Local induction of patterning and programmed cell death in the developing
*Drosophila* retina

David T. Miller and Ross L. Cagan*

Washington University School of Medicine, Department of Molecular Biology and Pharmacology, Box 8103, 660 South Euclid Avenue, St. Louis, MO 63110, USA

*Author for correspondence (e-mail: cagan@pharmdec.wustl.edu)

Accepted 8 April; published on WWW 19 May 1998

**SUMMARY**

Local cell signaling can pattern the nervous system by directing cell fates, including programmed cell death. In the developing *Drosophila* retina, programmed cell death is used to remove excess cells between ommatidia. Cell ablation revealed the source and position of signals required for regulating the pattern of programmed cell death among these interommatidial cells. Two types of signals regulate this patterning event. Notch-mediated signals between interommatidial precursors result in removal of unneeded cells. Cone cells and primary pigment cells oppose this signal by supplying a ‘life’-promoting activity; evidence is provided that this signal occurs through localized activation of the EGF Receptor/Ras pathway. Together, these signals refine the highly regular pattern observed in the adult retina.

Key words: *Drosophila melanogaster*, Programmed cell death, Apoptosis, Ablation, Retina, Cell patterning

**INTRODUCTION**

Recent years have brought an appreciation of the importance of selective cell death in the sculpting of epithelia. Naturally occurring cell death has been termed ‘programmed cell death’ (PCD) and involves activation of a basal ‘death machinery’, a cassette of death-related genes common to most apoptotic deaths examined (reviewed by Chinnaiyan and Dixit, 1996; White, 1996). This death machinery appears to be present in most cells, and various signals mediated through cell cycle control and especially cell-cell signaling are required to trigger its activation. Little is understood of the regulation of PCD during epithelial development, particularly in regard to how certain cells are chosen to die while others are spared. This paper examines the decision between differentiation and PCD in the developing pupal *Drosophila* retina.

Precise spatial control of PCD is particularly important in developing epithelia, where cell death plays a prominent role in patterning. For example, PCD is used to carve joints and digits from the distal limb, create lumina, and remove excess neurons (Ganan et al., 1996; Lund et al., 1996; Zhou and Niswander, 1996; Macias et al., 1997; Jacobson et al., 1997). It may also play a key role in epithelial morphogenesis including generation of the semicircular canals of the inner ear (Fekete et al., 1997) and neural tube closure (Weil et al., 1997). In the developing hindbrain, upregulation of MSX-2 is associated with maintaining the boundaries of rhombomeres (Maden et al., 1997); this expression is regulated by periodic expression of BMP-4. Both these molecules play a variety of roles during development which are independent of PCD, suggesting that some signals that regulate cell death may be common to other fate decisions as well. While these tissues have provided important insights into the regulation of PCD, their complexity has thus far made it difficult to determine how individual cells regulate PCD; presumably death is regulated through signals provided in a spatially restricted manner.

One candidate to provide this regulation is the ras signal transduction pathway. PCD in epithelia deprived of their integrin-mediated interaction with the extracellular matrix can be blocked by expression of oncogenic v-H-Ras (Frisch and Francis, 1994). In hematopoietic cells, which depend on their interaction with support cells for survival, overexpression of oncogenic H-Ras blocks PCD, while expression of dominant negative H-Ras enhances PCD (Kinoshita et al., 1997). This Ras-mediated block in PCD occurs, at least in part, via the Raf/MAPK pathway (Kinoshita et al., 1997). Adhesion-mediated PCD in cultured intestinal epithelial cells can be blocked by expression of Bcl-2 or the oncogenic c-H-ras (Rak et al., 1995). These experiments suggest that regulation of the ras signaling pathway can regulate PCD. However, the mechanism by which some cells respond to death-promoting signals while others do not is still unclear.

One important source of ras activation is the receptor tyrosine kinase (RTK) class of signaling receptors. RTKs have been implicated in a variety of local cell signaling events, and also play a role in PCD. For example, the survival of many cell types in the developing nervous system require the support of trophic factors such as nerve growth factor (NGF), which acts through a balance of p75 and the TrkA RTK to determine survival (reviewed by Levi-Montalcini, 1987; Snider, 1994). In the developing *Drosophila* retina, blocking the activity of the
epidermal growth factor receptor ortholog DER affects a variety of cell fate decisions, including ectopic loss of interommatidial cells (Freeman, 1994a, 1996).

