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Identification of the death zone: a spatially restricted region for programmed cell death that sculpts the fly eye

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Programmed cell death (PCD) sculpts many developing tissues. The final patterning step of the _Drosophila_ retina is the elimination, through PCD, of a subset of interommatidial lattice cells during pupation. It is not understood how this process is spatially regulated to ensure that cells die in the proper positions. To address this, we observed PCD of lattice cells in the pupal retina in real time. This live-visualization method demonstrates that lattice cell apoptosis is a highly specific process. In all, 85% of lattice cells die in exclusive 'death zone' positions between adjacent ommatidia. In contrast, cells that make specific contacts with primary pigment cells are protected from death. Two signaling pathways, _Drosophila_ epidermal growth factor receptor (dEgfr) and Notch, that are thought to be central to the regulation of lattice cell survival and death, are not sufficient to establish the death zone. Thus, application of live visualization to the fly eye gives new insight into a dynamic developmental process.
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The development of many tissues in metazoan organisms involves an overproliferation of cells balanced with programmed cell death (PCD) to attain the final size. PCD is also an important mechanism for patterning a number of complex tissues by removing cells in specific regions.[1–6]

The _Drosophila_ eye is sculpted by PCD and represents an ideal system to dissect the mechanism of developmentally regulated PCD.[7,8] Studies on the fruit fly circumvent some of the difficulties of studying developing organs in mammals, and benefit from the powerful genetics of this model organism coupled with the fact that the cell death pathway is evolutionarily conserved. The ~750 ommatidia of the fly eye are arranged in a stereotypical hexagonal pattern that is determined in late larval life and refined during mid-pupal development through selective PCD. Prior to the onset of PCD, an excess number of cells lie between ommatidial units; a subset of these seemingly identical cells is removed through cell death, leaving just the right number of cells to solidify the perfect hexagonal lattice of the eye.[9,10]

It is not known how PCD is regulated to ensure that the proper number of cells die.[7] In contrast to PCD in _Caenorhabditis elegans_, lattice cell death is not specified by lineage; therefore, cell death in the developing _Drosophila_ retina must be directed by positional information through cell–cell communication,[11,12] as is also the case in mammalian development. Several laboratories have demonstrated that signaling from the _Drosophila_ epidermal growth factor receptor (dEgfr/ DER/Torpedo/Flb)/ras pathway promotes lattice cell life,[13–16] in part through negative regulation of the cell death effector

head involution defective (HID).[17,18] During the period of lattice cell death, dEgfr is expressed by all lattice cells, and its soluble ligand, Spitz (Spi), is expressed by cone cells and primary pigment cells.[13] Counteracting the dEgfr/ras pathway is the Notch signaling pathway that directs lattice cell death.[13,19] Notch is highly expressed in lattice cells. An epistasis experiment demonstrated that Notch acts to promote apoptosis by inhibiting life signaling through dEgfr.[20]

In sum, the current belief is that all lattice cells receive life signals through the dEgfr/ras pathway with counteracting death signals through Notch.[7,20] How can these overlapping signals be resolved so that some of the cells die and some live? There are two likely scenarios: (1) life or death signals are propagated directionally, killing cells in specific regions until no cells remain to populate that region and (2) cells die randomly until the right number remain and the death signal is turned off. The distinguishing feature between these two models is the position of the dying lattice cell; scenario (1) is likely to have PCD occurring in stereotypical death regions, whereas in scenario (2) dying cells should be randomly placed. Previous studies using electron microscopy were unable to determine whether certain cell contacts promote life or death among lattice cells.[9,10]

To definitively address where a cell resides when it is instructed to die, we developed a new method to watch the apoptotic process occur in the living pupal eye. Our live-visualization experiments demonstrate that doomed lattice cells are not chosen randomly with respect to their spatial position. Rather, lattice cell PCD occurs in distinct 'death

zones'. Mechanosensory bristle groups influence the position of the death zone, but are not required for the establishment of a death zone. This novel method sets the stage for detailed studies of the dynamic signaling interplay between cells that results in spatially restricted PCD.

Results

Molecular apoptotic markers cannot distinguish the position of dying cells in the *Drosophila* retina. Specification of the 14 cells of each ommatidial unit (eight photoreceptor neurons, two primary pigment cells and four cone cells), as well as the mechanosensory bristle groups, begins during larval life and continues through early pupal development. By 18 h after pupariation formation (APF), the unique morphology of the primary pigment cells, cone cells and bristle groups is evident (photoreceptors lie below the plane of the image, Figure 1a). The remaining cells will form the interommatidial lattice of secondary pigment cells (touching two primary pigment cells) and tertiary pigment cells (touching three primary pigment cells), collectively termed 'lattice cells', that separate neighboring ommatidia and set the precise hexagonal pattern of the adult eye. Over the next few hours of pupal development, the lattice cells and bristle groups reorganize in an ordered fashion, bringing the ommatidia into a tighter configuration in which each is separated by a single layer of lattice cells in an end-to-end chain (Figure 1b and c). During this time, a few cells per ommatidium are removed through PCD.[21] By 24 h APF, the bristle groups have moved to occupy alternating vertices of the lattice (Figure 1c). Excess lattice cells are removed through PCD leaving just one lattice cell to form a secondary pigment cell stretched between tertiary pigment cells and bristle groups (Figure 1d).[7,9,10]

Our studies into spatial regulation of lattice cell death began by investigating whether molecular apoptotic markers identify dying cells early enough in the process to allow us to view their positions. Although both terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) and an antibody that recognizes a *Drosophila* activated caspase (anti-cleaved caspase 3 (CC3))[22] label dying lattice cells, neither could resolve the 'starting' position of the apoptotic cells because by the time these methods identified dying cells, they had lost their apical contacts and shrunk basally (Figure 1e and f; see also Supplementary Figure 1).

