Hth, a negative regulator of photoreceptor differentiation that is repressed by Dpp signaling (**Fig. 12.1B**; refs. *28*, *37*, and *40*). Another target for Wg that is likely to be relevant in this context 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 *(41)*.

Taken together, these results suggest that division of the eye disc between an anterior head field and a posterior eye field relies 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 *(6, 35)*.

1.2. Wg Patterns the Dorsal–Ventral Axis in the Eye Disc

In addition to establishing head primordia in anterior regions of the eye disc, Wg also contributes to distinguishing the dorsal and ventral domains. The GATA family transcription factor Pannier (Pnr) lies at the top of a regulatory cascade that leads to dorsal/ventral (D/V) compartmentalization of the eye disc and activation of the Notch receptor precisely along the D/V midline. It is not clear when dorsal-ventral differences are first established, as *pnr* transcripts are present in the dorsalmost region of the embryonic eye disc *(42)*, but a *pnr*-GAL4 line does not drive UAS-GFP expression in the dorsal eye disc until early second instar *(43)*. *wg-lacZ* is expressed in the dorsal peripodial membrane and dorsal margin cells beginning in the first instar *(8, 30, 44)*; at least at later stages, dorsal *wg* expression is dependent on *pnr (42)*. *wg* expression in the dorsal peripodial epithelium can be attributed to activation by Pnr of an eye-specific enhancer in the *wg* 3′ cis-regulatory region *(45)*.

Wg cooperates with Hh, which is also expressed dorsally in the late second instar eye disc, to activate the expression of the *Iroquois complex* (*Iro-C*) homeobox genes *araucan*, *caupolican* and *mirror* in the dorsal half of the disc *(8, 33, 42, 44, 46)*. The Iro-C proteins repress the expression of Fringe (Fng), a glycosyltransferase that makes the Notch receptor more responsive to one of its ligands, Delta (Dl), and less responsive to Serrate (Ser) *(47–49)*. 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 *(47-49)* (**Fig. 12.1A**). Regulation of the *Dl* and *Ser* expression patterns is likely to involve both the dorsal Iro-C proteins and the ventral determinants Lobe and Sloppy-paired *(50, 51)*. 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 later determines the initiation point of the MF.

An additional site of *wg* expression appears at the ventral margin of the early third instar eye disc *(8, 30, 44)*. Both Ser and its upstream activator Lobe are required for ventral eye development and growth *(43, 50)*. 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 (**Fig. 12.1B**; ref. *52*). 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 *(53, 54)*. This function of Wg is discussed in more detail in **Chapter 11** in **Volume 2**.

