Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis

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Summary

The *Drosophila* compound eye is formed by selective recruitment of undifferentiated cells into clusters called ommatidia during late larval and early pupal development. Ommatidia at the edge of the eye, which often lack the full complement of photoreceptors and support cells, undergo apoptosis during mid-pupation. We have found that this cell death is triggered by the secreted glycoprotein Wingless, which activates its own expression in peripheral ommatidia via a positive feedback loop. Wingless signaling elevates the expression of the pro-apoptotic factors *head involution defective*, *grim* and *reaper*, which are required for ommatidial elimination. We estimate that approximately 6-8% of the total photoreceptor pool in each eye is removed by this mechanism. In addition, we show that the retinal apoptosis previously reported in *apc1* mutants occurs at the same time as the peripheral ommatidial cell death and also depends on *head involution defective*, *grim* and *reaper*. We consider the implications of these findings for eye development and function in *Drosophila* and other organisms.

Supplemental data available online

Key words: *Drosophila*, Eye, Apoptosis, Wnt, *head involution defective*, *reaper*, *grim*

Introduction

The appropriate removal of excess cells is an important aspect of patterning and morphogenesis during development. Cell removal is achieved by localized induction of programmed cell death (PCD), or apoptosis. Classic examples include the sculpting of digits in the vertebrate limb bud (Zuzarte-Luis and Hurle, 2002) and the removal of excess neurons in the developing central nervous system (Oppenheim, 1991). Identifying which signaling pathways influence this life-or-death choice and the mechanisms by which they interact with the cellular apoptotic machinery remains a major goal of developmental biology.

Elimination of excess cells is essential for the formation of the compound eye of *Drosophila*. The fly eye consists of approximately 750 repeated units called ommatidia, which consist of eight photoreceptor neurons (R cells), four cone cells (which secrete the lens) and two primary pigment cells (Wolff and Ready, 1993). Differentiation of these cells is triggered by the passage of the morphogenetic furrow (MF). During late larval/early pupal stages, as R cells differentiate in regularly spaced clusters behind the MF, undifferentiated cells furthest from these clusters undergo PCD (Baker and Yu, 2001). One day after the start of pupation, after all the cells of each ommatidium have been specified and organized into an ordered array, an additional round of apoptosis reduces the number of interommatidial cells. The survivors of this trimming become secondary and tertiary pigment cells (Brachmann and Cagan, 2003). In both these cases, the epidermal growth factor receptor (EGFR)/Ras signaling pathway is thought to provide a survival signal (Baker and Yu, 2001; Miller and Cagan, 1998; Yu et al., 2002). The primary target of this survival signal is the pro-apoptotic factor Head involution defective (Hid) (Yu et al., 2002). Ras signaling inhibits Hid activity post-translationally (Bergmann et al., 1998) and downregulates *hid* transcript levels (Kurada and White, 1998).

Hid belongs to a group of proteins that promote PCD by inhibiting the activity and/or stability of inhibitor of apoptosis proteins (IAPs) (Martin, 2002; Shi, 2002). These IAP inhibitors share limited sequence similarity, most notably a short RHG domain at their N-termini that binds to IAPs (Shi, 2002). In flies, the founding members of this group were *hid*, *reaper* (*rpr*) and *grim* (Chen et al., 1996; Grether et al., 1995; White et al., 1994). More recently, an additional family member called *sickle* was identified in flies (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002), and SMAC/Diablo (Du et al., 2000; Verhagen et al., 2000) and HtrA2/Omi (Suzuki et al., 2001) have been identified in mammals.

In addition to the apoptotic events mentioned above, there is a later occurrence of PCD in the fly eye that is less well understood. A ring of apoptotic cells is found at the periphery of the eye at mid-pupation (Wolff and Ready, 1991). These dying cells at the edge of the ommatidial field contain photoreceptors, cone cells and primary pigment cells (Hay et al., 1994; Wolff and Ready, 1991).

We have found that Wg expression coincides with the mid-pupal ring of apoptosis. Wg signaling at the eye's perimeter activates Wg expression in peripheral ommatidia. Wg signaling

also induces the expression of *hid*, *rpr* and *grim*, and these genes are required for the elimination of approximately 80-100 perimeter ommatidia/eye. The ommatidia destined for PCD are often incomplete, probably resulting from an insufficient number of precursor cells at the edge of the eye for recruitment into clusters.

Under normal conditions, Wg signaling is tightly correlated with the stability of cytosolic Armadillo (Arm), the fly β-catenin homolog (Peifer et al., 1994; van Leeuwen et al., 1994). *apc1* is a fly homolog of the adenomatous polyposis coli (APC) tumor suppressor gene, which encodes a negative regulator of Arm/β-catenin stability (Polakis, 2000). Mutation of *apc1* results in *arm*-dependent mid-pupal apoptosis of all photoreceptors (Ahmed et al., 1998). We found that this PCD occurred at the same time as the peripheral ommatidial cell death and that it is also dependent on *hid*, *rpr* and *grim*. Mutations in the APC gene in humans cause retinal lesions in which photoreceptor neurons degenerate (Traboulsi et al., 1990). This and other results will be discussed in regard to the possible role Wnt induction of apoptosis may play in vertebrate eye development and disease.

Materials and methods

Drosophila genetics

Fly stocks were maintained on standard medium at 25°C unless otherwise indicated. Pupal developmental ages are expressed as hours after pupal formation (APF) with white pre-pupae defined as 0 hours APF. The P[*GMR-Gal4*], P[UAS-*wg*] stock was described previously (Cadigan et al., 2002). The UAS-*lacZ*, UAS-*p35*, UAS-wg^{ts} and wg^{IL} lines were from the Bloomington Stock Center (Indiana University) and UAS-ras^{V12} was from Mike Simon. hid^{P}, hid^{X14}, *Df(3L)H99*, *Df(3L)X25* and *Df(3L)XR38* were from Kristin White. The $apc1^{S76}$ and $apc1^{Q8}$ alleles (Ahmed et al., 1998) were from Yashi Ahmed and Eric Wieschaus. The fz^{9131}, $fz2^{M2-8}$ P[*FRT*, w^{+}]^{2A} chromosome was generously provided by Rob Howes and Roel Nusse. The P[*FRT, hs-neo*]^{82B}, $pygo^{10}$ chromosome was from David Parker. Clones of *fz, fz2* and *pygo* were generated in the eye using $Eye\text{-}Flp^{T12}$ (Newsome et al., 2000), provided by Barry Dickson. Clones were marked by *arm-lacZ* as previously described (Cadigan et al., 2002; Parker et al., 2002) or with *Ubi-GFPnls* (Davis et al., 1995).