The *Drosophila* retina represents a particularly accessible tissue to address issues of local cell-cell signaling. Correct pattern is achieved in the *Drosophila* retina in part through the temporal and spatial control of PCD. The mature retina is composed of an organized array of some 750 unit eyes, or ‘ommatidia’, each containing eight photoreceptor neurons, four cone cells, two primary pigment cells (1’s), and a hexagonal lattice composed of secondary/tertiary pigment cells (2/3’s) and sensory bristle organules (Fig. 1C). With the possible exception of the cells of the bristle organule, cell fates in the retina are not determined through lineage-based restriction but instead rely on local signals passed between cells (Zipursky and Rubin, 1994). These signals result in progressive recruitment of undifferentiated cells by their previously differentiated neighbors.

Creation of the interommatidial lattice of 2/3’s is the result of the final cell fate decision in the retina: some cells are recruited as 2/3’s while remaining excess cells are removed by PCD. Two different cell types have been proposed to be the major regulators of cell death in the retina: 1’s and cells of the bristle organule. 1’s were implicated as a potential regulator of PCD by experiments examining Notch loss-of-function alleles: reduction of Notch function led to loss of both 1’s and PCD, leading to the suggestion that 1’s direct PCD (Cagan and Ready, 1989a). Alternatively, bristles have been proposed as regulators of PCD in the retina due to clustering of apoptotic cells (detected by acridine orange staining) around bristle organules (Wolff and Ready, 1991). More recent experiments indicate that cell death can occur in the absence of bristles (Cadigan and Nusse, 1996; see Discussion), although their presence may still influence PCD.

Removing a gene by mutation has proved to be a useful method for assessing its role in directing differentiation, although mutant cells generally retain most of their potential repertoire of signals. The most complete test of a cell’s contribution to local signaling is to remove it by laser ablation, a technique which has been used in a variety of organisms to eliminate specific cells during early development (Sulston and White, 1980; Taghert et al., 1984; Montell et al., 1991). The developing *Drosophila* retina is an ideal candidate for ablation studies: its morphology has been described with single cell resolution; it contains a variety of powerful genetic and molecular tools; and many of the molecules required for its development are known (reviewed by Krämer and Cagan, 1994; Bonini and Choi, 1995; Kumar and Moses, 1997). Unfortunately, as with most neuroepithelia in the developing fly, the larval retina is optically shielded by more superficial cells and by cuticle (Girton and Berns, 1982).

In this paper, we identify a stage just prior to PCD in the pupal retina which is fully accessible to laser ablation. Using this technique, evidence is provided that the cone cells and 1’s provide a signal that promotes survival of cells in the interommatidial lattice. Further evidence is provided that this signal represents part of a balance between signals of the ras and Notch pathways, which appear to act in opposition to regulate the number of interommatidial cells permitted to remain.

**MATERIALS AND METHODS**

**Cell ablations**

Individual retinal cells in living *Drosophila* pupae were ablated with a nitrogen pulsed dye laser (Laser Science, Inc.). Staged pupae were immobilized on a piece of tape and the head portion of the pupal case was removed. Pupae were then mounted on their side in an ellipsoid hole cut out of a 1 mm thick bed of 2% agarose in PBS and coverslipped with oxygen-permeable halocarbon oil.

The laser beam was directed through the 100x objective of a Zeiss Axiosplan microscope with Nomarski optics. Beam intensity was modulated via neutral density filters, and impulse frequency was adjusted to deliver approximately 200 blasts over the course of 10-15 seconds. Ablations were judged successful if condensation of nuclear material was observed in the targeted cell. During laser microsurgery, cells of the eye were viewed through a low-light camera and video monitor.

The effects of cell ablation were assessed by cobalt sulfide histological staining of pupal eye discs 24 hours after ablation (Melamed and Trujillo-Cenoz, 1975). Most tracings were done using a video image directly from the microscope to allow refocusing and confirmation of cell positions. Since laser microsurgery was performed on only one eye of each pupa, examination of the non-ablated eye served as an internal control for each procedure. No alteration in the time course of retinal development was observed between ablation-treated eyes and non-ablated eyes.

**Ablation of primary pigment cells**

To assess the replacement of primary pigment cells following ablation, nascent 1’s were ablated prior to 24 hours after puparium formation (APF). A single cone cell within an ommatidium in a nearby region was also ablated to serve as a landmark for identifying ommatidia with ablated 1’s. Pupae were allowed to develop for an additional 24 hours before the eye discs were removed for cobalt sulfide staining.