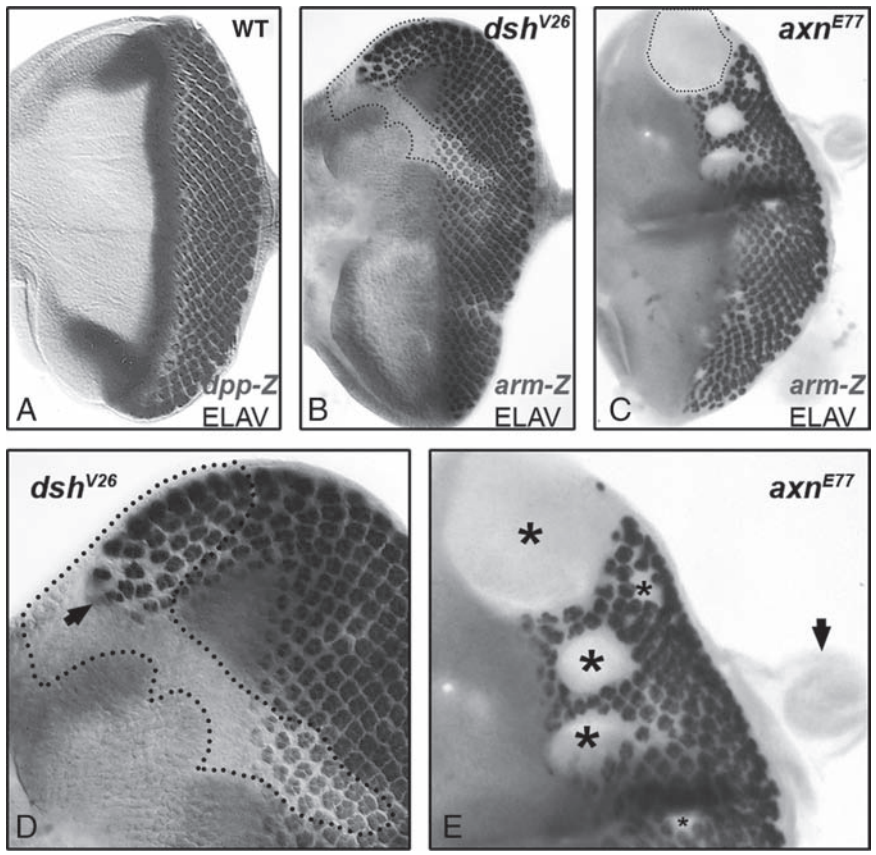


Fig. 12.2. Effects of genetic manipulation of the Wg pathway on photoreceptor differentiation. All pictures are third instar eye discs stained with X-gal to reveal β-galactosidase activity (gray) and with an antibody to the neuronal marker Elav (black). In each panel, anterior is to the left and dorsal 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 clones of cells abutting the anterior dorsal margin (clone boundaries indicated by dotted line), prevents Wg signal transduction and allows ectopic MF initiation resulting in 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 (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**). (**B**) is reprinted, with permission, from ref. *99*.

1.3. 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 results in very small eye discs *(15, 17)*, although it is possible that this size reduction is 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 Axn from clones of cells results in dramatic overgrowth, especially in clones that contact the posterior margin *(15, 32, 33)*. Such clones do not respect the normal boundaries of the eye disc, but form large rounded projections beyond these boundaries (**Fig. 12.2C,E**; refs. *32* and *33)*. 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 *(28, 55)*.

Final eye size must depend on the interplay between Wg and other growth regulators such as Dpp, Notch, and the Notch target Upd *(8, 47–49, 56–58)*. Expression of Wg or activated Arm throughout the eye disc reduces eye size in addition to blocking differentiation *(33, 36, 52)*, probably because it prevents the expression or action of these other factors. Because clones of cells in which Wg signaling is activated are still able to respond to molecules secreted by surrounding cells, Wg may have a mitogenic effect only in combination with additional growth regulatory signals.

1.4. Wg Surrounding the Eye Patterns the Peripheral Retina

As the MF progresses toward the anterior of the disc during the third instar, *wg* is transcribed in a thin stripe of cells along the posterior margin of the eye disc, adjacent to the retinal field *(17, 31)*. 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 is consistent with the transformation of *sgg* or *axn* mutant eye disc cells, in which Wg signaling is maximally active, into head cuticle *(31–33)*. This pattern of *wg* expression is maintained throughout pupation and in the adult head. Wg diffusion from this peripheral source establishes a gradient that patterns the peripheral retina *(31, 59)*.

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 *(59)*. 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; refs. *59* and *60)*. Finally, bristles are absent from the outermost ommatidial rings, but present in the remainder of the eye (**Fig. 12.1C**). A series of gain- and loss-of-function experiments demonstrated that a gradient of Wg signaling organizes the differentiation of these concentric features *(59)*.

During the pupal phase, the most peripheral ring of ommatidia is eliminated by apoptosis *(61–63)*, while the surrounding secondary and tertiary pigment cells survive and contribute to the PR *(63)*. Wg signaling at mid-pupation is required for the death of these 80–100 ommatidia that often lack the full complement of cells and might compromise peripheral vision *(62–64)*. Consistently, *wg* overexpression or loss of the negative Wg pathway component Adenomatous polyposis coli (APC) 1 in the retinal lattice is sufficient to elicit apoptosis of all photoreceptors at mid-pupation *(63, 65, 66)*. Programmed cell death of the peripheral ommatidia also requires the Snail group transcription factors Worniu (Wor), Escargot (Esg), and Snail (Sna), which are targets of the Wg signaling pathway *(64)*.

While high levels of Wg activity result in photoreceptor death, allowing only pigment cells to differentiate, intermediate 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 *(59, 60)*. Finally, low 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 (59, 67, 68)*. As endogenous *wg* is not essential for bristle repression, another *Drosophila* Wnt may contribute to this function *(67)*.

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 patterning 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 clones of cells are mutant for components of the Wg signaling pathway. This technique is described in the following sections.