Histology and scanning electron microscopy

Immunostainings were performed as described (Cadigan and Nusse, 1996). Rat anti-Elav (1:100) and mouse anti-Cut (1:75) were from the University of Iowa Hybridoma Bank. Affinity purified mouse anti-Pros (1:5000) was from Richard Carthew. Affinity purified rabbit anti-Wg (1:50) was prepared as described (Bhanot et al., 1996). Guinea pig anti-Sens (1:500) was kindly provided by Hugo Bellen and rabbit anti-Bar (1:50) by K. Saigo. Mouse anti-lacZ was purchased from Sigma (St Louis, MO). Cy3- and Cy5-conjugated secondary antibodies were from Jackson Immunochemicals (West Grove, PA) and Alexa Flour 488-conjugated secondaries were from Molecular Probes (Eugene, OR). TUNEL staining was performed as described (Wang et al., 1999) using the Cell Death Detection Kit (Fluorescein) from Roche Diagnostics (Indianapolis, IN). All fluorescent pictures were obtained with a Zeiss Axiophot coupled to a Zeiss LSM510 confocal apparatus.

In-situ hybridizations were performed as previously described (Cadigan et al., 1998). Probes were made by PCR on genomic DNA with the following oligos: 5′CTTGCAATTTCACTGGCGGCGATGTGT3′ and 5′GTAATACGACTCACTA TAGGGCGGCCAAGTGAAGCTCTGTGGTTTCTTC3′ for *hid*, 5′CGTTCGTTTTCCCG CCAAATGAGTCAG3′ and 5′GTAATACGACTCACTATAGGGCGCTCGTTCCTCCTCATGTGTCCATAC3′ for *grim*, 5′GACACCAGAACAAAGTGAACGAACTCG3′ and 5′GTAATACGACTCACTATAGGGCGTGTTGTGGCTCTGTGTCCTTGACTGCA3′ for *rpr*. Oligos on the reverse strands each has a T7 RNA polymerase site. Antisense dioxygenin probes were synthesized using the Ambion T7 Megascript kit with the Roche DIG RNA labeling mix.

Samples for scanning electron microscopy (SEM) were prepared as previously described (Cadigan and Nusse, 1996). All images were processed as Adobe Photoshop files.

Results

Wg activates apoptosis of ommatidia at the periphery of the eye

Fig. 1A shows that Wg was expressed in a ring around the mid-pupal eye, i.e. 42 hours after pupal formation (APF), coincident with the peripheral apoptosis previously reported (Fig. 1B) (Hay et al., 1994; Wolff and Ready, 1991). The highest level of Wg expression was found at the very edge of the eye, outside the field of Elav-positive R cells (Fig. 1E-H). Apoptotic nuclei were found in a region of lower Wg expression (Fig. 1E,F) and some of them were still slightly positive for Elav (Fig. 1G), indicating dying R cells.

To test whether Wg signaling was required for this perimeter PCD, we induced clones mutant for *wg*, the Wg receptors *frizzled* (*fz*) and *frizzled 2* (*fz2*), or the nuclear factor *pygopus* (*pygo*). Simultaneous removal of *fz, fz2* abolishes Wg signaling in all readouts examined (Bhanot et al., 1999; Chen and Struhl, 1999), and the same is true for *pygo* (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). Apoptosis was not observed at the edge of the eye in *fz, fz2* clones (Fig. 1N) or in *pygo* or *wg* clones (data not shown). Wg was still expressed inside the *pygo* and *fz, fz2* clones (Fig. 1Q,R and data not shown), indicating that Wg signaling was not required for the cells at the very edge to express Wg. In fact, Wg protein was found at a greater distance into the eye in *pygo* clones, which could be similar to the increased spread of Wg in the wing in the absence of Wg signaling (Cadigan et al., 1998).

Inside the mutant clones, extra R cell clusters were apparent (Fig. 1S,T), while apoptotic Elav-positive cells outside the clone were observed (Fig. 1S). Many extra photoreceptor clusters were also seen at the perimeter when the caspase inhibitor *p35* was expressed throughout the eye (GMR-Gal4::UAS-*p35*) (Fig. 1K), consistent with earlier reports (Hay et al., 1994). Thus Wg removed ommatidia at the edge of mid-pupal eyes by activation of apoptosis.

Wg induces its own expression in ommatidia destined to die

When PCD was blocked by expression of *p35* (Fig. 1J), a dramatic increase in Wg expression was observed (Fig. 1I), which corresponded to the perimeter ommatidia (Fig. 1L). In wild-type eyes at an earlier time (24 hours APF), Wg was expressed uniformly at the edge (Fig. 2A). By 28 hours APF, most of the perimeter of each eye displayed Wg expression in clusters (Fig. 2B), which corresponded to R cells (data not shown, but see Fig. 2F,G). This was more apparent at 32 hours APF (Fig. 2C), and Wg expression in the ommatidia started to fade by 36 hours APF (Fig. 2D). At 24 and 28 hours APF, almost all the apoptotic cells were in the interior (Fig. 2A,B),