Live visualization of the developing pupal retina unambiguously identifies dying cells. Ultrastructural analyses of the pupal eye demonstrated previously that a dying lattice cell loses its apical foothold before undergoing other structural changes.[9,10] Therefore, we needed to develop a method to identify dying cells before or at this point to definitively position the doomed cell before it dies. We took advantage of the fact that once the eye disc has everted in early pupal life, it is accessible to microscopic evaluation. Expression of a membrane-localized green fluorescent protein (GFP) molecule allowed visualization of the cells in the living pupal eye. Through time-lapse imaging, capturing a Z-series at each time point, the live-visualization
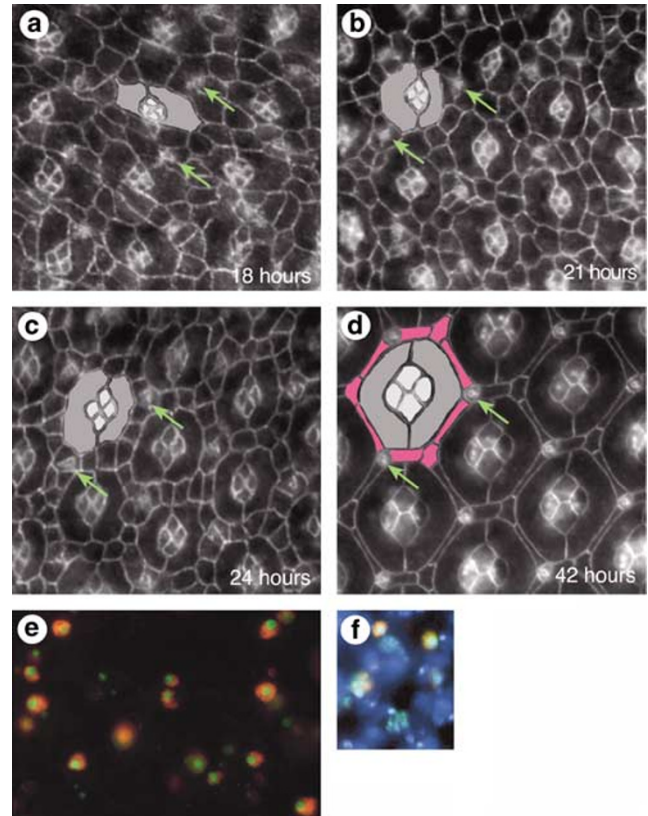

Figure 1 Apoptosis shapes the pupal eye. (**a–d**) Whole-mount pupal retinae dissected at developmental times are shown, immunostained with anti-armadillo. For clarity, an ommatidium is shaded in each panel. The primary pigment cells are dark gray, cone cells are light gray and examples of bristle groups are indicated by green arrows. Interommatidial lattice cells account for the remainder of cells. (**a**) 18 h APF, (**b**) 21 h APF, (**c**) 24 h APF, (**d**) 42 h APF. 1°, primary pigment cells; cc, cone cells; 2°, secondary pigment cells; 3°, tertiary pigment cells. Collectively, the 2° and 3° cells are referred to as the interommatidial lattice (pink). (**e**, **f**). Lattice cell death is apoptotic, but dying cells cannot be positioned using molecular markers. (**e**) TUNEL labeling of apoptotic nuclei in green and CC3 immunostaining in red of a 26 h APF pupal retina demonstrating that dying cells are small bodies that are TUNEL positive and CC3 reactive. (**f**) Higher magnification view of another disc demonstrating that shrunken, dying cells lie in the lattice cell nuclear layer and that fragmented nuclei are still TUNEL positive but may no longer be CC3 reactive (DAPI, blue; TUNEL, green; CC3, red). Anterior is to the left

method unmistakably discerned dying cells as they lose their apical footprint and begin to shrink below the surface. An example of a series of time-lapse images is shown in Figure 2a (discussed below). Thus, the live-visualization method fulfills the requirement of identifying dying cells at the first structural indication of death.