2. Materials

2.1. Useful Tools to Study Wg Signaling in the Drosophila Eye

Drosophila strains	Comment	References
wg^{CX4}	amorph	*69*
wg^{1}	viable hypomorph	*70*
wg^{IN}	not secreted	*71*
wg^{IL114}	temperature sensitive : 17°C (P) → 25°C (R)	*72*
$wg^{l\text{-}12}$	temperature sensitive : 17°C (P) → 25°C (R)	*73*

***Drosophila* strains**	**Comment**	**References**
wg$^{en\text{-}11}$	enhancer trap, amorph	*74*
arr$^{12\text{-}1}$	loss of function	*75*
fz^{15}	amorph	*76*
fz2^{C1}	loss of function	*77*
dsh^{V26}	amorph	*78*
axn^{E77}	loss of function	*33*
sgg^{D127}	amorph	*79*
dAPC^{Q8}	loss of function	*80*
arm^{1}	amorph	*81*
pan^{2}/ dTCF^{2}	amorph	*82*
act5c>y^{+}>wg	FLP-out	*83*
tub>w^{+}>wg	FLP-out	*53*
tub>CD2, y+> flu-wg	FLP-out, HA-tagged	*84*
GMR-wg	expressed posterior to MF	*53*
GMR-wg^{ts}	temperature sensitive : 16.5°C (P) → 25°C (R)	*59*
*sev>w^{+}>arm**	activated form	*53*
UAS-wg	GAL4-responsive promoter	*85*
UAS-wg^{ts}	temperature sensitive : 16.5°C (P) → 25°C (R)	*86*
UAS-GFP-wg	GFP-tagged	*87*
UAS-Nrt-flu-wg	tethered, HA-tagged	*88*
UAS-dTCF$^{\Delta N3}$/ UAS-dTCF^{DN}	dominant negative	*82*
UAS-arm	GAL4-responsive promoter	*80*
UAS-arm^{K45}	activated form	*89*
UAS-arm^{S10}	activated form	*90*
UAS>CD2, y^{+}>flu-Δarm	N-terminal deletion, activated	*88*
UAS-sgg	GAL4-responsive promoter	*91*
UAS-sgg^{S9A}/ UAS-sgg^{act}	activated form	*36*
UAS-axn	GAL4-responsive promoter	*92*
wg2.11-lacZ	reporter	*45*
arm-lacZ	reporter	*93*

***Drosophila* strains**	**Comment**	**References**
wg-GAL4	GAL4 expressed in *wg* pattern	*94*
arm-GAL4	GAL4 expressed in *arm* pattern	*95*
Antisera		
Anti-Wg	Developmental Studies Hybridoma Bank	
Anti-dAPC		*84*
Anti-Arm	Developmental Studies Hybridoma Bank	
Anti-Axn		*92*
Anti-Elav	Developmental Studies Hybridoma Bank (recognizes photoreceptor nuclei)	

2.2. Fly Stocks

1. *y,w, ey-FLP ; + ; FRT82B, arm-lacZ / TM6B.*
2. *y,w ; + ; FRT82B, axn^{E77}/ TM6B.*
3. *y,w, arm-lacZ, FRT19A ; + ; ey-FLP / TM6B.*
4. *y,w, dsh^{V26}, FRT19A / FM7.*

2.3. Dissection

1. Two pairs of fine forceps (Dumont # 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 using forceps.
5. Petri dishes (35 × 10 mm).
6. Polystyrene microwell mini trays, 60 wells (Nunc).
7. Microscope slides, cover slips (18 × 18 mm), and nail polish.
8. A flat metal plate that can fit in an ice bucket.

2.4. Solutions

1. 0.1 M phosphate buffer, pH 7.2: mix 1 M Na_2HPO_4 and 1 M NaH_2PO_4 in a 72:28 ratio, respectively, and add 9 volumes of water.
2. PEM: 0.1 M PIPES pH 7.0, 2 mM $MgSO_4$, 1 mM EGTA; it is usually kept as a 2X stock at 4°C.
3. Freshly prepared fixative: either 4% (w/v) formaldehyde in PEM: for 10 mL, mix 5 mL of 2X PEM, 1 mL of H_2O and 4 mL of 10% (w/v) methanol-free formaldehyde (Polysciences) OR 2% (w/v) formaldehyde in PLP: Prepare a phosphate buffer solution by mixing 75 mL of 0.1 M phosphate buffer, pH 7.2, 2.5 mL of 1 M Na_2HPO_4, and

122.5 mL of H_2O. To 15 mL of this, add 1 mL of water and 0.27 g lysine. Just before use, add 50 mg sodium periodate and 4 mL of 10% (w/v) methanol-free formaldehyde.