**Cone cell ablations**

Ablation of all four cone cells led to ectopic death of interommatidial precursor cells (IPCs) in the region of the ablation. In order to quantitate PCD, a line was drawn bisecting the ten ommatidia surrounding the four that were ablated, i.e. the ‘ablation zone’. The number of interommatidial cells in this ‘ablation zone’ was compared between control eyes and eyes where cone cells were ablated prior to 22h APF in various genetic backgrounds. Cells bisected by the line were always counted.

**Photoreceptor ablations**

Ablation of all eight photoreceptors (R1-8) would result in collapse of the ommatidium. Instead, we ablated two to three R cells in each of four ommatidia facing an interommatidial region (n=3) to create a small patch of IPCs devoid of their normal neighboring R cells. Within the ommatidium at the anterior edge of this space we ablated R4/S/6; at the equatorial (toward the retina midline) edge, R3/4; at the posterior edge, R1/2/3; at the polar (away from the midline) edge, R1/6/7.

**Fly strains**

LX37 is an ‘enhancer trap’ line in which P element-mediated genomic insertion of a lacZ reporter gene results in expression in IPCs and later in 2/3’s; expression appears to be strongest in the condensed nuclei of cells undergoing apoptosis (D. T. M. and R. L. C., unpublished data). LX37/CyO flies contain a normal retina, and PCD occurs on a normal time course. The identity of apoptotic cells was confirmed by their position just above or below the plane of the epithelium and their condensed chromatin within refractive nuclei, all hallmarks of retinal apoptosis. Ablations on LX37 pupae were performed between 18 and 22 hours APF, and the eyes of these pupae were dissected at 28-29
hours APF, the time when PCD is peaking in the retina. Ablation did
not provoke premature PCD: cobalt sulfide staining at 26 hours APF
– prior to the normal onset of PCD – revealed that IPCs remained in
the ablated patch up to the time of PCD (not shown).

The N\textsuperscript{fa-g\textsubscript{2}} allele, also known as N\textsuperscript{fa-g\textsubscript{2}}, is a member of an allelic
series of eye-specific Notch mutations which affect pigment cell
development. Shellenbarger and Mohler (1975) demonstrated that
N\textsuperscript{fa-g\textsubscript{2}} affects Notch function only in the pupal eye. Phenotypically,
N\textsuperscript{fa-g\textsubscript{2}} is indistinguishable from N\textsuperscript{fa-g\textsubscript{3}} (Lindsley and Zimm, 1992;
unpublished results). N\textsuperscript{fa-g\textsubscript{2}} and N\textsuperscript{fa-g\textsubscript{3}} flies were obtained from the
Bloomington Drosophila Stock Center.

Dras\textsuperscript{val12} is described by Fortini et al. (1992). Death of IPCs was
blocked in hs-Dras\textsuperscript{val12}/CyO flies by a 1-hour heat shock at 37°C
starting at 26 hours APF. \(\lambda\)-DER is a fusion of the dimerization
domain of the lambda repressor to the intracellular domain of DER;
the result is spontaneous activation of DER (Queenan et al., 1997).
Overexpression of \(\lambda\)-DER was accomplished with the Gal4-UAS
system by growing UAS-\(\lambda\)-DER flies at 18°C for 48 hours
(approximating 29 hours APF at 25°C), expressing Gal4 on a heat-
shock promoter for 1 hour at 37°C to activate \(\lambda\)-DER expression, and
then staining the tissue at 80 hours APF (approximating 40 hours APF
at 25°C) with cobalt sulfide to assess PCD.

**Immunohistochemistry**

Immunostaining for DER and Spitz was performed at 28 hours APF
with a guinea pig polyclonal antibody to DER (1:150) and a rat
polyclonal antibody to Spitz (1:20; gifts from B. Shilo). Detection of
Spitz was enhanced with the biotinyl-Tyramide kit (Dupont-NEN).

**RESULTS**

**Retinal development**

The first cell types to emerge in the developing retina are the
photoreceptor neurons and (non-neuronal) cone cells, which arise
within the retinal neuroepithelium of the mature larva. The larva then
undergoes pupation as the retina evaginates (‘disc eversion’) and is
repositioned to lie distally against the pupa’s cuticle. This feature has allowed us to directly visualize
and ablate cells within the live pupa. Soon after disc eversion,
the 1°s emerge to enwrap the cone cells (22-24 hours APF).
They establish direct contact with the remaining undifferentiated
cells which lie between ommatidia, which this paper will refer to as interommatidial precursor cells or IPCs
(Fig. 1A,B). Finally, a hexagonal lattice is formed between
ommatidia as IPCs are directed into one of two fates: 2°/3° or
PCD. The result is a precise hexagonal array of ommatidia,
each surrounded by nine 2°/3°s and three bristles (Fig. 1C).

