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Canonical Wingless Signaling Regulates Cone Cell Specification in the *Drosophila* Retina

Julia B. Cordero[†] and Ross L. Cagan^{*}

Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, New York

Abstract

Correct tissue patterning during development involves multiple morphogenetic events that include specification of different cell fates, cell proliferation, cell death, and coordinated changes in cell shape, position, and adhesion. Here, we use the *Drosophila* retina to explore the molecular mechanisms that regulate and integrate these various events. In a previous report, we found that *wingless* (*wg*) was required to induce a previously unknown surge of cell death (“early death”) in the pupal retina. Here, we show that *wg* is also required to induce the more widely studied mid-pupal cell death (“late death”) in a process that involves regulation of DIAP1. Furthermore, our data suggest that *wg* has a previously unreported role in specifying the glial-like cone cells. This activity requires canonical *Wg* signaling and is linked with Notch pathway activity. Our work broadens the role of canonical *Wg* signaling to encompass multiple patterning steps in the emerging *Drosophila* retina.

Keywords

wingless; cone cells; retina; *Drosophila*

INTRODUCTION

The *Drosophila* retina has proven to be a useful system for studying the events and signaling pathways regulating cell fate determination and patterning within developing tissues and organs. The highly organized and repetitive structure of the pupal retina provides a platform to study these issues at single-cell resolution. Early patterning signals lead to creation of an array of approximately 750 ommatidial units that will eventually grow to 14 cells each: 8 photoreceptor neurons and 6 support cells: 4 cone cells and 2 primary pigment cells (1°s; Fig. 1J). Photoreceptors and cone cells are specified during the larval stages (Wolff and Ready, 1991b). Next, 0–42 hr after puparium formation (APF), ommatidia are re-organized into a precise hexagonal array by the maturation of the interommatidial cells into an interweaving honeycomb of secondary and tertiary pigment cells (2°/3°s; Fig. 1J). Through local cell–cell signaling, 2°/3°s are organized into a hexagonal array by local cell rearrangements and removal of excess cells through programmed cell death (Cagan and Ready, 1989b; Wolff and Ready, 1991a; Reiter et al., 1996). We have previously reported a role for Wingless (*Wg*) in regulating aspects of cell death in these later patterning processes (Cordero et al., 2004).

^{*}Correspondence to: Ross L. Cagan, Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029. ross.cagan@mssm.edu.

[†]Dr. Cordero’s present address is The Beatson Institute for Cancer Research, Cancer Research UK, Glasgow, G61 1BD, UK.

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Wg is a member of the Wnt family of secreted proteins, which regulate a wide variety of biological processes including embryonic development, tissue self-renewal, cell proliferation, and cell differentiation (Logan and Nusse, 2004). Mutations that lead to mis-regulation of Wnt signaling result in severe developmental abnormalities as well as diseases including cancer in a wide variety of tissues (Clevers, 2006). Wnts bind to and activate Frizzled (Fz) receptors at the cell surface, which in turn activates the cytosolic protein Dishevelled (Dsh). Downstream of Dsh, the Wnt pathway branches into “canonical” and “non-canonical” signaling pathways (Bejsovec, 2005). Canonical Wnt pathway signaling is characterized by activation of Dsh and inactivation of a destruction complex that allows β -catenin to accumulate in the nucleus. Nuclear β -catenin binds TCF/Pangolin to activate Wnt-dependent transcription (Jones and Bejsovec, 2003; Seto and Bellen, 2004).

The role of the canonical Wg/Wnt pathway in early *Drosophila* eye cell fate specification has not been fully explored. Most work on Wg signaling in the *Drosophila* eye has focused on regulation of ommatidial planar cell polarity (PCP). This process requires the activities of downstream pathway components but apparently not Wg itself (Mlodzik, 1999; McNeill, 2002). Hyper-activation of the Wg pathway by using a gain-of-function allele of *wingless* (*wg*) or by mutations in the inhibitor Adenomatous Polyposis Coli (APC) resulted in retinal degeneration induced by death of photoreceptor cells during late stages of eye development (Ahmed et al., 1998; Brunner et al., 1999). Additionally, *wg* overexpression blocked proneural gene expression in the early pupal eye (3–4 hr APF; Cadigan and Nusse, 1996). Finally, loss of function data showed that *wg* from the dorsal and ventral lateral edges of the larval eye opposes *hh* and *dpp* by blocking neuronal differentiation anterior to the morphogenetic furrow (Ma and Moses, 1995; Treisman and Rubin, 1995; Cadigan et al., 2002).