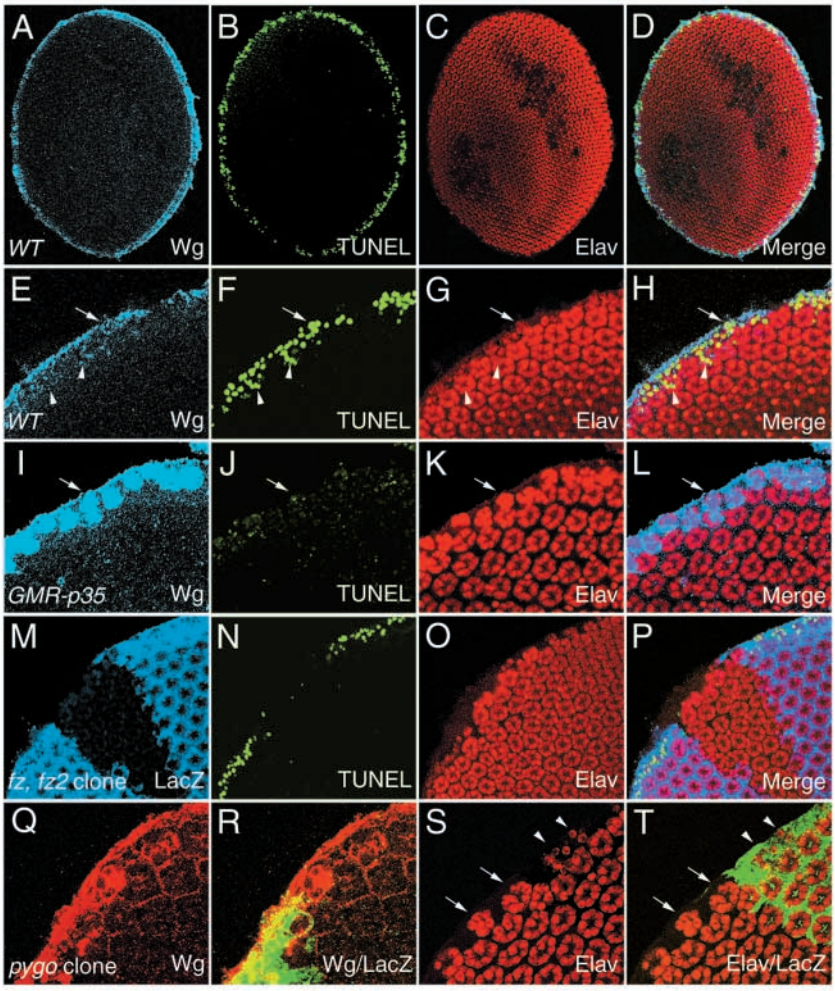

Fig. 1. Wg signaling eliminates R cell clusters from the edge of the eye through apoptosis. All micrographs are of 42 hours APF eyes. (A-D) Wild-type eye stained for Wg protein (blue), TUNEL (green) and Elav (red). Wg staining is coincident with the apoptotic cells at the edge of the eye. (E-H) Close-up of the eye perimeter in (A-D). Most of the Wg protein is adjacent to the apoptotic cells, with lower levels in the dying cells, some of which are still Elav-positive (arrows). The edge of this eye is slightly curved, making some of the TUNEL-positive nuclei appear to be present in interior Elav-positive photoreceptors in the optical stack (arrowheads). (I-L) GMR-Gal4::UAS-*p35* eyes stained as in wild type. Apoptosis is not observed (J) but a large accumulation of Wg protein is observed (I) coincident with photoreceptors (L; arrow indicates one example). (M-P) Clone mutant for the Wg receptors *fz* and *fz2*. Clonal marker (LacZ) is shown in blue, TUNEL in green and Elav in red. No apoptosis is observed inside the *fz, fz2* clone. (Q-T) Clones mutant for the Wg signaling component *pygo* stained in red for Wg (Q,R) or Elav (S,T). Clonal marker (LacZ) is shown in green. Wg expression is still present in the *pygo* clone. Extra R cell clusters in a *pygo* clone are apparent (arrows) while decaying clusters are seen in the adjacent $pygo^{+}$ tissue (arrowheads).

corresponding to the elimination of inter-ommatidial cells (Hay et al., 1994; Miller and Cagan, 1998). TUNEL-positive nuclei at the edge of the eye were apparent at 32 hours APF, and their number increased by 36 hours APF (Fig. 2D), although they were still far from the numbers seen at 42 hours APF. These data indicate that Wg expression in the peripheral ommatidia preceded PCD. If apoptosis was blocked, elevated Wg expression persisted in the ommatidia that were to be eliminated (Fig. 1I).

This induction of Wg expression in the perimeter ommatidia was dependent on Wg signaling. In *pygo* clones, the expression of Wg in ommatidia did not occur (Fig. 2F), although the expression in non-ommatidial cells at the very edge of the eye was still present. Similar results were observed in *fz*, *fz2* clones (data not shown). This Wg pattern inside the clones was very similar to the wild-type pattern at 24 hours APF (Fig. 2A).

An alternative explanation for the lack of Wg-positive ommatidia in the *pygo* clones at 30 hours APF is that these mutant cells were developmentally delayed. In such a scenario, Wg expression would occur later and eventually cause PCD. However, Wg was not found in perimeter ommatidia of *pygo* clones at 36 hours APF (data not shown) or 42 hours APF (Fig. 1R and data not shown). Consistent with this, no sign of apoptosis was observed in mutant clones at 48 hours APF (Fig. 2J) or 54 hours APF (data not shown), long after control ommatidia had undergone PCD. Perimeter ommatidia were still found in the *pygo* clones at 54 hours (Fig. 2L) and likely persisted until adulthood (data not shown and Fig. 5H). There was no detectable developmental lag in cells unresponsive to Wg. Rather, these cells were unable to initiate autoregulation of Wg expression or Wg-dependent PCD.

The induction of Wg expression in the ommatidia destined for elimination suggested that Wg is required for PCD at this time. To confirm this, we attempted to use the conditional mutant wg^{IL}, which encodes a temperature-sensitive protein (Baker, 1988). However, even at 16.5°C, where wg^{IL} is wild-type for most Wg activities (Baker, 1988; Cadigan and Nusse, 1996), very few Wg-positive ommatidia or subsequent apoptosis was observed (see Fig. S1 at http://dev.biologists.org/supplemental/), precluding the use of this allele to determine the critical phase for Wg activity in regulating PCD. As an alternative, the wg^{ts} gene was expressed under the control of the *GMR* promoter (Fig. 3). When reared at the restrictive temperature (25°C), GMR-wg^{ts} eyes had a normal pattern of apoptosis (Fig. 3D). If shifted to 16.5°C at 24 hours APF and cultured until an equivalent stage, considerable R cell apoptosis was observed (Fig. 3A,B). Less PCD was evident in shifts at 30 hours APF (Fig. 3C) and shifts at 36 hours APF resulted in almost no ectopic apoptosis (data not shown). These data demonstrate that Wg expression at 24 hours APF can induce R cell apoptosis, consistent with the expression pattern of endogenous Wg.

Wg activates *hid*, *grim* and *rpr* expression at the edge of the eye and these genes are required for perimeter apoptosis