Each cell type in the developing retina can be recognized by
the position of its nucleus. Typically, nuclei are first found in
the basal part of the neuroepithelium and rise apically as a cell
begins its differentiation. During early pupal stages, cone cell
nuclei are arranged as an apical ‘cloverleaf’ at the center of
each ommatidium and several microns above the photoreceptor
nuclei; two 1° nuclei form an apical ring around the cone cells;
and the IPC nuclei are found basally between ommatidia (these
nuclei are slow to rise apically except bristle nuclei, which are
found at an intermediate level early in their differentiation).
This stereotyped arrangement permitted identification and
ablation of each cell type.

Once the ablation was performed, pupae were permitted to
develop for an additional 24 hours to allow for establishment
of all cell types; retinas were then removed and stained with
cobalt sulfide to highlight each cell type at the surface. In each
experiment, the non-ablated partner was used as an internal
control. The effects of ablation were limited to the target cell,
with little apparent collateral damage to neighboring cells as
assessed by their normal subsequent development.

**Ablated 1°s are replaced**

Disc eversion is complete by 18 hours APF at 25°C; the first
indication of 1° differentiation is the apical migration of its
nucleus at 22-24 hours APF. In initial studies, laser ablation of
a 1° at this stage resulted in its rapid replacement (Cagan and
Ready, 1989a).

Previous work demonstrated that loss of pupal Notch activity
blocks both 1° differentiation and PCD, leading to the
suggestion that 1°s promote PCD (Cagan and Ready, 1989a).
However, it is not clear whether loss of 1°s leads necessarily to a block in PCD or whether Notch affects each process independently. Cell ablation provided a more direct assessment of the role of 1°s on PCD. To assess whether 1°s promote PCD they were ablated 24-27 hours APF, a stage after the point in their development when replacement can occur. Removal of a single 1° resulted in misplaced and, surprisingly, missing 2°/3°s in the vicinity of the ablated 1°s, resulting in fewer 2°/3°s surrounding the ommatidium. Bars, 5 μm.

Cone cells induce the 1° fate and regulate PCD

One potential strategy for early removal of 1°s was to determine which cell type is required to direct the 1° fate. Morphology and genetic manipulations have indicated that cone cells, which directly abut developing 1°s, are the most likely source of such an inductive cue (Cagan and Ready, 1989a,b). Ablation of the four cone cells from a single ommatidium at 18-22 hours APF prevented recruitment of any 1°s to that ommatidium (Fig. 3A). Sparing just one of the cone cells, however, was sufficient to induce 1-2 1°s (n=7; Fig. 3B). No difference was found in the ability of any of the four cone cells to induce a 1°. Ablation of photoreceptor neurons had no effect on 1° differentiation (not shown). Thus, the presence of cone cells is necessary for the recruitment of 1°s. Importantly, these results indicated that removal of cone cells would provide a reliable and early means of removing 1°s.

Loss of cone cells and 1°s results in ectopic PCD

In order to more clearly examine the effects of signals from 1°s on PCD, all four cone cells were removed from four neighboring ommatidia within a ‘square’. Within this square are contained 48±3 (n=3 pupae) IPCs (the precursor cells that would normally differentiate as 2°/3°s or die; see Materials and Methods for method of counting). Ablation of the sixteen resident cone cells between 18-22 hours APF left most of these IPCs without neighboring cone cells or 1°s. Normally, approx. 25% of these cells would undergo PCD, leaving the 37 surviving cells to form the interweaving hexagonal lattice of 2°/3°s. Again, loss of 1°s led to an increase in PCD: approx. 65% of the potential 2°/3°s within the ablated region were eliminated as assessed 24 hours later with cobalt sulfide staining and with the interommatidial cell marker LX37 (see below). This massive loss of 2°/3°s was followed by a rearrangement which brought the apices of the surrounding, unablated ommatidia together to approximate a ‘normal’
The same ablation procedure was followed by expression of the adjacent to a cone cell and contained an apically-positioned nucleus. A cell was scored as a 1° if it was randomly selected control region of the same retina. Bars, 5 μm. The five bristle cells in the center (arrow) have converged at a point previously occupied by four complete ommatidia. Following ablation of sixteen cone cells from four ommatidia, IPCs were dying throughout the ablated patch (black arrow). The same ablation procedure, death of interommatidial cells was blocked in Notch1 flies. The dashed line (see A) outlines 51 2°/3° pigment cells. A cell was scored as a 1° if it was adjacent to a cone cell and contained an apically-positioned nucleus. The same ablation procedure was followed by expression of the gain-of-function Dras1val12 at 26 hours APF (just prior to the onset of PCD); hyperactivation of the ras pathway was sufficient to prevent death and rescue the 2°/3° fate in most or all IPCs. The dashed line contains 58 cells as compared to 60 cells within an analogous randomly selected control region of the same retina. Bars, 5 μm.