In a previous report, we described a Wg-dependent stage during which significant levels of cell death occur (Cordero et al., 2004). We christened this wave of programmed cell death (PCD) “early death” to distinguish it from the previously characterized mid-pupal “late death” (Cagan and Ready, 1989b; Wolff and Ready, 1991a). This provided the first evidence that *wg* plays a role in organizing the interommatidial lattice. Here, we show that *wg* is also required to induce mid-pupal cell death (“late death”) in the retina through post-transcriptional regulation of the inhibitor of apoptosis, DIAP1. More surprisingly, we determine that Wg pathway activity is also required earlier for the specification of cone cell fate in the larval retina in a process that involves functional interaction with Notch. This work indicates that Wg is acting in a broader manner in the eye than previously suspected.

RESULTS

Wg Induces Cell Death in the Mid-pupal Retina

To test the role of *wg* in mid-pupal cell death (“late cell death”) and to distinguish it from its role in early cell death (Cordero et al., 2004), we used animals carrying a temperature-sensitive allele of *wg* in *trans* to a null. Animals were either kept at the permissive temperature (Fig. 1A,B) or switched to the nonpermissive temperature just after the wave of early death had passed (Fig. 1C,D). We observed a significant decrease in cell death and concomitant increase in IPC number in *wg^{ts/+}* retinas shifted to the nonpermissive temperature (Fig. 1C,I). Similar results were observed in clones of the null allele *wg^{CX4}* (Fig. 1E,F; Supp. Fig. S1C,C', which is available online).

Caspases are involved at each stage of cell death in the pupal retina (Rusconi et al., 2000; Cordero et al., 2004). Cell death is initiated upon degradation of inhibitors of apoptosis (DIAP) proteins (Steller, 2008). Consistent with a role for *wg* as a pro-death signal in the retina, clones of *wg^{CX4}* showed increased levels of the anti-apoptotic protein DIAP1/Thread (Fig. 1G,H). A *thread-lacZ* reporter line revealed no change in transcript levels upon loss of *wg* (not shown),

suggesting that the regulation of DIAP1 was posttranscriptional. Together with previous reports (Cordero et al., 2004; Lin et al., 2004), our results suggest that *wg* is required to induce each stage of developmental cell death in the pupal retina.

Wingless Regulates Cone Cell Development

Wg has been previously linked to apoptotic cell death in the eye and our results extend this work. Remarkably, clones of genotypically *wg^{CX4}* cells also exhibited multiple patterning defects. In particular, we frequently observed abnormalities in the shape, organization and number of cone cells (Fig. 2A). Defects in 1°s were occasionally observed, possibly as a consequence of abnormal cone cell induction (Miller and Cagan, 1998). Staining with the cone cell-specific marker Cut (Blochliger et al., 1993) confirmed the cone cell defects (Fig. 2B). The role of Wg in cone cell differentiation is separable from its role in PCD in the *Drosophila* pupal retina: using the temperature sensitive *wg^{ts}* mutation, pupal shifts led to a block in PCD without defects in cone cells (Cordero et al., 2004; Fig. 1).

These results were unexpected: previous work in the larval disk only reported a requirement for Wg activity in blocking neuronal differentiation anterior to the morphogenetic furrow (Ma and Moses, 1995; Treisman and Rubin, 1995; Cadigan et al., 2002). Expression of *wg* was only unambiguously detectable in the antennal disc and at the edges of the larval retina (Treisman and Rubin, 1995; Fig. 2F). Nevertheless, we observed defective cone cells in ommatidia throughout the larval eye epithelium; Figure 2A shows an example of a centrally located *wg^{CX4}* clone. Importantly, targeted down-regulation of *wg* by RNA interference within the larval eye field (*GMR >wgIR*) (Fig. 2C; Supp. Fig. S1E,E') or clones of genotypically *wg^{CX2}* cells (Fig. 2D) resulted in cone cell aberrations comparable to the defects observed within *wg^{CX4}* clones. Over-expression of *GMR >wgIR* yielded a consistent but milder phenotype, presumably due to the timing and/or levels of *wg* knockdown. Again, *wg^{CX2}* clones exhibited cone cell defects whether the clones occupied the center (Supp. Fig. S1A) or edge (Fig. 2D) of the retina in a manner similar to *wg^{CX4}*.