4. PTX: 0.1 M phosphate buffer, pH 7.2, 0.2% (v/v) Triton X-100. For some antibodies, other detergents such as saponin may be preferable.
5. Normal donkey serum (Jackson Immunoresearch) or goat serum if goat secondary antibodies are used.
6. Rat anti-Elav (Developmental Studies Hybridoma Bank) and HRP-conjugated donkey anti-rat (Jackson Immuno-research).
7. Freshly prepared diaminobenzidine (DAB) solution: for 250 µL staining solution, add 225 µL of 0.1 M phosphate buffer, 0.2% (v/v) Triton to 25 µL 5 mg/mL DAB; add 5 µL of 1% (w/v) cobalt chloride for intensification and 2.5 µL of 0.3% H_2O_2 (freshly diluted from a 30% stock). For double staining with X-gal, include 5 µL of 1% (w/v) nickel ammonium sulfate and 6 µL of 1% (w/v) cobalt chloride in the DAB solution. DAB is toxic; handle with gloves and deactivate used tips, etc., overnight in bleach before disposal. The 5 mg/mL solution should be stored in 25 µL aliquots at –80°C.
8. X-gal staining buffer: For 50 mL, mix 1.8 mL 0.2 M Na_2HPO4, 0.7 mL 0.2 M NaH_2PO4, 1.5 mL 5 M NaCl, 50 µL 1 M $MgCl_2$, 3.05 mL 50 mM $K_3Fe(CN)_6$, 3.05 mL 50mM $K_4Fe(CN)_6$, and H_2O to 50 mL.
9. 8% X-gal solution in dimethylformamide (store in aliquots at –80°C).
10. 80% glycerol (v/v; Roche molecular biology grade) in PBS.

3. Methods

3.1. Genetics

The FLP/FRT method is used to generate, during development, clones of homozygous mutant cells in the eye of a fly heterozygous for a mutation affecting Wg signaling *(96)*. The presence of Flipase recombination target (FRT) sites near the centromere allows exchange between the mutant and wild-type chromatids on homologous chromosomes. This recombination is catalyzed by the Flipase enzyme (FLP), which is specifically expressed in the developing eye under the control of the *eyeless* promoter *(97)*. Chromatid segregation at mitosis may result in a homozygous mutant cell, which will proliferate to form a mutant clone (**Fig. 12.3**). In the third instar eye disc, the presence of the ubiquitously expressed *armadillo-lacZ (arm-Z)* transgene

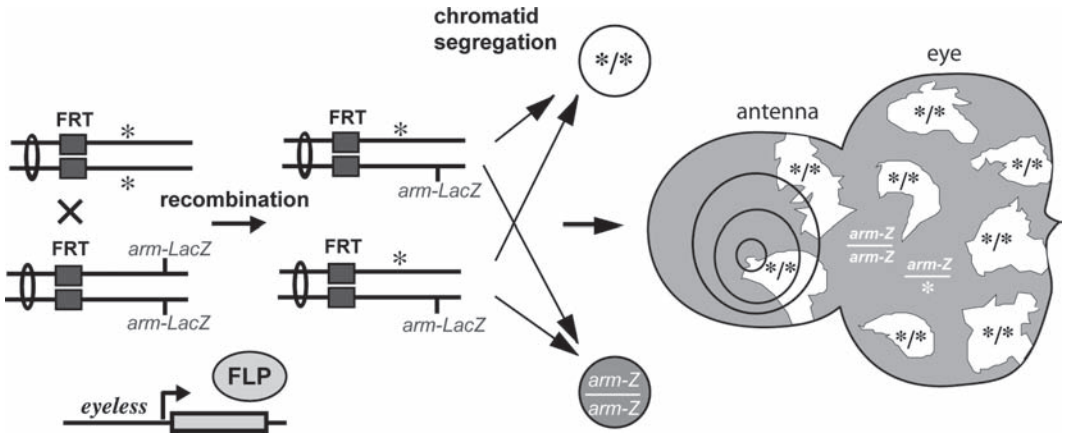


Fig. 12.3. Mitotic recombination using the FLP/FRT system. In cells heterozygous for a mutation (*/+), the FLP/FRT system enables mitotic recombination between homologous chromatids, resulting in the production of one homozygous mutant (*/*) daughter cell and one wild type (+/+) daughter cell. In third instar eye discs mosaic for the mutation, a P element carrying the *arm-lacZ* marker allows homozygous mutant clones (*/*) to be recognized by their lack of β-galactosidase activity (gray).