Fig. 4. Ablation of cone cells in four adjacent ommatidia at 20 hours APF. Tracings are to the right (A) or below (C,D) cobalt sulfide stained tissue. (A) Removal of 16 cone cells (and by consequence their associated 1°s) from four wild-type ommatidia at 20 hours APF results in extensive loss of 2°/3°s. A dashed line bisects the 10 ommatidia which had surrounded the four ablated ones. Normally, this region would contain 37 2°/3°s when viewed after the completion of PCD; now it contains only 16 cells. Note that these remaining cells are tightly associated with 1°s from the 10 unablated ommatidia. The five bristle cells in the center (arrow) have converged at a point previously occupied by four complete ommatidia. (B) Matching the ablation procedure from (A), an LX37 enhancer trap line was stained for β-galactosidase activity to highlight 2°/3° photoreceptor neurons, cone cells, or 1°s are necessary for Photoreceptor core of each ablated ommatidium remained beneath the surface.

This result is consistent with the model that 1°s do not promote cell death within the interommatidial lattice of IPCs but instead repress it. However, our ablation protocol does leave ommatidia with cone cells and 1°s at the periphery of the ablated patch, and it is also possible that 2°/3° precursor cells are signaled stepwise to die by these 1°s. If this were the case, then PCD should first occur next to these 1°s at the periphery of the ablated patch, with the pattern of PCD then radiating inward toward the center as these perimeter ommatidia are brought together. Importantly, 1°s should provide only local signaling, since long-range signals would interfere with lattice formation in distant ommatidia; consistent with this view, ablation of a 1° affected only cells in the adjacent interommatidial lattice.

To determine the pattern of PCD, all cone cells from four ommatidia were ablated at 18-22 hours APF as described above. Cell death was then examined at 28-29 hours APF, the developmental stage at which PCD is maximal. To visualize IPCs, a fly line containing the enhancer trap marker LX37 was used, which stains the nuclei of all IPCs but is expressed especially strongly in apoptotic nuclei (Fig. 4B). After ablation, dying cells were observed throughout the ablated region but primarily at the center. They separated from the epithelium to lie both above and below it in clusters of 5-10 apoptotic cells (Fig. 4B). Their identity as apoptotic cells was further indicated by their condensed chromatin within refractive nuclei, hallmarks of retinal apoptosis. Thus, death can occur many cell diameters from the nearest cone cells and 1°s, indicating that 1°s are required not to direct PCD but to rescue IPCs into the 2°/3° fate and organize them into a proper interommatidial lattice.

Finally, although the photoreceptor neurons do not maintain an apical presence in the young pupa, they do contact the 2°/3°s more basally and could affect their organization into a proper lattice. However, ablation of a series of photoreceptors facing a defined lattice region (see Materials and Methods) had little effect on the number of 2°/3°s. Some displacement of local cells was observed due to changes in ommatidial size (not shown); in addition, cone cell clusters were often misrotated indicating their position within the ommatidium is held by particular photoreceptor neurons. Thus, neither the photoreceptor neurons, cone cells, or 1°s are necessary for initiating interommatidial PCD. By the process of elimination, therefore, the signals necessary for PCD arise within the interommatidial lattice itself. These signals are mitigated by the 1°s and perhaps the cone cells.