Cells die in very specific positions within the interommatidial lattice – the 'death zone'. We carefully analyzed a visual field from three pupal retinae and tabulated the location of each apoptotic cell with respect to the neighboring ommatidia (roughly 130 apoptotic events per retina). The results are presented in a graph that depicts the mean of the three experiments (Figure 2b). The time course shown in Figure 2a is a randomly chosen region from one of these retinae (the schematic defines horizontal and oblique regions as they are used throughout this study). Remarkably,
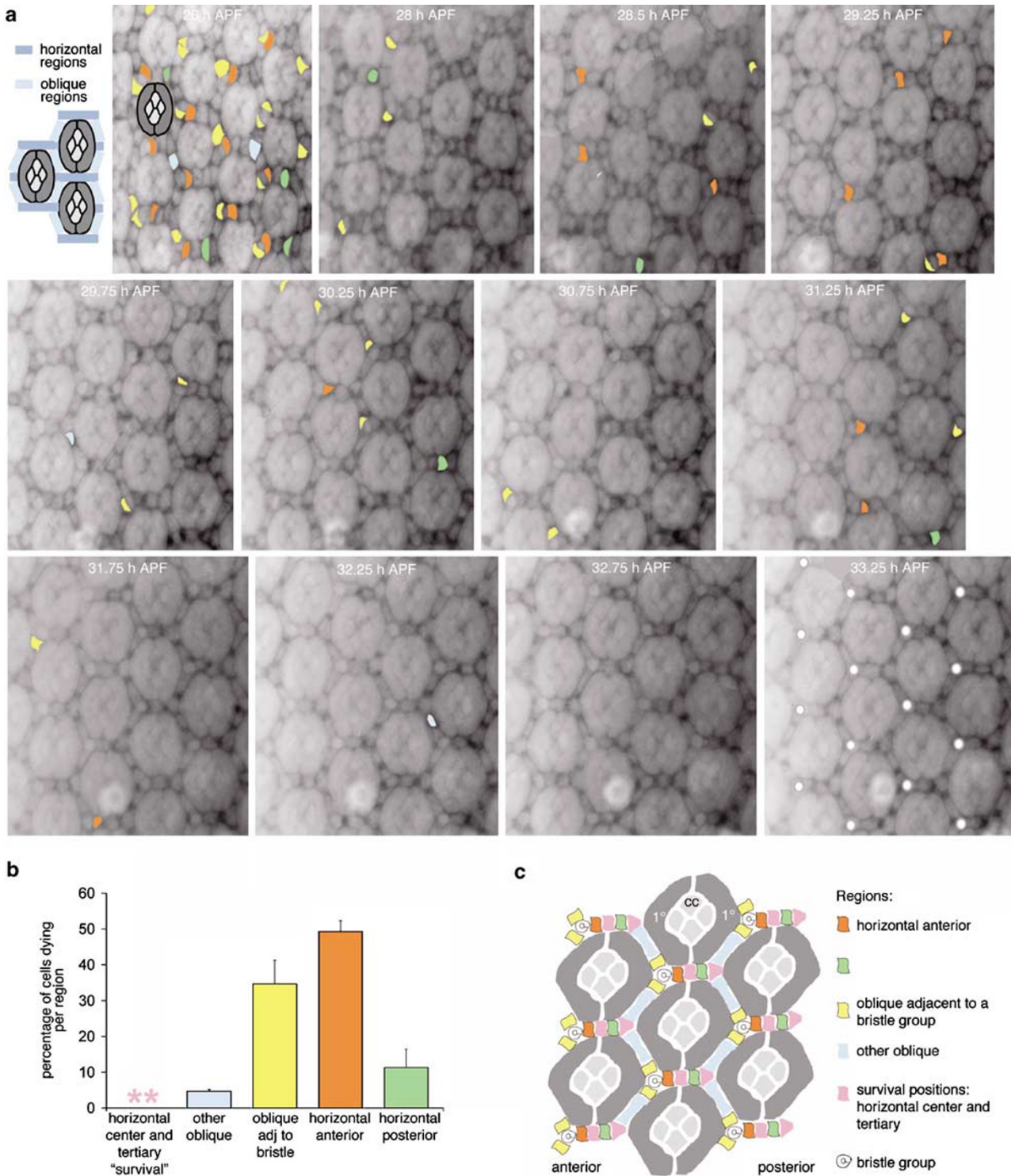
Figure 2 Lattice cell apoptosis is spatially regulated. Time-lapse images of the developing pupal retina, beginning at 26 h APF. **(a)** The image is inverted so that the GFP-labeled membranes are black. The increase in size of the imaged region is owing to pupal development. Dying cells are highlighted on the image just prior to their demise. Cells that die in the horizontal anterior region are in *orange*. Cells that die in oblique regions adjacent to bristle groups are *yellow* and the few that die in the horizontal posterior and other oblique regions are *green* and *light blue,* respectively. On the first image, all cells that will die over the time course have been identified in the appropriate color. The bristle groups are apparent from the earliest time point at positions close to their final positions and are identified by white circles on the final image. An ommatidial unit has been drawn in for orientation. **(b)** Percentage of cells observed dying graphed relative to specific regions. Colors correlate to those used in (**a**) and (**c**). Pink asterisks highlight that no cells died in these regions. **(c)** Schematic of the pupal retina, colors correlate with (**a**) and (**b**). Each colored rectangle corresponds to a region in which cells will either be more likely to live (pink) or die (orange and yellow). Note that cells that die adjacent to a bristle group may die above or below the bristle group as drawn. Live imaging of the pupal eye had no effect on the viability of the pupa nor did it lead to any visible morphological change in the adult eye. Anterior is to the left

roughly one-half of all apoptotic cells occupy a horizontal anterior position as they begin to undergo death (orange in Figure 2a–c). Another large percentage of dying cells (35%) occupies an oblique position adjacent to a bristle group (yellow in Figure 2a–c). In contrast, dying cells in the other oblique regions (light blue) or horizontal posterior position (green) account for only 5% and 11% of apoptotic cells, respectively (Figure 2a–c). Previous electron microscopy studies had noted that many dying cells were located near bristle groups;[9,10] our results confirm this and solidify the observation by quantifying it. The two regions, horizontal anterior and oblique adjacent to a bristle group, are therefore defined as the 'death zone' where 85% of apoptotic lattice cells will be located.