The pro-apoptotic gene *hid* is required for the apoptosis of

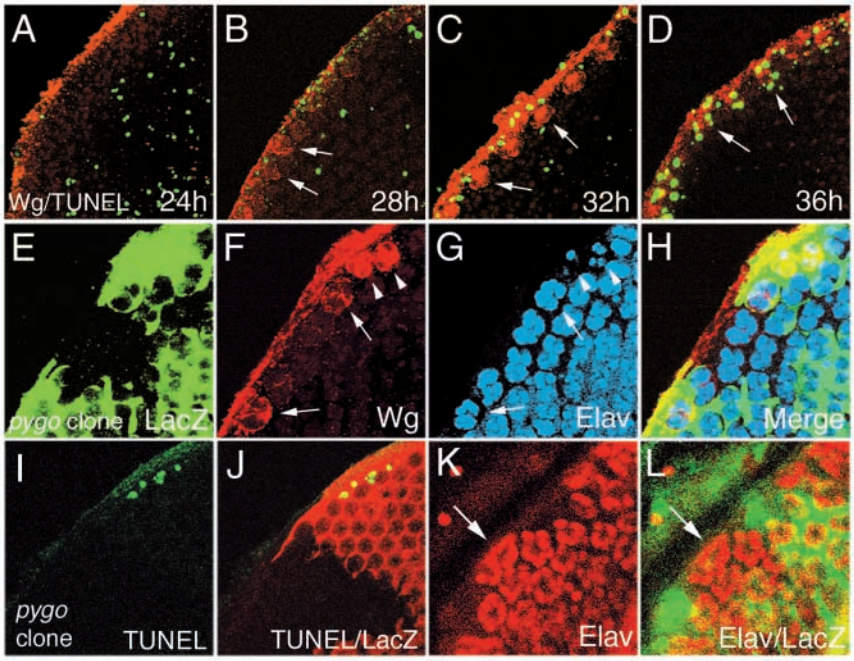

Fig. 2. Accumulation of Wg in ommatidial clusters precedes PCD and depends on Wg signaling. (A-D) Wild-type eyes showing time course of Wg protein expression (red) and TUNEL (green) at 24 hours (A), 28 hours (B), 32 hours (C) and 36 hours APF (D). At 24 hours APF, Wg is expressed in a uniform ring at the eye's edge. Starting at 28 hours and peaking at 32 hours APF, Wg expression is found in clusters (arrows). These clusters are fading at 36 hours APF. TUNEL-positive nuclei in the eye interior at 24 and 28 hours APF correspond to dying inter-ommatidial cells. TUNEL-positive nuclei begin to appear at the edge at 32 hours APF and increase by 36 hours APF in the Wg-positive clusters. (E-H) 32 hours APF eye containing a clone of *pygo* stained for Wg (red), Elav (blue) and clonal marker LacZ (green). Wg expression overlaps with Elav outside the clone (arrows) but not in cells lacking *pygo*. Note that some of the Wg clusters contain Elav-clusters in a different focal plane (arrowheads), suggesting that they are already undergoing apoptosis. (I,J) *pygo* clone at 48 hours APF stained for TUNEL (green) and clonal marker lacZ (red). No TUNEL-positive nuclei are observed in the clone. (K,L) *pygo* clone in 54 hours APF pupal eye stained for Elav (green) and clonal marker lacZ (red) showing two perimeter ommatidia inside the clone (arrow) that have not undergone PCD.

undifferentiated and inter-ommatidial cells during larval and early pupal development (Kurada and White, 1998; Yu et al., 2002). Therefore, we explored the role of *hid* in mid-pupal ommatidial PCD. The hid^{P} allele is a transposon insertion allele in the *hid* ORF (Grether et al., 1995). In hid^{P} homozygotes, apoptosis was still observed at the perimeter (Fig. 4B), although it was distinct from wild type in that high Wg expression was still found in the apoptotic ommatidia and the Elav staining in the perimeter clusters still appeared normal (see Fig. S2 at http://dev.biologists.org/supplemental/). This suggests that the rate of ommatidial apoptosis is significantly reduced in *hid* mutants.

Three other genes in the fly genome, *rpr*, *grim* and *sickle*, are known to encode proteins with limited sequence similarity to Hid (Shi, 2002; Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002). These proteins promote apoptosis by inhibiting the activity and/or stability of IAPs (Martin, 2002, Shi, 2002; Srinivasula et al., 2002). Therefore, we explored whether functional redundancy could account for the partial inhibition of Wg-dependent apoptosis in *hid* mutants.

The *H99* deficiency completely removes *hid*, *grim* and *rpr* but not *sickle* (White et al., 1994; Christich et al., 2002; Wing et al., 2002). The *X25* deletion removes *hid* and *grim* while the *XR38* deficiency removes *rpr* and *sickle* (Peterson et al., 2002). Clones of *X25* showed a strong reduction of apoptosis 60% of the time (arrowhead in Fig. 4C,D), with the reminder having a much smaller reduction in TUNEL (arrow in Fig. 4C,D). *H99*/+ heterozygotes displayed a phenotype at the eye's perimeter similar to that of *hid* mutants, while *H99*/*XR38* eyes displayed slightly less TUNEL signal than in *H99*/+ (data not shown). In *H99* homozygous clones, there was almost no apoptosis (Fig. 4F) and extra R cell clusters were observed (Fig. 4G; see arrows). No evidence for a developmental lag was observed in *H99* clones, with Wg-positive ommatidia appearing at the same time as controls (Fig. 4J) and perimeter R cells persisting until 54 hours APF with no sign of PCD (Fig. 4L and data not shown).

The above data suggest that all three pro-apoptotic factors in the *H99* interval are required for the *wg*-dependent PCD at the edge of the eye. *hid*, *grim* and *rpr* transcripts were all

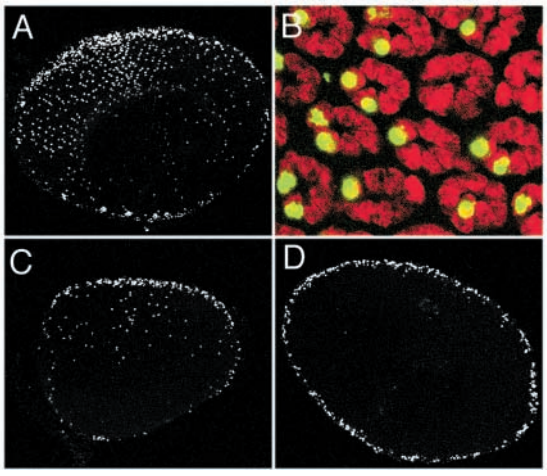

Fig. 3. Ectopic Wg expressed at 24 hours APF can induce R cell PCD. GMR-Gal4::UAS-wg^{ts} eyes were stained for TUNEL (green) and Elav (red). Animals were reared at 25°C (where wg^{ts} is inactive) and shifted to 16.5°C at 24 hours APF (A,B), 30 hours (C) or not shifted (D). After the shift to the lower temperature (activating Wg activity), animals were cultured until the equivalent of 42 hours APF (development occurs 2.7× slower at the lower temperature). A shift down at 24 hours APF results in considerable apoptosis in the interior R cells (A,B) while less TUNEL-positive cells are observed when shifted at 30 hours APF. No ectopic PCD was observed in eyes cultured continuously at 25°C (D).