Notch directs PCD in the interommatidial lattice

To provide further evidence for signaling within the interommatidial lattice and to assess the nature of these signals, we examined the role of two signaling pathways in our ablation paradigm. Previous work has implicated the Notch signaling pathway in all cell fate decisions in the developing retina including patterning of the 2°/3° lattice. Notch encodes a large transmembrane signaling receptor which is expressed exclusively in IPCs during the stage of PCD (Koooh et al., 1993). Alleles that reduce Notch activity in the young pupa both prevent 1° differentiation and block PCD (Cagan and Ready, 1989a), which led to the suggestion that 1°s promote...
death in neighboring IPCs. However, cells in the position of 1’s were observed to demonstrate some differentiation (Cagan and Ready, 1989a). In addition, our evidence indicates that 1’s actually promote the 2°/3° fate at the expense of PCD. Either the Notch-mediated block in PCD is due to partial differentiation of 1’s which can then block PCD, or Notch is in fact required to promote PCD and its role in 1° differentiation is separable. To determine whether Notch has a direct role in PCD, we reduced its function in the absence of 1’s.

The Nfα-s2 mutation is one of a series of alleles that remove Notch activity specifically in pigment cells, resulting in loss of both 1° differentiation and PCD (Shellenbarger and Mohler, 1975; Cagan and Ready, 1989a; unpublished results). Sixteen cone cells were ablated prior to 22 hours APF in four ommatidia as described above to create a ‘zone’ that lacked cone cells and 1’s. The number of 2°/3’s which subsequently differentiated was then compared between the zone receiving ablation and unablated Nfα-s2 controls. Ablated Nfα-s2 retinæ retained 51±3 2°/3’s (n=5; Fig. 4C), a number similar to those retained in unablated controls (56±8; n=5). The ability of Notch mutations to block PCD in the absence of 1’s, and Notch’s exclusive expression within the IPCs during the 2°/3° versus PCD decision, indicates that it acts directly within the interommatidial lattice to promote PCD.

**Ras signaling promotes the 2°/3° fate at the expense of PCD**

Our experiments indicate that Notch signaling promotes PCD in the interommatidial lattice. This suggests that other signals are required to balance this death signal by blocking death in a subset of maturing IPCs. One pathway which can act in opposition to Notch is the ras signal transduction pathway (Baker and Rubin, 1992; Miyamoto et al., 1995; Matsuo et al., 1997); ras is required for a variety of cell fate decisions in the developing retina (Wassarman et al., 1995). To test the role of ras signaling during PCD, flies were used in which an inducible heat shock promoter was fused to the activated Ras form Dras1Val12. A 1-hour pulse of Dras1Val12 throughout the retina beginning at 26 hours APF rescued IPCs from PCD. Early removal of cone cells and 1’s in four neighboring ommatidia had no effect on this rescue (Fig. 4D). This result indicates that ras signaling acts to prevent PCD and/or promote the 2°/3° fate. With regard to PCD, therefore, ras acts in opposition to Notch signaling.

This ras-mediated rescue of cells is similar to, and epistatic to, the rescue provided by cone cells and 1’s. Are the two signals linked?

**DER signaling promotes the 2°/3° fate at the expense of PCD**

The ras pathway has been demonstrated to be activated by a variety of extracellular stimuli, including signaling through receptor tyrosine kinases (RTKs). In the developing retina, the EGF receptor ortholog DER is an RTK that regulates a variety of cell fate determination steps including 2°/3° determination. Consistent with the results described above for activation of Dras1, loss of DER activity leads to a loss of 2°/3’s, presumably due to an excess of PCD (Freeman, 1996). To determine whether DER signaling is sufficient to block PCD, flies containing an activated form of DER (λ-DER) fused to an inducible heat shock promoter received a 1-hour heat shock. Expression of λ-DER throughout the young pupa partially blocks PCD, indicated by ectopic 2°/3’s in the mature pupa. Asterisks in the tracing to the right demonstrate five examples of pairs of 2’s in positions where normally only one is present. Cell types are represented by colors as defined in Fig. 1C. (B) DER expression was observed primarily in the 2°/3° cell lattice during the stage of maximal PCD (28 hours APF). (C) Spitz expression is complementary to DER at 28 hours APF, with expression highest in cone cells (large arrow) and bristle organules (small arrow) and lower levels in 1’s. (D) A model for the spatial regulation of PCD in the Drosophila pupal retina. IPCs help organize themselves by activating the Notch signaling pathway to promote PCD. If cone cells and 1’s are removed, this signal is sufficient to kill all IPCs. Cone cells and 1’s oppose this signal through localized secretion of Spitz, which activates DER/Ras signaling in IPCs and helps guide them into the 2°/3° fate. Bars, 5 μm.
Ablation permits removal of individual cells, an important condition for testing the role of specific cells in the patterning process. Using this method, we tested previous hypotheses regarding cell fate induction in the pupal retina. The importance of local cell-cell communication was further confirmed in these studies, but the specific nature of the cell-cell interactions was often unexpected.