The time-lapse images reveal that dying cells are replaced by adjacent lattice cells that will, in turn, die should too many cells remain in the region. Additionally, these experiments identify survival positions. No cells die in the tertiary position or in the horizontal face if contacting all four primary pigment cells (survival positions are pink in Figure 2c, see also Supplementary Figure 2). By the end of the period of PCD, these horizontal center cells will stretch to occupy the horizontal face between the tertiary cell and the bristle group. Thus, the live-visualization method is the first to unambiguously demonstrate that PCD in the pupal eye is nonrandom.

Bristle groups influence the placement of the death zone. Because apoptotic cells were often found next to the bristle group, we next investigated whether lack of bristle groups would alter the pattern of lattice cell apoptosis. Flies ectopically expressing *wingless* in the eye (P[*sev-wg*] flies) have normal eyes except that mechanosensory bristle groups are replaced with tertiary cells[23] (Figure 3a). We confirmed the absence of bristle groups (Figure 3b and c – compare with Figure 3d). Because ectopic Wg protein in the pupal eye might influence lattice cell apoptosis, we investigated whether the *sev* promoter drives expression of *wg* in the mid-pupal P[*sev-wg*] eye. No Wg protein expression was observed in P[*sev-wg*] or control wild-type retinae at 24 or 28 h APF (data not shown).

Live imaging was employed to investigate whether removal of bristle groups influenced the placement of the death zone. The lack of bristle groups had a dramatic effect on the pattern of dying cells in the pupal retina (Figure 3f and g). The vast majority of dying cells (80–90%) occupy a position either posterior (green) or anterior (orange) in the horizontal face between ommatidia. In contrast to eyes with bristle groups, few cells die in oblique positions (light blue). Thus it is clear that, without bristle groups, the death zone moves to encompass both anterior and posterior regions of the horizontal face, straddling the horizontal center 'survival' position, and is no longer associated with oblique positions. This result establishes that the bristle group, although not required for patterning of the eye through apoptosis, influences the spatial placement of the death zone.

Signaling through the *Drosophila* Egfr and Notch receptors is active in all lattice cells. Loss of Notch signaling leads to ectopic life of lattice cells.[13,19] Conversely, a block in dEgfr signaling leads to ectopic death of lattice
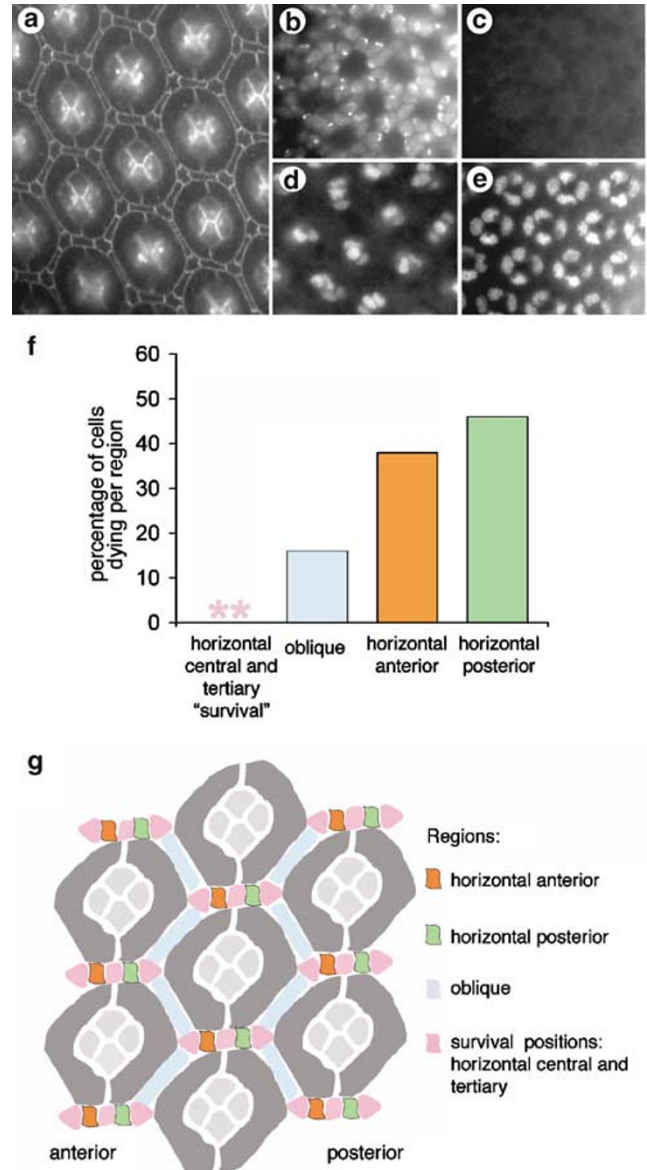

Figure 3 Spatial regulation of lattice cell PCD is altered in the absence of bristle groups. Characterization of the P[*sev-wg*] retina. **(a)** Anti-armadillo immunostain of a 42 h APF P[*sev-wg*] retina showing no patterning defects and a replacement of all bristle groups with tertiary cells. **(b, c)** DAPI staining of 26 h APF P[*sev-wg*] eye disc identifying lattice cell nuclei **(b)** and lack of immunostaining with an antibody specific for cut **(c)**. All four bristle nuclei of a wild-type 26 h APF pupal eye disc are identified by anti-cut **(d)**. **(e)** Cone cell nuclei are cut-positive in P[*sev-wg*] retina from **(b)** and **(c)**, as an internal control for the antibody used. **(f)** Percentage of cells observed dying graphed relative to specific regions in P[*sev-wg*] retinae demonstrating that ∼85% of cell death occurs in two specific regions, but in contrast with wild type, these two regions are now both in the horizontal face. Colors correlate with those in Figure 2 and in **(g)**. Pink asterisks highlight that no cells died in these regions. Shown is the average of two experiments. **(g)** Schematic of the pupal P[*sev-wg*] retina. Colors correlate with **(f)**. Anterior is to the left