Finally we tested the allele *wg^{IN67}*, which encodes a secretion defective protein that is trapped in the endoplasmic reticulum and gives rise to wing defects (van den Heuvel et al., 1993; Supp. Fig. S1D,D'; data not shown). Genotypically *wg^{IN67}* eyes did not exhibit any detectable cone cell phenotype (Supp. Fig. S1B). Furthermore, *wg^{IN67}* clones also failed to show expected defects in morphogenetic furrow progression (not shown) or pupal processes required to pattern the eye edge (Chen and Struhl, 1999; Lin et al., 2004). These results suggest that *wg^{IN67}* may retain residual function that is sufficient to achieve correct eye but not wing patterning. A similar result was obtained in clones of the temperature sensitive allele *wg^{IL114}*, which also results in defective protein secretion (not shown). To further confirm the *wg* expression pattern in the retina we used anti-Wg antibody to detect protein expression (Fig. 2G,H). Wg staining essentially confirmed the expression pattern observed with the *lacZ* reporter line (Fig. 2F,G). Of note, extended (48 hr) antibody incubation yielded low levels of staining in the posterior part of the eye disc (Fig. 2H), but the fidelity of this staining was difficult to confirm.

Together these data indicate that (i) Wg acts as a local or short-range signal in the retina to regulate cone cell development and (ii) Wg acts at levels below detection by conventional tissue staining.

Frizzled and Components of the “Canonical” Wingless Pathway Mimic the *wg* Cone Cell Phenotype

Wnt/Wg proteins bind to and activate Frizzled (Fz) receptors at the cell surface. *Drosophila* has two Frizzled receptors that act in part redundantly (Chen and Struhl, 1999). Double mutant clones for *fz1* and *fz2* exhibited cone cell defects that phenocopied defects observed in

genotypically *wg* tissue (Fig. 2E, Supp. Fig. S1F,G). These data suggest that Fz proteins act as receptors to mediate Wg activity during cone cell development.

We next tested whether components of the “canonical” Wingless pathway were required for cone cell development in the *Drosophila* retina. The canonical signaling pathway includes transcriptional regulation by Armadillo/ β -catenin and its co-factors TCF/Pangolin and Pygopus (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002; Jones and Bejsovec, 2003; Seto and Bellen, 2004). Overexpression of a dominant-negative version of TCF (*TCF^{DN}*) has been widely used to down-regulate canonical Wg signaling (van de Wetering et al., 1997; Cadigan et al., 2002). Overexpressing *TCF^{DN}* in the eye (*GMR>TCF^{DN}*) resulted in severe morphogenetic defects in the retina including aberrant cone cell number and morphology (Fig. 3A). The adult eyes of *GMR>TCF^{DN}* animals were ‘glossy’ with poorly defined lens facets (Fig. 3A, inset). ‘Glossy’ phenotypes are characteristic of retinas with faulty lens secretion due to defective cone cells (Cagan and Ready, 1989a; Fu and Noll, 1997). The cone cell defects observed in *GMR>TCF^{DN}* retinas overlapped with the ones observed in genotypically *wg* and *fz1, fz2* clones (Fig. 2) but the overall phenotype of *GMR>TCF^{DN}* retinas was consistently stronger. TCF is active as a transcriptional repressor in the absence of (Wg) signaling and these additional defects may reflect a loss of constitutive TCF function. Alternatively, it is possible that overexpression of *TCF^{DN}* affects pathways other than Wg signaling.

To better assess the role of TCF in cone cell development we targeted *TCF^{DN}* specifically to the primaries and cone cells using the driver *sparkling-gal4* (*spa>TCF^{DN}*; Jiao et al., 2001). The retinas of *spa>TCF^{DN}* animals showed mild but consistent defects that were exclusive to the cone cells (Fig. 3B). The lower penetrance of the phenotype is likely due to the lower strength of the *sparkling-gal4* driver. Finally, clones of *pygopus* (*pygo*) also showed cone cell defects reminiscent of the other pathway components but milder: most commonly, *pygo* ommatidia exhibited abnormal cone cell morphology but normal cell number with a few exceptions (Fig. 3C,D). Of note, we did not observe increased staining of the cell death marker Caspase-3 (data not shown), indicating that this is not likely due to death of emerging cone cells. The milder phenotype observed upon *pygo* loss is consistent with previous work reporting that loss of *pygo* resulted in partial loss of Wg signaling (Parker et al., 2002). Furthermore, results in mice suggest that *pygo1* and *pygo2* requirement for Wnt signaling is context dependent (Jessen et al., 2008).