Establishment of 1°s

Induction and patterning of the pigment cells appears to require a combination of signals. Previous work suggested that an ‘equivalence group’ of two to three 1° precursors interact to select a single 1°, and that this interaction requires Notch signaling (Cagan and Ready, 1989a,b). Early ablation of 1°s resulted in their replacement, providing further evidence for this view. Replacement appeared to occur at a similar position, as the newly made 1° was generally correctly positioned. Apparently, once a cell is established as a 1° it provides a signal to prevent neighboring cells from differentiating as 1°s as well, a mechanism which limits the number of 1°s. This form of ‘lateral inhibition’ has been observed in other fate decisions, both in the fly and elsewhere (Skeath and Carroll, 1992; Doe and Goodman, 1985; Sulston and White, 1980). In addition, removal of all four cone cells results in loss of both 1°s, indicating cone cells provide an additional signal required to select a single 1° from the adjoining equivalence group. Therefore, establishment of the 1° fate appears to require at least three steps: (i) establishment of an equivalence group; (ii) establishment of one cell as the 1°; (iii) repression of the 1° fate in other cells within the group which are then free to differentiate as 2°/3°s or die.

In a normal ommatidium, the two 1°s are positioned pairwise across the anterior/posterior axis, leading to the suggestion that the anterior and posterior cone cells play the leading role in determining the number and position of 1°s (Cagan and Ready, 1989b). However, ablation of any combination of cone cells yielded no distinction in the ability of any one cone cell type to induce the 1° fate. This surprising result suggests that either interactions between cone cells are required to determine which will induce 1°s, or 1°s determine their own position without regard to cone cells. One of the most interesting unresolved issues which has emerged from our ablation studies is the nature of the spatial cues that select the position of cells. This issue of patterning will be further discussed below with regard to the interommatidial lattice.

Development of 1°s requires at least signaling through the Notch and ras signaling pathways. Loss of Notch activity results in too many 1°s, consistent with a role for Notch in suppressing additional 1°s. The ligand responsible for this signal appears to be the transmembrane ligand Delta, which is expressed in all four cone cells (Parks et al., 1995). A reduction in DER activity decreases the number of 1°s (Freeman, 1996), while early overexpression of activated Dras1Val12 increases the number of 1°s (D. T. M. and R. L. C., unpublished results) supporting a role for the DER/Ras pathway in inducing 1°s. The observed early expression of the DER ligand Spitz in cone cells suggests it may play a role in this process. Therefore, the balance of Notch and DER signaling may help determine the 1° fate, although it is unclear how these signals could provide the spatial precision required to select specifically two 1°s to enwrap the cone cell quartet.

Assessing models for patterning and PCD

Our ablation evidence indicates that a balance of signals is also responsible for selecting between the 2°/3° and PCD fate. Ablations proved was particularly useful in exploring this issue, as several models have been proposed as to the source of the ‘life-versus-death’ signal. Several lines of evidence point to local signaling as an important regulator of PCD. Evidence from C. elegans has shown that PCD can be initiated by signals received from a neighboring cell: removal of the U.1p or U.rp cell is required for the linker cell in the male gonad to die, indicating these cells provide a killing signal (Sulston et al., 1980). In addition, an important regulatory step during T cell maturation involves signaling through Fas/Fas Ligand, which can remove cells through PCD by direct cell-cell contact (reviewed by Nagata, 1997).

Loss of Notch activity in the young pupa blocked both 1° specification and PCD, leading to the proposal that 1°s direct death in the neighboring IPCs (Cagan and Ready, 1989a) in a manner similar to the role of U.1p/U.rp. However, direct removal of 1°s resulted in the death of neighboring IPCs. This indicates that cone cells and 1°s actually provide a ‘life’- or 2°/3°-promoting signal. The role of Notch appears to be two-fold: (i) it is required for the differentiation of 1°s; and (ii) it promotes PCD in IPCs in a manner that is independent of its function in 1°s. Loss of Notch prevents PCD in a manner that can override any loss of signaling from neighboring cone cells or 1°s.