cells, whereas hyperactivation of dEgfr signaling rescues all lattice cells from death.[13–16] As we determined that apoptosis of lattice cells is spatially restricted to the death zone, we hypothesized that we could visualize changes in Notch or dEgfr signaling in lattice cells that are destined to die. Specifically, we expected to see a reduction in dEgfr

signaling or an induction of Notch signaling in cells in the death zone regions: horizontal anterior or oblique adjacent to bristle groups. We chose the Notch target gene, enhancer of split (E(spl)), as a marker for active Notch signaling[24] and phosphorylated mitogen-activated protein kinase (P-MAPK) as an indicator of active dEgfr signaling. To show specificity for the anti-E(spl) and anti-P-MAPK antibodies used, we ectopically expressed dominant-negative (DN) dEgfr

Figure 4 Lattice cells transduce Notch and dEgfr signals. (**a–c**) Notch signaling as determined by E(spl) immunostaining. (**a**) E(spl) immunostaining of lattice cell nuclei. (**b**) DAPI of the same region as in (**a**). (**c**) Close-up view of a different region demonstrating that dying nuclei are not E(spl) positive (arrows); nuclei in green, E(spl) in red. Thus, E(spl) may be a caspase cleavage substrate. Healthy nuclei that are E(spl) negative are bristle group nuclei. (**d–i**) dEgfr signaling as determined by an antibody specific for P-MAPK (anti-dpERK). (**d**) Lattice cells contain cytoplasmic P-MAPK. (**e**) DAPI of the same region as in (**d**). (**f**) Overlay of (**d**) and (**e**); nuclei in green, P-MAPK in red. The bristle group does not maintain cytoplasmic P-MAPK (white arrowheads mark a few bristle groups). (**g**) P-MAPK immunostaining demonstrating that lattice cell nuclei are negative and that at least two of the bristle group nuclei contain P-MAPK (the bristle nuclei are above the plane of the lattice cell nuclei and thus, slightly out of the focal plane). (**h**) DAPI of the same region as in (**g**). (**i**) Overlay of (**g**) and (**h**); nuclei in green, P-MAPK in red. Ommatidial schematics are included in (**a**), (**d**) and (**g**) to orient the reader. Anterior is to the left. (**j**, **k**) Reduction in N signaling by ectopic expression of a dominant-negative Notch, N[ECN] substantially reduces E(spl) reactivity. (**j**) E(spl) immunostaining of *hs-N[ECN]* retina, not subjected to heat shock. (**k**) E(spl) immunostaining of *hs-N[ECN]* retina 1 h after heat shock. (**l**, **m**) Reduction in dEgfr signaling by ectopic expression of a DN dEgfr greatly reduces antibody reactivity for P-MAPK. (**l**) P-MAPK immunostaining (anti-dpERK) of *hs-DN-dEgfr* retina, not subjected to heat shock. (**m**) P-MAPK immunostaining of *hs-DN-dEgfr* retina 45 min after heat shock. Note: For each pair (**j** and **k**, **l** and **m**), the same exposure time was used for imaging

(*hs DN-dEgfr*) or DN Notch (*hs-N[ECN]*) to reduce signaling of each pathway (Figure 4j–m). At 26 h APF, each visual field in the following experiments investigates a number of dying cells as well as those preparing to die (see Figure 1e and Supplementary Figure 1).

The antibody specific for E(spl) established that *all* lattice cells of 26 h APF fixed pupal retinae robustly express E(spl), indicating an activated Notch signaling pathway in all lattice cells (Figure 4a and b). Dying, condensed nuclei lose E(spl) immunoreactivity, suggesting that E(spl) may be a caspase target (Figure 4c). Similarly, the antibody specific for P-MAPK (anti-dpERK) demonstrated that *all* lattice cells maintain activated MAPK at similar levels regardless of position (Figure 4d–f). Intriguingly, only cytoplasmic P-MAPK was detected in the lattice cells, although at least two of the bristle group cells display nuclear P-MAPK and not cytoplasmic P-MAPK (Figure 4f, arrowheads and g–i). This suggests that dEgfr signaling does not modulate transcription of nuclear targets, but instead leads to cytoplasmic P-MAPK.

Because P-MAPK is directly regulated by dEgfr signaling, we expected to see a reduction in P-MAPK levels in cells signaled to die. We did not see this (although a slight change in P-MAPK levels or a qualitative change in the protein would not be detected), implying that loss of life signaling through dEgfr is not the apoptotic inducer. Yu *et al.*[20] proposed that survival of lattice cells is promoted by inhibition of Notch activation, preventing Notch antagonism of dEgfr activity. Despite the fact that E(spl) protein may not respond to rapid changes in Notch signaling, our data clearly refutes this idea because all lattice cells, whether living or dying, are transducing a Notch signal.