Taken together these results suggest that *wg* regulates cone cell number and morphogenesis through the canonical Wg pathway.

Canonical Wg Signaling Regulates Cone Cell Specification

The pupal retinal phenotypes we observed upon down-regulation of *wg* and its downstream effectors could be due to either defects in the initial specification of cone cells or failure to maintain cone cells after they were specified. To distinguish between those possibilities we more closely analyzed cone cell phenotypes within the larval retina, the stage at which cone cells first emerge.

Clones of genotypically *fz1, fz2* (Fig. 4A–C) ommatidia displayed reduced cone cells at the earliest stages of cone cell specification as assessed with the cone cell-specific marker Cut. Clones of *wg^{CX4}* and *wg^{CX2}* showed nearly identical phenotypes (Fig. 5E–G, not shown). As already evidenced by their pupal phenotype (Fig. 3C,D) clones of *pygopus* (*pygo*) showed milder cone cell defects in larval eye discs (Fig. 4G–I). Importantly, *GMR>TCF^{DN}* larval retinas also showed defective specification at the earliest steps of cone cell development as visualized by decreased number of Cut positive cells (Fig. 4F). We confirmed these observations with a second cone cell specific marker, DPax2 (Fu and Noll, 1997; Fig. 4E).

Together, these data indicate that cone cells require canonical Wg pathway activity for their initial specification. Of note, and unlike Notch and EGFR loss of function mutants (Flores et al., 2000), no general down-regulation of cone cell markers was observed in any of the Wg pathway mutant contexts analyzed. Cone cell markers were lost only when cone cells were missing. This suggests that neither DPax2 nor Cut are transcriptional targets of Wg signaling in the retina. This is in contrast to the wing disc where, indirectly, *wg* regulates Cut expression (Micchelli et al., 1997).

Cone cell specification in the larval retina is dependent on correct prior development of photoreceptor neurons (Greenwald and Rubin, 1992; Chang et al., 1995; Dickson et al., 1995; Lai, 2002). Furthermore, hyper-activation of Wg signaling resulted in defective photoreceptor specification and cell death (Ahmed et al., 1998; Brunner et al., 1999; Cadigan et al., 2002) as well as concomitant loss of cone cells (Supp. Fig. S5). Hence, defects in photoreceptor differentiation could explain the observed cone cell defects in Wg pathway loss of function mutants. To assess this possibility we examined photoreceptor specification in *GMR>TCF^{DN}* larval ommatidia, which exhibit strongly abnormal cone cell specification (Fig. 4; Supp. Fig. S2F). *GMR>TCF^{DN}* retinas showed no detectable defects in photoreceptor specification as assessed by photoreceptor cell type-specific markers *H214*, *svp*, and *mδ0.5* (Supp. Fig. S2).

To more closely explore this issue, we examined the dynamic specification of R7 versus the neighboring anterior cone cell. Precursors of these two cell types compose the “R7 equivalence group” (Greenwald and Rubin, 1992; Chang et al., 1995; Dickson et al., 1995; Lai, 2002). Precursors of R7 and cone cells are initially marked by low levels of the transcription factor Prospero; local signals eventually promote high Prospero levels to help designate one cell as R7 (Xu et al., 2000). No significant changes in the levels or dynamic expression pattern of Prospero were observed in genotypically *pygo* or *fz/fz²* backgrounds or in the presence of *TCF^{DN}* (Supp. Fig. S3). Together these data are consistent with the view that canonical Wg signaling in the retina acts specifically on cone cells and does not alter earlier cell fate specification.

Wingless From Photoreceptors Is Required for Cone Cell Specification

To better understand the role of Wg signaling during cone cell specification we next assessed the spatial distribution of components of the canonical pathways as well as the source of the ligand Wg. Consistent with our phenotypic analysis, Pygo was found to be strongly expressed in the nuclei of cone cells and photoreceptor neurons as determined by colocalization with Cut and Elav, respectively (Fig. 5A,B). The specificity of the anti-Pygo antibody (de la Roche and Bienz, 2007) was confirmed by loss of staining in *pygo* null clones (Fig. 5C,D). We attempted to detect anti-TCF (de la Roche and Bienz, 2007) localization in the eye disc but were unable to see unambiguous staining (not shown).