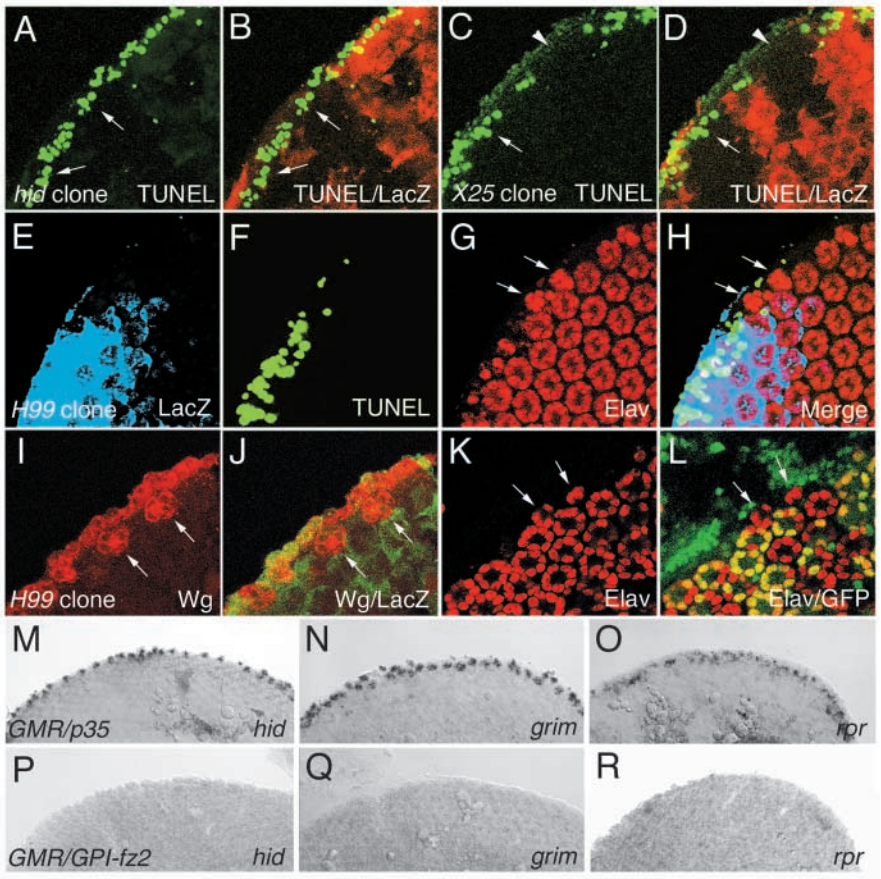

Fig. 4. Requirement of *hid*, *grim* and *rpr* for perimeter apoptosis. All eyes are 42 hours APF except where noted. (A,B) *hid*^{P} mutant clone stained for TUNEL (green) and clonal marker LacZ (red). No detectable reduction in apoptosis is observed. (C,D) *Df(3L)X25* deficiency clone stained as for (A,B). In one clone there is little reduction in TUNEL (arrow), while a strong reduction is observed in the other (arrowhead). (E-H) *Df(3L)H99* clone stained for clonal marker LacZ (blue), TUNEL (green) and Elav (red). Very little TUNEL is observed inside the clone (F) and extra R cell clusters are apparent (arrows in G). (I,J) *Df(3L)H99* clone at 30 hours APF stained for Wg (red) and clonal marker lacZ (green). Wg-positive ommatidia are present inside the clone. (K,L) *Df(3L)H99* clone at 54 hours APF stained for Elav (red) and clonal marker GFP (green). Perimeter ommatidia are still observed inside the clone at this late time. (M-O) GMR-Gal4::UAS-*p35* eyes stained for *hid* (M), *grim* (N) or *rpr* (O) transcripts. Elevated expression of all three genes is observed at the edge of the eye. (P-R) GMR-Gal4::UAS-*GPI-fz2* eyes stained for *hid* (P), *grim* (Q) or *rpr* (R). No elevated expression of these genes was observed at the edge of the eye.

elevated at the periphery in GMR-Gal4::UAS-*p35* eyes (Fig. 4M-O). The expression pattern was clustered in a similar pattern to that found for Wg expression. The perimeter expression of all three genes was completely abolished in GMR-Gal4::UAS-*GPI-fz2* eyes (Fig. 4P-R). GPI-Fz2 is known to be an efficient inhibitor of Wg signaling (Cadigan et al., 1998) and blocked peripheral apoptosis as completely as p35 (data not shown). These results strongly suggest that *hid*, *grim* and *rpr* are expressed in the Wg-positive peripheral ommatidia and that Wg signaling is required for this expression.

Perimeter ommatidia are often incomplete

Ommatidia are formed by the sequential recruitment of cells into clusters. The process starts with the specification of evenly spaced R8 neurons, which then recruit four additional neurons (R2-R5). After a wave of mitosis, R1 and R6 are added and then specification of R7 completes the eight R cell complement. Four cone cells and two primary pigment cells are then added (Wolff and Ready, 1993).

Staining with the general neuronal marker Elav revealed that many ommatidia at the edge of the eye were incomplete (Fig. 5B,D and data not shown). We examined the composition of the peripheral ommatidia using cell-specific markers. Senseless (Sens) is a marker for R8 (Frankfort et al., 2001), Bar for R1/R6 (Higashijima et al., 1992), Prospero for R7 (Kauffmann et al., 1996) and Cut for cone cells (Blochlinger et al., 1993). Wild-type or GMR-Gal4::UAS-*p35* eyes were stained for these proteins at 5 or 30-32 hours APF, before perimeter apoptosis occurred, with similar results. This indicates that PCD did not influence the perimeter ommatidia composition before 32 hours APF. All the ommatidia containing at least two Elav-positive cells had one R8 and almost all single Elav-positive cells were Sens-positive (data not shown). Some peripheral ommatidia were missing either R1 or R6 (Fig. 5A,D) and many had fewer than four cone cells (e.g. asterisk in Fig. 5C). We conclude that many of the ommatidia destined to die are incomplete, perhaps from lack of cells at the edge of the eye field for recruitment into the developing clusters. Interestingly, most (>80%) perimeter ommatidia had an R7 cell, even if they had fewer than the normal complement of R cells (arrowheads in Fig. 5B).

The incomplete ommatidia were clearly eliminated, since they were not found at 42 hours APF (data not shown). To determine whether complete ommatidia are also eliminated, we used Wg expression at 32 hours APF as a marker for ommatidia that will undergo PCD. Many Wg-positive ommatidia had an R7 cell (Fig. 5B,E) and four cone cells (Fig. 5C,F). Occasionally, Wg-positive ommatidia appeared to have the normal number of Elav-positive R cells (arrow in Fig. 5B,E). The Wg-positive cone cells clusters were often smaller than normal (Fig. 5C,F; arrowheads), although occasionally we observed some of near normal size (arrows in Fig. 5C,F). These cells are capable of secreting lens, as evidenced by the presence of surface ommatidia of reduced size at the edge of adult eyes containing large clones of *pygo* or *fz*, *fz2* (Fig. 5H and data not shown). The clone shown is at the ventralmost portion of the eye, where inhibition of Wg signaling did not result in expansion of the eye field at the expense of head anlage (data