(93) distal to the FRT sites provides a way to discriminate clones of homozygous mutant cells, which lack β-galactosidase activity, from heterozygous or homozygous wild-type cells (**Fig. 12.3**). Photoreceptor differentiation within clones identified by the absence of X-gal staining can be monitored using an antibody to the neuronal marker Elav *(98)*.

In freshly yeasted food vials:

1. Cross 6-8 *y,w, ey-FLP; FRT82B, arm-lacZ / TM6B* females with 4-6 *y,w; FRT82B, axn^{E77}/ TM6B* males.
2. Cross 6-8 *y,w dsh^{V26}, FRT19A/FM7* females with 4-6 *y,w, arm-lacZ, FRT19A/Y; eyFLP/TM6B* males.

Allow the flies to lay eggs for 2 to 3 days and transfer to a fresh vial. Approximately 1 week later, select non-*Tubby y,w, ey-FLP/y,w or* Y*; FRT82B, arm-lacZ / FRT82B, axn^{E77}* third instar larvae from **cross 1**. Select non-*Tubby y,w, arm-lacZ, FRT19A / y,w, dsh^{V26}, FRT19A ; eyFLP /* + third instar female larvae from **cross 2**.

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 half way 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. 12.4A**). Usually the eye-antennal discs will remain

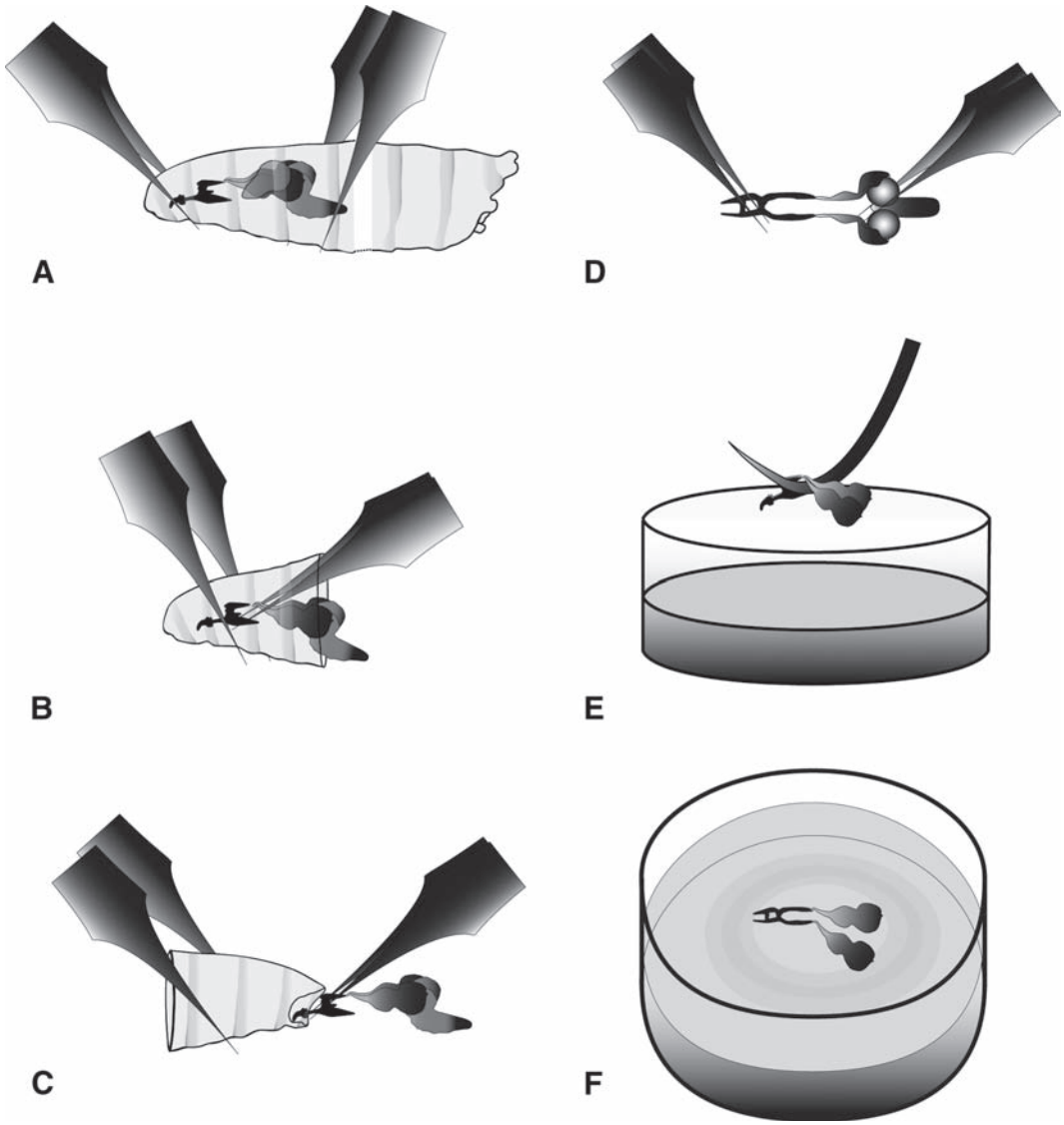


Fig. 12.4. Cartoon of the dissection protocol for eye disc staining. (**A**) Using forceps, open the larva by pulling on the mouthhooks (black). (**B–C**) Remove the larval cuticle and extract the eye-antennal discs attached to the mouthhooks. (**D**) Pin down the brain between the two hemispheres and pull on the mouthhooks to separate the eye discs from the brain. (**E**) Pick up the discs with a tungsten hook placed under the antennal discs, with the apical, convex side facing downward. (**F**) Deposit the discs on the surface of the fix, allowing the discs to flatten due to surface tension.

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. 12.4B,C**). To remove the brain, hold the mouthhooks with one pair of forceps and pin the brain to the dissection dish with one point of a second pair of forceps placed between the two hemispheres. Gently

pull on the mouthhooks to break the optic stalk that links the eye disc to the brain (**Fig. 12.4D**). 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. 12.4E**). Deposit the discs on the surface of the fix solution (usually 4% formaldehyde [v/v] in PEM) in a 35 × 10 mm Petri dish placed on a flat metal plate in an ice bucket (**Fig. 12.4F**). Fix the discs for 25–35 minutes on ice (*see* **Note 2**).
4. Using the hook, transfer the eye discs attached to the mouthhooks into a 30 mm Petri dish filled with PTX. Wash the discs in this solution for 15 minutes on ice.