To determine which cells in the retina provide the source of Wg, we made small MARCM-based clones of the null allele *wg^{CX4}* and analyzed the resulting phenotypes in the larval and pupal retina (Fig. 5E–N). Loss of Wg activity in photoreceptors was sufficient to cause defects in cone cell specification (Fig. 5E–G,K,L). Ommatidia featuring clones only in cone cells (Fig. 5M,N) or interommatidial cells (not shown) showed no defects. Figure 5H–J shows an example of defective ommatidia with clones in photoreceptors and interommatidial cells.

Together these data suggest that Wg secreted by photoreceptors activates signaling in the cone cells to regulate their specification.

Wg Signaling Cooperates With Notch During Cone Cells and Photoreceptor Specification

To date, two major pathways have been reported to regulate cone cell specification in the *Drosophila* retina, Notch (N) and Epidermal Growth Factor Receptor (EGFR) signaling (Flores et al., 2000). No genetic interaction was observed between multiple components of canonical Wg signaling and EGFR signaling (not shown). Reduction of Notch pathway activity is required for each step in cell fate induction including cone cell specification (Cagan and Ready, 1989a). Down-regulation of *Notch* using a *sevenless-gal4* driven RNA interference construct (*sev>NIR/+*; Fig. 6A,D,G) resulted in an expected significant loss of cone cells (Fig. 6D). We did not observe significant defects in photoreceptors (Fig. 6G) presumably due to the timing of the *sevenless-gal4* driver. Adult *sev>N^{IR}* eyes showed glossy retinas and significant signs of necrosis manifested by black patches of tissue (Fig. 6A). Of interest, removing one genomic copy of *wg* (Fig. 6B,E,H) or *pygo* (Fig. 6C,F,I') significantly suppressed both, the cone cell and necrotic phenotypes of *sev>N^{IR}* retinas.

We further tested the functional connection between Notch and Wg signaling using the classic *Notch* allele *N^{spl-1}* (Fig. 6J–M; Supp. Fig. S4). *N^{spl-1}* retinas displayed significant loss of photoreceptor neurons, severe loss of cone cells, and adult eyes of reduced size (Fig. 6J,K; Supp. Fig. S4). Consistent with our previous results, removing one genomic copy of *wg* or *pygo* significantly suppressed the adult eye as well as cone cell specification phenotype of *N^{spl-1}* animals (Fig. 6M; Supp. Fig. S4). Importantly, reducing *wg* activity also led to dominant suppression of photoreceptor defects (Fig. 6J,L), indicating that Wg signaling can contribute to photoreceptor differentiation in the context of abnormal Notch signaling.

To better understand the epistatic relationship between Notch and Wg signaling we tested whether Notch activity was altered in the presence of reduced Wg activity. We failed to observe any significant changes in Notch signaling activity—monitored by levels of the Notch pathway reporters E(spl) and *Su(H)-LacZ*—in retinas carrying *wg* clones or over expressing *TCF^{DN}* (not shown). Together, our data indicates a functional link between Wg and Notch pathway signaling during cone cell specification. It also suggests a subtler requirement for Wg activity during photoreceptor specification.

DISCUSSION

Taken together our results have uncovered a previously unknown role for Wg and components of the canonical pathway during cell fate specification in the larval retina. We examined known candidate targets, and a role for Wg in cone cell fate specification appears to occur by novel mechanisms. Of note, recent work identified *phyllopod* (*phyl*)—an essential player in the balance between photoreceptor and cone cell specification (Chang et al., 1995; Dickson et al., 1995)—as a negative regulator of Wg and Notch signaling in *Drosophila* wing and eye disc (Nagaraj and Banerjee, 2009). Loss of *phyl* led to up-regulated Wg and Notch signaling along with ectopic cone cells and concomitant loss of R1 and R6 photoreceptor fates (Nagaraj and Banerjee, 2009). Perhaps Wg acts with Phyllopod to set this balance; however, we failed to detect ectopic photoreceptors to compensate for the loss of cone cells in our studies (Supp. Fig. S2), leaving open the question as to which factors act with Wg during cone cell specification. Finally, our data indicate that Wg does appear to have a role in photoreceptor differentiation at least in the context of altered Notch signaling (Fig. 6). Though impaired canonical Wg signaling is not sufficient to detectably affect photoreceptors on its own, our data do indicate that it can act on photoreceptor neurons when Notch signaling is compromised (Fig. 6). These results are of relevance to studies on ommatidial PCP, a process demonstrated to require Notch but not canonical Wg signaling (Fanto and Mlodzik, 1999; Mlodzik, 1999; McNeill, 2002). It remains possible that Notch/Wg cooperation also exists in a similar context and this interaction should be considered when exploring a role for each pathway during early ommatidial emergence.