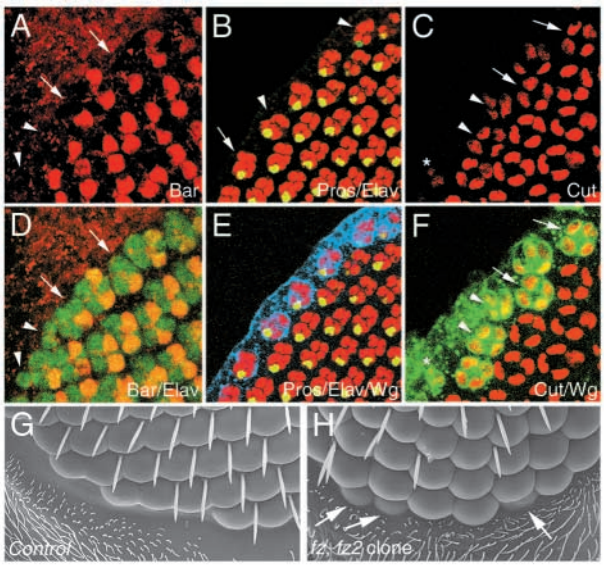

Fig. 5. Ommatidia destined to die are often incomplete. (A,D) Wild-type eye at 5 hours APF stained for Bar (red) and Elav (green). Bar is a marker for R1 and R6. Several ommatidia at the edge contained only one positive Bar cell (arrows), while smaller Elav-positive clusters contained none (arrowheads in D). (B,E) GMR-Gal4::UAS-*p35* eye at 32 hours APF stained for Elav (green), Prospero (Pros; red) and Wg (blue). Pros is a marker for R7 and Wg for ommatidia that will later undergo PCD. About 20% of the Wg-positive ommatidia do not contain an R7 (data not shown). Most of the Wg-positive ommatidia containing an R7 lack some R cells (arrowheads,) but occasionally they appear to have a full complement (arrow). (C,F) GMR-Gal4::UAS-*p35* eye at 32 hours APF stained for Cut (red) and Wg (green). Cut is a marker for cone cells. Some Wg-positive ommatidia had four cone cells of normal or near normal size (arrows, compared with more interior ommatidia), but most four cone cell clusters appeared smaller than normal (arrowheads). Often fewer than four cone cells were observed per ommatidia (asterisk). (G) SEM of adult control eye showing the periphery of the ventral eye. (H) SEM of an adult eye containing a large *fz, fz2* clone covering the ventral portion of the eye showing three smaller ommatidia never seen in controls (arrows).

not shown), as is observed when Wg signaling is blocked dorsally (Heslip et al., 1997). Small extra ommatidia are also observed at the edge of adult GMR-Gal4::UAS-*p35* eyes (data not shown) (Hay et al., 1994).

The R cell apoptosis seen in *apc1* mutants depends on *hid*, *grim* and *rpr*

Mutants in the *apc1* gene, which encodes a negative regulator of Wg signaling, cause R cells to undergo PCD during mid-pupation (Ahmed et al., 1998). In addition, expression of an activated form of Arm results in a similar phenotype (Ahmed et al., 1998; Freeman and Bienz, 2001). We found that R cell apoptosis in *apc1* mutants occurred at the same time as the perimeter cell death in wild type, with TUNEL-positive cells arising around 35 hours APF (data not shown) and peaking at 42 hours APF (Fig. 6E). All Elav-positive cells were eliminated by 54 hours APF (data not shown). Adult *apc1* mutants survive to adulthood and have slightly smaller eyes (K. Cadigan, unpublished observations) with no photoreceptors (Ahmed et al., 1998).

To test whether apoptosis in *apc1* mutants is similar to that which occurs at the perimeter, we examined whether *hid*, *grim* and *rpr* were required for this PCD. We found a slight reduction in the amount of TUNEL signal in *hid*, *apc1* mutant eyes and the signal was greatly reduced in a *H99*/+ background (data not shown). Apoptosis was completely abolished in *H99* mutant clones (Fig. 6G–I). As in the case of the perimeter apoptosis, the PCD found in *apc1* mutants appeared to depend on *hid*, *grim* and *rpr*.

Interestingly, we found no elevation of Wg expression in the interior of *apc1* mutant eyes, even when PCD was completely blocked in *H99* mutant clones (data not shown). Thus, the elevated Wg signal in the absence of *apc1* was not sufficient to activate the Wg autoregulatory loop.

Discussion

Wg signaling induces apoptosis through activation of *hid*, *grim* and *rpr* expression

Elevated Wg signaling has previously been reported to induce apoptosis in the photoreceptor neurons of the mid-pupal eye (Ahmed et al., 1998; Freeman and Bienz, 2001). However, the physiological relevance and mechanism of this Wg signaling-dependent PCD were not clear. In this report, we demonstrated that endogenous Wg induced apoptosis at the edge of mid-pupal eyes. Wg signaling induced the expression of the apoptotic activators *hid*, *grim* and *rpr* (Fig. 4M-R). Using a combination of different deficiencies in the *hid*/*grim*/*rpr* region, we demonstrated that all three genes were required for Wg-dependent perimeter ommatidia PCD (Fig. 4A-F). This is distinct from the apoptosis that occurs earlier in eye development, where removal of *hid* is sufficient to prevent PCD (Kurada and White, 1998; Yu et al., 2002). Coordinated regulation of these three genes has not previously been reported, and it will be interesting to determine whether the activation by Wg signaling is direct or occurs through an intermediary.

Mid-pupal apoptosis at the eye's perimeter depends on Wg signaling

During mid-pupation (36-44 hours APF), ommatidia at the edge of the eye are eliminated through PCD that depends on Wg signaling. Our results indicate the following order of events, summarized in Fig. 7. The ring of Wg expression bordering the eye anlage is initially established around 6 hours APF (Cadigan et al., 2002) (data not shown). At 24 hours APF, after eversion of the pupal eye, Wg is found in cells at the edge of the eye that are distinct from the perimeter ommatidia (Fig. 2F-H and data not shown). Several hours later, between 28–32 hours APF, Wg expression is found in some of these ommatida (Fig. 2B,C). The establishment of this pattern depends on the ommatidial cells being able to transduce the Wg signal (Fig. 2E-G). Wg signaling then activates the expression of *hid*, *grim* and *rpr* and apoptosis ensues. Under normal conditions, Wg expression is largely faded from apoptotic ommatidia by 36 hours APF (Fig. 2D). When apoptosis is blocked, the positive feedback loop of Wg expression is unchecked, and Wg and the pro-apoptotic factors can accumulate to high levels (Fig. 1I; Fig. 4M-O).