Spitz secreted from cone cells and primary pigment cells rescues lattice cells from death. Alongside our experiments investigating dEgfr signaling, we tested the proposed model that sSpi from cone cells and primary pigment cells could block apoptosis in lattice cells. We investigated which cells can influence lattice cell death through Spi secretion using the 'FLP-out' technique[25,26] to ectopically express sSpi or mSpi (the full-length molecule) in random cells of the pupal retina. Expression of mSpi from single cells in the retina had no effect on retinal patterning (data not shown), consistent with other experiments in which mSpi expressed in the eye was inactive owing to lack of processing.[16,27]

In instances where a single lattice cell expressed sSpi, lattice cells multiple cell diameters from the sSpi-expressing cell were rescued from death, as expected from a secreted ligand that moves outward symmetrically in all directions (Figure 5a and b). Note, however, that it is unlikely that sSpi is the spatial regulator as (1) sSpi presumably moves radially outward and (2) experimentally determined levels of P-MAPK (Figure 4) indicate that all lattice cells receive the same level of dEgfr ligand.

Spi secreted from primary pigment cells rescues lattice cells nearby (Figure 5d). Additionally, sSpi produced by cone cells can traverse the primary pigment cell, leading to ectopic lattice cells (Figure 5c). Because sSpi was turned on in the marked cells as the end-to-end rearrangement was beginning, the
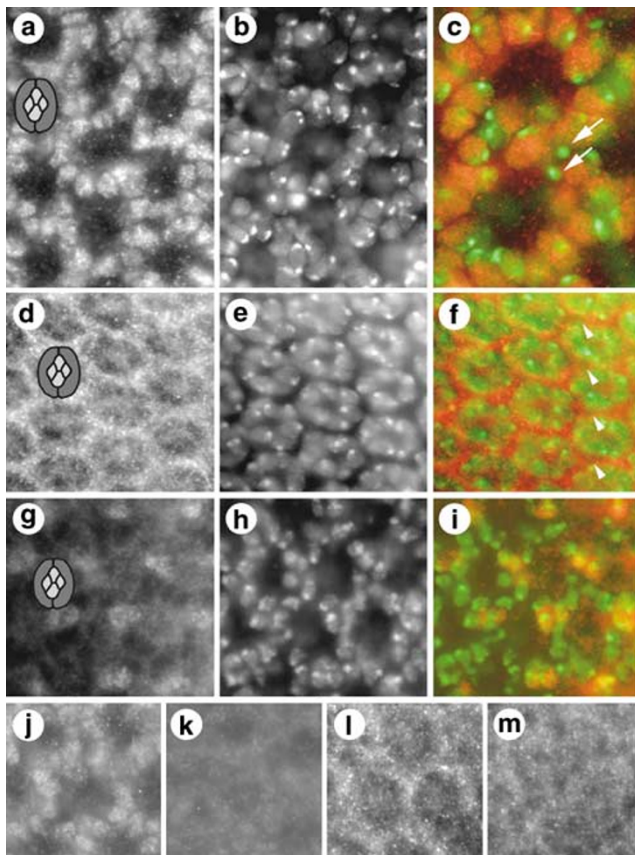
pattern of rescued lattice cells may reflect a group of cells that were clustered.

Rescued lattice cells appear to be attracted toward the sSpi cell (refer to Figure 5a and b); thus, sSpi may be important for the normal end-to-end reorganization of lattice cells seen at ~24 h APF. Occasionally, we observed very small cells in the death zone that expressed sSpi (Figure 5e). These cells may have been signaled to die, but were subsequently rescued by sSpi expression (expressed from 21 h APF onward). This intriguing observation suggests that the cell shape change occurs before the point-of-no-return in cell death and implies that increased P-MAPK signaling can stop the apoptotic process once it has begun.

Lattice cells that reside in the death zone prior to the period of cell death have a lower dEgfr signaling threshold. We next investigated how blocking of dEgfr
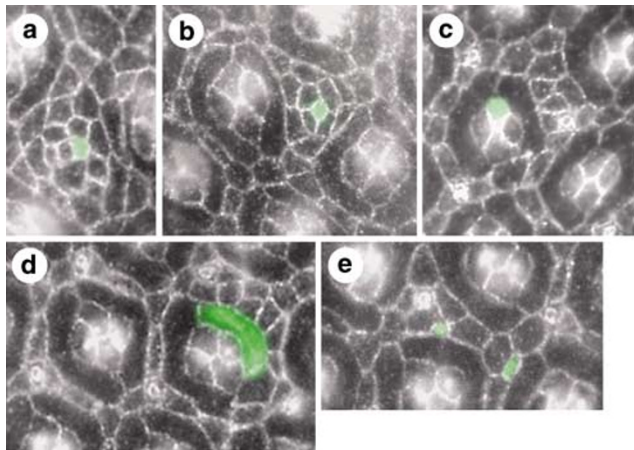

Figure 5 Secreted Spitz rescue cells from PCD several cell diameters away. 'FLP-out' experiments to constitutively drive expression of sSpi from single cells. *hs-FLP; Act5C>y⁺>Gal4/+ ; UAS-sSpi/+* pupae were heat shocked at 37°C as described for each and dissected at 42 h APF. Anti-armadillo immunostains with constitutive sSpi-expressing cells identified by coexpression of GFP (green). **(a, b)** From heat shock at 5.75 h APF for 5 min. **(c, d, e)** From heat shock of 21 h APF pupa for 20 min