The genetic program that regulates eye development shows remarkable conservation among species (de Jongh et al., 2006). In *Drosophila*, cone cells secrete the overlying lens required for proper vision (Cagan and Ready, 1989b). Multiple human ocular pathologies are caused by mutation of known Wnt pathway genes and genes that may be targets of this pathways (de Jongh et al., 2006). Furthermore, targeted loss and targeted loss of canonical Wnt signaling through deletion of β -catenin in vertebrate models results in severe defects in lens morphogenesis (Smith et al., 2005). The precise role of the different components of the pathway in this process remains unclear, and *Drosophila* cone cells represent an exquisitely sensitive and relatively simple model system to identify conserved gene networks that are required for normal lens formation.

EXPERIMENTAL PROCEDURES

Fly Strains

Crosses were conducted on standard dextrose media and incubated at 25°C unless otherwise noted. The following stocks were kindly provided to us by our colleagues: *wg^{IL114}*, *wg^{CX4}*, and *UAS-TCF^{DN}* (Amy Bejsovec); *pygo^{S123}* (Mariann Bienz); *wg^{CX2}* (Jessica Treisman); *fz^{h51}*, *fz^{2c1}* (Gary Struhl); *sev-gal4,UAS-NIR* (Marek Mlodzik); *N^{spl-1}* (Nick Baker); *DPax2-lacZ* (Utpal Banerjee); *UAS-CD4: GFP, hsFLP; gal80, FRT40A; tub-gal4/TM6B* (MARCM 40A) line (Andreas Bergmann); and *spa-gal4* (Markus Noll). *UAS-wgIR* and *UAS-NIR* were obtained from the Vienna *Drosophila* RNAi Centre. The remaining fly lines were obtained from the Bloomington Stock Center.

Experimental Genotypes

1. *wg^{ts/-}* experiments: *wg^{IL114}/wg^{CX4} FRT40A*
2. *wg^{CX4}* clones: *hs-FLP1; ubi-nGFP FRT40A/wg^{CX4} FRT40A*
3. *wg^{CX2}* clones: *hs-FLP1; ubi-nGFP FRT40A/wg^{CX2} FRT40A*
4. *pygo^{S123}* clones: *yw hs-FLP122/w-; tub-gal4 UAS-GFPnls FRT82B tub-gal80/FRT82B pygo^{S123}*
5. *TFC^{DN}* overexpression: Figure 3A: *GMR-gal4/w-; UAS- TFC^{DN}/+*; Figure 3B: *spa-gal4/w-; UAS-TFC^{DN}/+*; Figure 4E: *GMR-gal4/w-; UAS- TFC^{DN}/DPax2-lacZ*.
6. *fz^{h51}*, *fz^{2c1}* clones: *hs-FLP/w-; fz^{H51} fz^{2C1}, ri, FRT2A/2x Ubi-GFP FRT2A*
7. *wg^{CX4}* MARCM clones: *UAS-CD4: GFP, hsFLP; tub-gal80, FRT40A/wg^{CX4} FRT40A; tub-gal4/+*
8. *Notch/wg* genetic interactions: Figure 6A,D,G: *w-; sev-gal4,UAS-NIR/+*; Figure 6B,E,H: *w-; sev-gal4,UAS-NIR/wg^{CX4} FRT40A*; Figure 6C,F,I: *w-; sev-gal4,UAS-NIR/FRT82B pygo^{S123}*; Figure 6J,K: *N^{spl-1}/Y; +/+*; Figure 6L,M: *N^{spl-1}/Y; wg^{CX4} FRT40A/+*

Clonal Analysis and Temperature Sensitive Experiments

Discrete clones of mutant tissue were generated by FRT-mediated recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993). *pygo* clones were generated using the MARCM technique (Lee and Luo, 1999). Recombinant clones were induced by heat shocking larvae 72 hr after egg laying (AEL) for 1 hr at 37°C. MARCM clones of *wg^{CX4}* were generated by performing 3 heat shocks of 30 min each.