A second model has suggested that PCD in the interommatidial lattice requires extensive morphological rearrangements of the IPCs. The IrreC-rst locus encodes a member of the Immunoglobulin (Ig) superfamily which can mediate homophilic adhesion. Specific mutations in IrreC-rst lead to a failure of IPCs to reorganize as a single row between 1°s and a subsequent loss of PCD (Reiter et al., 1996). This has led to the suggestion that cells may first need to reorganize to receive the proper death cue (Reiter et al., 1996). However, when the cone cells and 1°s from four ommatidia were removed by ablation, widespread PCD was observed. The IPCs remaining in the ablated region could not rearrange to contact a 1°, indicating that 1° contact does not promote PCD. Instead, IrreC-rst may promote PCD in a manner independent of a cell’s contacts or position.

A third model has proposed that bristle organules may play a central role in promoting PCD. This is based on the observation that many apoptotic bodies are observed near the bristles (Wolff and Ready, 1991); bristles form within the interommatidial lattice in the midst of the IPCs. Our observation that Spitz is expressed at high levels in at least one bristle cell is consistent with this observation. However, bristles
are not absolutely required for PCD, as loss of bristles results in an otherwise normally patterned lattice (Cadigan and Nusse, 1996). Instead, we favor the idea that bristles use Spitz for their own proper positioning: each bristle group must move several cell diameters anteriorly from its point of birth to its final lattice position (Cagan and Ready, 1989b), and it may use Spitz to rescue IPCs located on its posterior side, effectively moving itself anteriorly. Bristle organules are originally composed of four cells arranged in a line, and it will be interesting to determine whether, for example, the posterior-most bristle cell (the socket-forming tormogen) expresses Spitz.

**Multiple signals pattern the interommatidial lattice**

Ablations permitted us to identify the cells and the nature of the signals that pattern the interommatidial lattice by regulating the decision between the 2°/3° fate and PCD. Removal of cone cells and 1°s resulted in massive ectopic death of IPCs. This PCD was blocked when Notch activity was reduced or when DER/Ras activity was increased. During the period of PCD, Notch is expressed throughout the IPC lattice (Kooh et al., 1993), whereas its ligand Delta is expressed in a subset of IPCs (Parks et al., 1995). DER is also expressed throughout the lattice, but its ligand Spitz is expressed in a complementary pattern in the cone cells, 1°s, and bristles (Figure 5C). Together these data suggest a simple model, in which the combination of Notch and DER signaling determine patterning.

Loss in activity of Notch (Cagan and Ready, 1989a; this paper) or its ligand Delta (Parks et al., 1995) prevents PCD. The Notch pathway is commonly used to provide local signaling for a variety of cell fates, and we propose that it is used in the lattice to direct PCD at the expense of the 2°/3° cell fate. This model would predict that without further signaling all IPCs would enter the death program, and our ablation studies confirm this prediction: removal of 1°s and cone cells result in massive apoptotic death. Therefore, 1°s (and perhaps cone cells) provide a signal which rescues some IPCs from the PCD decision.

The action of Notch appears to be opposed by DER. Loss of DER activity resulted in fewer 2°/3°s (Freeman, 1994a, 1996); activation of DER or Dras1 promoted cell survival even after removal of cone cells and 1°s. Together, these results support the idea that the DER/Ras pathway normally provides trophic support which is used to regulate PCD in the interommatidial lattice. This trophic support may be mediated by Spitz, a secreted DER ligand which can act across multiple cell diameters (Freeman, 1994b). In this view, the diffusion properties of Spitz regulate the balance between the 2°/3° fate and PCD. Our data support a more general model for the progressive recruitment of cells into the growing lattice through Spitz/DER signaling (Freeman, 1996).

One interesting question not addressed in this work is the nature of a signaling process which can create exactly nine 2°/3°s to enwrap the ommatidium. Two primary pigment cells form a circle around a quartet of cone cells; simple diffusion of Spitz from these cells cannot explain formation of a perfect hexagonal lattice in which each interommatidial cell is placed in its correct position (Fig. 1C). For example, by what mechanism is each 1° contacted by four 2°s and not three or five? Perhaps the answer lies in the identification of mechanisms that can refine these diffusible signals.

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**REFERENCES**


Maclas, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Rosen, M. A. and We thank Jim McCarter and Tim Schell for assistance with laser ablations; N. Perritom for hs- Dras1 flies; B. Shilo for antibodies to DER and Spitz; T. Schupbach for lambda-DER flies; J. Gordon, P. Taghert, I. Boime, R. Kopan, D. Ornitz, and E. M. Johnson for helpful comments on the manuscript. D. T. M. was supported by Pharmacology Training Grant T32GM07805. This work was supported by NIH R01-EY10717. --