Our data cannot distinguish whether Wg from the edge cells or the induced Wg in the perimeter ommatidia is required for activating PCD, since the clones we examined removed *wg*

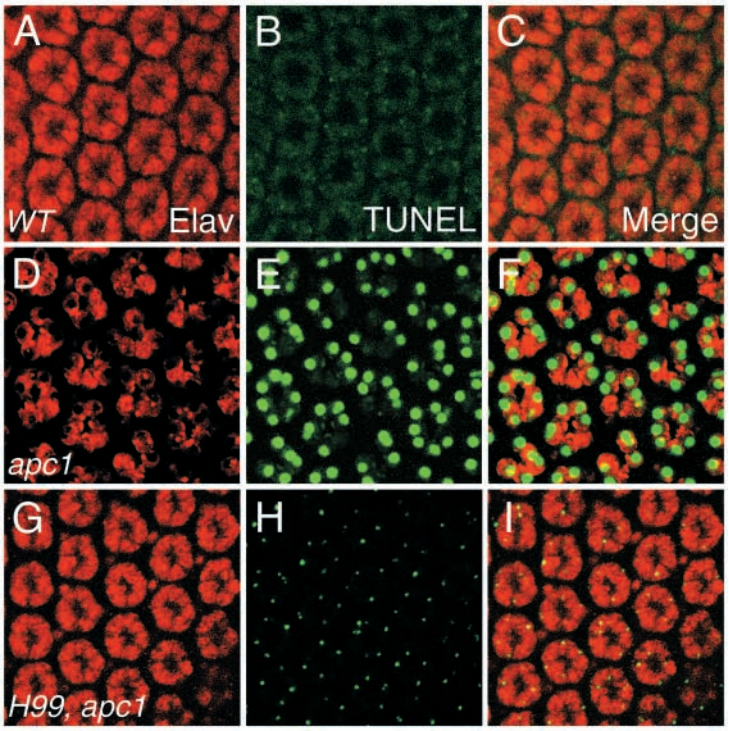

Fig. 6. The R cell apoptosis in *apc1* mutants is absent in *H99* clones. All micrographs are of the interior of eyes at 42 hours APF. (A-C) Wild-type eye stained for Elav (red) and TUNEL (green). No PCD is apparent. (D-F) $apc1^{S76}/apc1^{Q8}$ mutant eye stained as above. Many of the Elav-positive cells are TUNEL-positive. (G-I) Large *Df(3L)H99* mutant clone in an $apc1^{S76}/apc1^{Q8}$ mutant background stained as above. No cells outside the clone are visible in the field shown. There is a complete block of apoptosis and the Elav pattern is identical to wild type.

activity from both cell types. However, the expression of Wg in the ommatidia precedes the appearance of TUNEL-positive nuclei (Fig. 2B,D) and the level of Wg expression is at similar levels as found in the edge cells (Fig. 2C). Therefore, we favor a model in which Wg expression in the ommatidia is necessary for apoptosis to occur.

Incomplete ommatidia are not found at the edge of wild-type eyes (data not shown) (Hay et al., 1994). Therefore, the partial ommatidia observed during early/mid-pupation are destined to die. These ommatidia are also marked by Wg expression at 30-32 hours APF (Fig. 2C,F). The smallest of these clusters has just one cell, usually expressing a marker for the R8 cell type (data not shown). All ommatidia containing more than one R cell contain an R8, but many are missing either R1 or R6 (Fig. 5A,D). Around 20% of the ommatidia destined to die were lacking an R7 cell (data not shown). There are also Wg-positive clusters containing 0-3 cone cells, instead of the normal four (Fig. 5C,F and data not shown). We suspect that the clusters at the perimeter of the eye field simply run out of surrounding cells to recruit.

While most of the eliminated ommatidia appear to have been incomplete, we believe that some did possess the full complement of neurons and support cells. Some Wg-positive ommatidia appeared to contain eight R cells (Fig. 5B,E), and ones containing four cones cells were not uncommon (Fig. 5C,F). Many also possessed two primary pigment cells (data not shown). Almost all the Wg-positive ommatidia that possessed four cone cells appeared significantly smaller than normal (arrowheads in Fig. 5C,F and data not shown). These correspond to the 'stunted ommatidia' previously reported (Hay et al., 1994; Wolff and Ready, 1991). These ommatidia probably give rise to the smaller ommatidia observed at the edge of adult eyes containing *fz*, *fz2* or *pygo* clones (Fig. 5H and data not shown).

How many ommatidia are eliminated by Wg signaling during development? There are approximately 80-100 Wg-positive ommatidia in GMR-Gal4::UAS-*lacZ* or GMR-Gal4::UAS-*p35* eyes at 32 hours APF. These estimates do not include the 1-2 cell clusters that are difficult to distinguish. Assuming an average of five R cells/Wg cluster, 400 to 500 R cells would be eliminated per eye. There are about 6000 photoreceptors in the adult eye. This calculation suggests that between 6.2 and 7.7% of the pupal photoreceptors are removed by Wg signaling during mid-pupation.

Besides the elimination of ommatidia described in this study, Wg signaling at the perimeter of the eye has also been shown to specify the pigment rim epithelia (Tomlinson, 2003) and to induce Homothorax expression, which is necessary for specification of the dorsal rim inner photoreceptors, which are specialized for detection of polarized light (Wernet et al., 2003). Thus, Wg signaling has multiple roles in refining the perimeter of the developing eye.

The function of ommatidia removal

What purpose does the elimination of these perimeter ommatidia achieve? The answer probably lies in the connections between the six outer R cells of each ommatidium and their post-synaptic targets in the laminal layer of the optic ganglia. The lamina is organized into units called cartridges, which underlie each ommatidium and form synapses with the outer R cells. Because of the precise arrangement of the

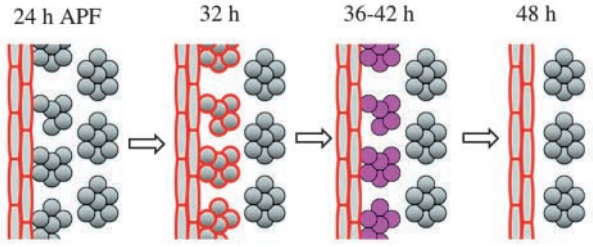

Fig. 7. Cartoon of Wg-induced PCD at the eye's perimeter. Non-ommatidial cells at the edge of the eye are depicted as oblongs and ommatidial cells as circles. A red outline indicates Wg expression and purple represents *hid*, *grim* and *rpr* expression. At 24 hours APF, Wg is found only in the edge cells. Between 28-32 hours APF, Wg expression is induced in the perimeter ommatidia, which are often lacking the full complement of cells. Wg signaling is necessary but not sufficient for this induction of ommatidial Wg expression. By 36 hours APF, Wg expression in the ommatidia is fading and the expression of the pro-apoptotic genes *hid*, *grim* and *rpr* are triggering apoptosis, causing the elimination of these ommatidia. See text for further details.

photoreceptors in the ommatidia and the curvature of the eye, a single line of sight is perceived by six different outer photoreceptors (R1-R6) residing in six different ommatidia. These R cells do not innervate the underlying cartridge; rather, each cell forms a synapse with a distinct adjacent cartridge. In this way, visual excitation in the curved surface of the retina is transformed into a smooth topographic map in the optic ganglia (for reviews see Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2002).