For $wg^{ts/-}$ experiments, $wg^{IL114/wg^{CX4}}$ pupae were grown at 18°C through 42 hr APF (equivalent to approximately 24 hr APF at 25°C) and then shifted to 25°C for 4–5 hr before dissection. As controls, $wg^{IL114/wg^{CX4}}$ pupae were kept at 18°C until dissection.

Immunofluorescence and TUNEL Staining

Pupal and larval retinas were dissected in PBS and processed for immunofluorescence as described previously (Brachmann et al., 2000). Primary antibodies used were: mouse anti-Armadillo N27A1 (1:3), mouse anti-Cut (1:100), rat anti-ElaV (1:500; Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti- β -gal (1:2,000; Cappel), rabbit anti-green fluorescent protein (GFP; 1:2,000; provided by Pam Silver), rabbit anti-Pygo (1:50; Mariann Bienz), rabbit anti-Wg (1:500; Roel Nusse), and mouse anti-DIAP1 (1:100; from Bruce Hay). Secondary antibodies: Cy5 (1:50; Jackson Laboratories), Alexa 488 (1:200), and Alexa 594 (1:100; Molecular Probes).

TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine-triphosphate nick end-labeling) was performed using the TMR-Red In Situ Cell Detection Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol.

Imaging

Images were captured using a Leica TCS SP5 and Nikon A1R confocal microscopes using the manufacturers software. Adult eyes were imaged in a Leica Z16 with Montage Software. Images were processed in Photoshop CS to adjust brightness and contrast.

Cell Counts

Cells were counted as described previously (Cordero et al., 2004). Hexagons of retinas from at least four different animals were counted. A minimum of 20 hexagons was scored for each condition. Statistical analysis was done using Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Wg induces programmed cell death (PCD) in the mid-pupal retina. **A–D:** wg^{IL114}/wg^{CX4} pupae were either kept at the permissive temperature (A,B) or shifted to the non-permissive temperature through late-stage cell death (C,D). Retinas were stained with anti-Armadillo to label cell membranes (B,D; $\times 63$ magnification) and TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling) to detect apoptotic nuclei (A,C; $\times 10$ magnification). **E–H:** Twenty-eight hour after puparium formation (APF) retinas containing wg^{CX4} clones. Clonal tissue was marked by the absence of green fluorescent protein (GFP; F,H; green) or outlined by dashed line (E,G). TUNEL staining marks the apoptotic nuclei (E,F; red) and DAPI (4',6-diamidine-2-phenylidole-dihydrochloride) staining labels the nuclei of all cells (F; blue). Anti-DIAP1 antibody staining (G,H; red). **I:** Interommatidial cell counts from control and experimental pupal retinas expressed as the average number of IPCs per hexagon $\pm$ SD. **J:** Tracing of a wild-type pupal retina depicting the different cell types present in mature ommatidia: cone cells (c), primary pigment cells (1°), secondary pigment cells (2°), and tertiary pigment cells (3°). Sensory bristles are seen as “bulls eye” organules located at alternating vertices between ommatidia; photoreceptors lie below the plane of view. Anterior is to the right.

Fig. 2.

Early loss of Wingless signaling results in cone cell defects. **A–E:** Forty-two hour after puparium formation (APF) retinas with loss of function clones of *wg* (A,B,D), *fz* plus *fz2* (E), or overexpressing a *wg* RNAi transgene (*wg^{IR}*) under the eye-specific driver *GMR-gal4* (C). The clonal tissue was marked by the absence of green fluorescent protein (GFP; A,B,D,E; green) and outlined by dashed line (A,B,D,E). Cell outlines were visualized by staining with anti-Armadillo (A,C–E; red). Cone cell nuclei were labeled with anti-Cut antibody (B; red). Arrows indicate ommatidia with abnormal number of cone cells. Asterisks in D highlight two incomplete ommatidia at the edge of the retina as has been previously described upon loss of *wg* (Lin et al., 2004). Anterior is to the right. **F–H:** Wg expression in L3 eye-antennal discs by *lacZ* reporter (F) and anti-Wg antibody (G,H). H is a high magnification picture of the boxed area in G.

Fig. 3.