5. Transfer the discs to the antibody solution. A 60-well (10 × 6) microwell tray can be used for up to six different samples that will require 10 wells each (*see* **Note 3**). Up to 15 pairs of discs of one genotype can be stained together in a single well. Place a folded wet Kimwipe in the tray to maintain its humidity. Incubate the discs in primary antibody diluted in PTX, 10% (v/v) 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 within the microwell tray at each step. Wash 3 × 5 min in PTX at room temperature. Incubate in secondary antibody diluted 1:200 in PTX, 10% (v/v) serum, for at least 2 hours at 4°C. Wash 3 times for 5 minutes in PTX at room temperature.
7. 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.
8. Stop the reaction by transferring the discs to PTX.
9. Proceed with X-Gal staining if desired. Incubate in an Eppendorf tube containing prewarmed (65°C) X-gal staining buffer with a 1:40 dilution of 8% X-gal (7.5 µL in 300 µL) at 37°C (*see* **Note 4**). For *arm-lacZ* the staining time is usually 2 to 3 hours, but staining should be checked under a dissecting microscope. Wash in PBS, 0.1% (v/v) Triton X-100, rocking for 1 hour, to remove crystals of X-gal that may have become attached to the discs.
10. Mount discs in 80% (v/v) glycerol in PBS. Deposit a 20-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 5**).
11. View discs on a compound microscope using Nomarski optics and a 20X or 40X objective lens (*see* **Note 6**).

4. Notes

1. Keeping the mouthhooks attached to the eye-antennal discs provides a safe way to handle both discs from one larva 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 (**Fig. 12.3F**). 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 minutes 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. If the staining buffer cools down to room temperature, X-gal crystals will form and will be difficult to remove from the discs. To avoid this, prewarm the staining buffer at 65°C for at least 30 minutes. Add X-gal and transfer the tube to a 37°C water bath close to the microscope. Use the hook to place discs directly into the tube, removing it from the water bath for as short a time as possible.
5. To improve the quality of the mounting, drag each disc toward the same edge of the glycerol drop with its apical surface upward, until the disc immobilizes and flattens. Then gently place the cover slip 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 cover slip with forceps while observing the discs through the dissecting microscope to avoid using too much force.
6. Dorsal and ventral sides of the eye-antennal disc can be easily identified because the central circular fold within the antennal disc is closest to the ventral side (**Fig. 12.1B**).

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