Figure 6 Lattice cells with reduced dEgfr signaling can survive, whereas lattice cells in the death zone reveal a sensitivity to reduced dEgfr signaling. **(a–c)** 'FLP-out' experiments to constitutively express DN-dEgfr in individual cells. **(a)** Anti-armadillo outlines membranes of cells in a *hs-FLP; Act5C>y⁺>Gal4/UAS-DN-dEgfr; UAS-DN-dEgfr/+* retina heat shocked at 20 h APF for 5 min at 37°C and dissected at 42 h APF. Note that occasional lattice cells are missing. **(b)** Cells constitutively expressing DN-dEgfr are identified by coexpression of GFP. **(c)** Overlay of **(a)** and **(b)** with GFP in green. **(d)** *lattice Gal4; UAS-DN-dEgfr* retina demonstrating loss of lattice cells and occasional primary pigment cells (lattice cell Gal4 drives in a subset of photoreceptor cells and peripodial cells in the larval eye, but expresses primarily in lattice cells with occasional weak expression in primary pigment cells in 24–26 h APF pupal retinae (data not shown)). **(e–g)** Temporal expression of DN-dEgfr using *hs-Gal4*, each heat shocked 40 min at 37°C at 23.5 h APF. **(e)** *hs-Gal4* control retina. **(f, g)** Regions from two different *hs-Gal4, UAS-DN-dEgfr* retinae. Death zone lattice cells are more sensitive to reduction in dEgfr signaling, regardless of whether bristle groups are present. **(h)** A *hs-Gal4, UAS-DN-dEgfr* retina heat shocked for 80 min at 37°C at 24 h APF, note the almost complete loss of lattice cells with the longer heat shock. **(d–h)** Anti-armadillo immunostains

signaling in individual cells would affect lattice cell apoptosis. Using the FLP-out technique, a dominant-negative form of dEgfr (DN-dEgfr,[16]) was expressed in random single cells exclusively during the time of death. Unexpectedly, we observed that individual lattice cells expressing high levels of DN-dEgfr are still able to populate the fully formed lattice (Figure 6a–c). This is in opposition to data in which global
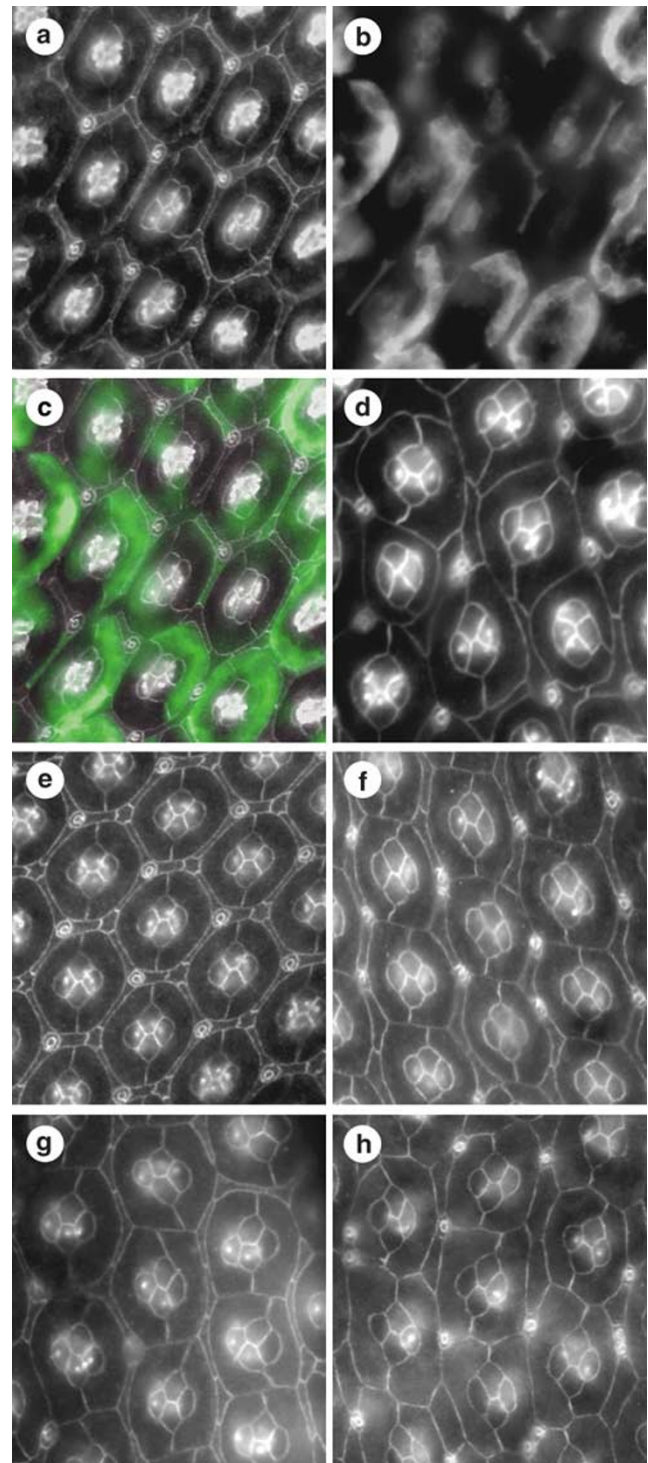
expression of *DN-dEgfr* was shown to lead to cell death in virtually all lattice cells[16] (and see Figure 6h). In our experiments, we also observed patterning defects indicative of excessive cell death. This suggests that individual lattice cells have differing thresholds for life signaling.

We reasoned that a major difference between our FLP-out experiment and previous experiments that reduced dEgfr signaling in the entire retina was that we did not alter dEgfr signaling in any cells that neighbor a lattice cell expressing DN-dEgfr. Therefore, we ectopically expressed DN-dEgfr primarily in all lattice cells with the result that almost no lattice cells survived (Figure 6d). This demonstrates that when dEgfr signaling is removed from all lattice cells, extensive apoptosis occurs. In contrast, removal of dEgfr signaling in single cells does not necessarily lead to that cell's death.