Canonical Wingless signaling is required for cone cell development. **A,B:** Forty-two hour after puparium formation (APF) retinas overexpressing a dominant-negative version of Pangolin/TCF under the control of *GMR-gal4* (A) or the cone-cell driver *sparkling-gal4* (*spa-gal4*) (B). (A; inset) Adult eye from animal overexpressing dominant-negative TCF under *GMR-gal4*. B, insets: *spa-gal4*, *UAS-gfp* retinas in which the *spa* expression domain was visualized by green fluorescent protein (GFP; green). B, right inset: Note colocalization with the cone cell nuclei, stained with anti-Cut (red). B; left inset: Spa is also expressed in primary pigment cells. DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) stains cell nuclei (blue). **C,D:** 42 hr APF retina containing a loss of function *pygo* clone generated by MARCM. Clonal tissue was positively marked with GFP (C; green) or outlined by dashed line (D). Cell outlines were visualized by staining with anti-Armadillo (A–D). Arrows point to ommatidia with abnormal cone cell number and arrowheads to ommatidia with abnormal cone cell morphology. Asterisks in D indicate ommatidia with three primary pigment cells enveloping the cone cell quartet; normal ommatidia contain two. Anterior is to the right.

Fig. 4.

Canonical Wingless signaling regulates cone cell specification. **A–C:** Third-instar larval (L3) retina containing a clone of genotypically *fz.fz2* cells. Clonal tissue was marked by the absence of green fluorescent protein (GFP; A; green) or outlined by a white dashed line (B,C). Cone cell nuclei were stained with anti-Cut antibody (A,B; red). DAPI (4',6-diamidino-2-phenylidole-dihydrochloride) stains the nuclei of all cells (C; blue). **D–F:** Control L3 retina (D) or retinas overexpressing dominant-negative TCF under the control of *GMR-gal4* (E,F). Cone cell nuclei were visualized with anti-Cut antibody (F) or a DPax2-LacZ reporter line (D,E). **G–I:** L3 retina with a loss of function *pygo* clone generated by the MARCM technique. Clonal tissue was positively marked with GFP (G; green) or outlined with a white dashed line (H,I). Anti-Cut antibody (G,H; red); DAPI (I; blue). Dashed lines encircle examples of ommatidia showing either wild-type (D) or defective cone cells (A,B,E,F,H). Anterior is to the right.

Fig. 5.

Photoreceptors provide Wingless activity to regulate cone cell specification. **A,B:** Pygo localization (green) in L3 eye discs. Cone cells were stained with anti-Cut (A; red) and anti-ElaV marks photoreceptors neurons (B; blue). Insets show Pygo localization in the nuclei of cone cells (A; orange) and photoreceptors (B; light blue). **C,D:** L3 retina showing MARCM clones for a null allele of *pygo* and stained with anti-Pygo antibody (C; red, D; white). The clonal tissue was positively marked with green fluorescent protein (GFP; C; green) or outlined with a yellow dashed line (D). **E–G:** L3 retina carrying MARCM *wg^{Cx4}* clones (green). Anti-Cut antibody (F,G; red); ElaV (E,G; blue). Asterisks mark clonal ommatidia. Dashed lines encircle examples of ommatidia showing defective cone cells (F). **A–G:** Anterior is to the left. **H–N:** Thirty-two hour after puparium formation (APF) retinas carrying MARCM *wg^{Cx4}* clones (green). Cell outlines were labeled with anti-Armadillo antibody (H,J,K,M; red). DAPI (4',6-diamidino-2-phenylidole- dihydrochloride) labels all cell nuclei (I,L,N; blue). Asterisks mark clonal ommatidia. Anterior is to the right.

Fig. 6.

Reduced Wg pathway activity suppressed *Notch* mutations in the eye. **A–I:** Adult (A–C) and L3 (D–I) eye phenotypes resulting from *sevenless*-driven overexpression of Notch RNAi (*sev-N^{IR}*) alone (A,D,G) or in a *wg* (B,E,H) or *pygo* (C,F,I) heterozygous background. **J–M:** L3 retinas from animal carrying the *split* allele of *N* alone (J,K) or under a *wg* heterozygote background (L,M). L3 retinas were stained with anti-Cut antibody to visualize the cone cell nuclei (D–F,K,M) and anti-Elav to visualize photoreceptor neurons (G–I,J,L). Anterior is to the right.