Because there is not a one-to-one relationship between ommatidia and lamina cartridges, a problem arises at the edge of the eye, where there are not enough adjacent cartridges for perimeter ommatidia to form synapses with. The development of the optic ganglia is stimulated by the projection of axons from the overlying ommatidia during larval and early pupal development (Meinertzhagen and Hanson, 1993). The peripheral-most ommatidia are thought to induce underlying cartridges before they are eliminated. Thus, there are extra laminal targets at the eye's edge for the remaining perimeter outer R cells to innervate (Meinertzhagen and Hanson, 1993). Removal of ommatidia by *wg*-dependent PCD should minimize the number of incorrect connections that would compromise the fly's peripheral vision.

What is the signal triggering the Wg autoactivation circuit?

The cells at the edge of the eye express Wg from 6 hours APF (Cadigan et al., 2002). However, Wg from these edge cells does not activate Wg in the adjacent ommatidia until 26-32 hours APF. Wg signaling alone is not sufficient to trigger Wg autoactivation, since elevated Wg expression is not observed in *apc1* mutant eyes (data not shown), even though the pathway is activated enough to induce R cell apoptosis (Fig. 6E) (Ahmed et al., 1998). There must be some other signal(s) that triggers the *wg* self-activation in the perimeter ommatidia.

The edge cells expressing Wg could also express another signal that would allow the Wg signal to activate Wg expression in the ommatidia at the appropriate time. This simply moves the problem back one step; i.e. what then triggers the expression/activation of this signal? An alternative is the presence of a signal that counteracts Wg signaling until 24-28 hours APF. One candidate is Ras signaling, which can block the activity of Wg in the eye (Freeman and Bienz, 2001; Hazelett et al., 1998). It has been previously suggested that elevated Ras signaling during larval and early pupal stages prevents overexpression of a stablized form of Arm from inducing PCD until mid-pupation (Freeman and Bienz, 2001). However, we found that overexpression of an activated form of *ras* did not block the perimeter apoptosis (H. Lin and K. Cadigan, unpublished). Therefore, we think it unlikely that a decrease in the Ras pathway is the signal allowing Wg to initiate the apoptotic cascade.

As discussed above, some eliminated ommatidia appear to have the full complement of cell types, so incompleteness does not appear to be the signal. However, the ommatidia destined to die are almost always smaller than normal (Fig. 5C,F and data not shown). Small size combined with Wg from the edge cells could initiate Wg expression in the ommatidia.

Another candidate for the trigger is the failure of synapse formation between the R cell neurons and their targets in the optic ganglia. Projection of the R cell axons into the ganglia is complete by early pupation, but the formation of synapses between the outer R cells and the lamina neurons does not occur until 24-38 hours APF (Clandinin and Zipursky, 2002). Perhaps the R cells from perimeter ommatidia cannot find enough post-synaptic targets because they are at the edge of the field. The absence of a retrograde signal from neurons in the optic ganglia could lead to the accumulation of Wg in these ommatidia.

Implications for eye development in other organisms and human disease

The expression pattern of Wg in the eyes of other arthropods suggests that it may function in a similar fashion as in *Drosophila melanogaster*. The flour beetle *Tribolium castaneum* is a primitive holometabolous insect species, in which the adult eye forms at the end of larval development from lateral head ectoderm known as optic placodes. During early pupation, the beetle ortholog of *wg* is expressed in a ring around the optic placodes (Friedrich and Benzer, 2000). In the crustacean *Mysidium columbiae* (mysid), retinal morphogenesis occurs in a posterior-to-anterior direction as in *Drosophila* (Duman-Scheel et al., 2002). As this occurs, the mysid ortholog of *wg* is expressed in a half ring around the posterior of the developing eye (Duman-Scheel et al., 2002). It will be interesting to determine whether this ring becomes complete as retinal differentiation proceeds and whether apoptosis occurs at the edge of the eye in beetles and mysids.

The finding that *Pax6* orthologs in flies, mice and humans are necessary for eye development has suggested a common origin for eyes (Halder et al., 1995). However, vertebrate and insect eyes have completely different morphologies, as well as distinct embryonic origins. This has led to the idea that a common 'primitive unit' consisting of light-sensing photoreceptors has been recruited for organogenesis several times during animal evolution (Gehring and Ikeo, 1999). However, many interesting parallels exist in the development of insect and vertebrate eyes, perhaps because of utilization of genetic circuits that were developed before the divergence of these species (Pichaud et al., 2001).

The periphery of the vertebrate eye contains the ciliary marginal zone (CMZ), which contains undifferentiated retinal progenitor cells, which can differentiate to add neurons and glia to the periphery of the eye (Reh and Levine, 1998). In the developing chick eye, Wnt2b is expressed in the CMZ. Blocking Wnt signaling causes premature differentiation of neuronal progenitors (Kubo et al., 2003). Whether Wnt signaling acts in part through inducing apoptosis in the CMZ remains to be examined.

A possible connection between Wnt signaling and PCD in insect and vertebrate eyes is also suggested by similarity in APC mutations. In flies, *apc1* mutants have retinal apoptosis (Ahmed et al., 1998) (Fig. 6). Some humans heterozygous for mutations in the *APC* gene also display retinal lesions, termed congenital hypertrophy of the retinal pigment epithelium (CHRPE). While an increase in the thickness of the retinal pigment epithelium is the most consistent feature of the condition in humans (Buettner, 1975; Traboulsi et al., 1990) and in mice carrying a similar mutation (Marcus et al., 2000), photoreceptor degeneration is also observed in several lesions. Because these lesions are probably due to somatic inactivation of the wild-type *APC* allele, they are variable and difficult to

study systematically. A more direct comparison with the fly *apc1* mutants is needed, perhaps with conditional knockouts of the mouse *APC* gene.

We have shown that in the fly eye, Wg induces apoptosis by activating the expression of *hid*, *grim* and *rpr*. These proteins then inactivate the caspase inhibitor IAP through an RHG domain at their N-termini (Shi, 2002). *hid*, *grim* and *rpr* have no obvious orthologs in vertebrates, although Smac/Diablo and HtrA2/Omi possess an RHG at their N-termini and inhibit IAP activity (Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). If Wnt signaling induces PCD in the vertebrate eye, does this occur through activation of Smac and/or HtrA2 expression? A similar parallel has been found in p53-induced apoptosis, where *rpr* is activated in flies (Brodsky et al., 2000) and HtrA2 in human cell culture (Jin et al., 2003). The involvement of Wnt signaling in the human eye though the induction of IAP inhibitors clearly deserves further consideration.

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