We hypothesized that a combination of signals regulated temporally might dictate whether a cell is sensitive to loss of dEgfr signaling. Therefore, we activated expression of *DN-dEgfr* in short pulses during pupal development (Figure 6e–h). Strikingly, a pulse of 40 min at 23.5 h APF specifically removed lattice cells in the horizontal face, leaving the precise number of oblique lattice cells in proper positions (Figure 6f and g). At this time point, no lattice cells have achieved the survival position of touching four primary pigment cells. A longer pulse resulted in loss of all lattice cells (Figure 6h). Thus, lattice cells that reside in the death zone are more sensitive to reduction in dEgfr signaling.

Discussion

PCD occurs in the death zone. We have used the *Drosophila* pupal retina as a model system to study spatial regulation of PCD. Apoptosis of a subset of lattice cells in the mid-pupal retina is the final patterning step in sculpting the fly eye. The static nature of ultrastructural studies made it impossible to determine whether dying lattice cells occupied specific positions between ommatidia.[9,10] Live visualization of the pupal retina is advantageous because it not only allows snapshots to capture specific events but also allows one to identify an apoptotic cell, and essentially look back in time to assign the cell's position before death.

Using time-lapse imaging of the eye of the living pupa, we identified dying cells as they lost their apical footprint, one of the earliest death events. We determined that 50% of apoptotic lattice cells occupied a position next to the bristle group and in the horizontal face between ommatidial units. Furthermore, 35% of dying lattice cells were located next to the bristle group along an oblique face. Thus, a remarkable 85% of apoptotic lattice cells died in two very specific regions between ommatidial units and adjacent to bristle groups.

However, bristle groups cannot be the origin of the death signal as removal of bristle groups does not block apoptosis. Instead, in the absence of bristle groups, the pattern of lattice cell death changed dramatically. Lattice cells at either the anterior or posterior portion of the horizontal face now had roughly an equal chance of dying. Together, the two horizontal regions accounted for 85% of the lattice cells undergoing PCD. We conclude that lattice cell PCD is regulated spatially so that apoptosis removes cells occupying the anterior and posterior horizontal positions between ommatidia. We have termed this region the 'death zone'. Between these two points of position-specific PCD, a single cell is protected from apoptosis. Apoptosis in the death zone ends when only this cell remains in the horizontal face between ommatidia.

How could bristle groups so dramatically affect the pattern of PCD? One possibility is that the bristle groups attract cells to their death in the death zones. Miller and Cagan[13] showed that bristle groups secrete Spi and our data (Figure 5) demonstrate that lattice cells respond by surrounding the source of secreted Spi. Just prior to the cell death period, there are on average 1.6 times more cells in the regions around the bristle groups than in other vertices (CBB, unpublished results). Additionally, in the absence of bristle groups, more cells appear to populate the horizontal regions prior to death (at the expense of the oblique regions).

The Notch and Egfr signaling pathways in pupal retinal PCD. Survival of lattice cells depends on a signal, hypothesized to be secretion of Spi, from primary pigment cells and/or cone cells.[13] Our experiments confirm that lattice cells transduce a dEgfr signal, but the interpretation is complicated by the fact that we were unable to detect P-MAPK in lattice cell nuclei, suggesting that none of the cells respond transcriptionally to dEgfr signaling. Instead, we identified P-MAPK in the cytoplasm of *all* lattice cells, including those in the death zone. It was recently reported that retaining P-MAPK in the cytoplasm in cells in the larval eye disc is important for normal development.[28,29] In the current study, it is unlikely that blocking of nuclear MAPK signaling by 'cytoplasmic hold' is required to induce apoptosis because ectopic transcriptional repression of activated MAPK targets by expression of the activated form of *aop* had no effect on apoptosis in the pupal retina (CBB, unpublished results). Taken together, these experiments argue that dEgfr signaling in lattice cells serves solely to activate P-MAPK in the cytoplasm. Cytoplasmic P-MAPK may suppress apoptosis through phosphorylation of cytoplasmic proteins such as HID.

In light of the fact that Notch is upstream of dEgfr, a new model was put forth: primary pigment cells and/or cone cells promote survival by inhibiting Notch activation and its disruption of dEgfr signaling in lattice cells.[20] We were able to directly test this model because our live-visualization data predicted the pattern of death signals. Our data do not support this hypothesis as *all* lattice cells, whether living or dying, are transducing a Notch signal; surviving lattice cells show no downregulation or inhibition of Notch signaling. We instead propose that Notch dampens the Spi-activated dEgfr survival signaling in all lattice cells. Thus, experimental removal of Notch (i.e. heat shock of *N^{ts1}*) would cause a general increase of dEgfr signaling (and survival of all cells). Conversely, loss of dEgfr signaling would lead to PCD, the default pathway. This model predicts that Notch transcriptionally activates a gene whose protein product keeps dEgfr signaling poised. A second scenario is one in which the Notch protein may itself influence the life or death decision of a cell. That Notch acts through a noncanonical pathway has been suggested previously.[30–33] In this model, Notch might function to activate

PCD only in death zone cells, acting as a third signal as described below.

Cell–cell signaling defines the death zone. How is the death zone molecularly defined? Cells in the horizontal region are more sensitive to a reduction of dEgfr signaling, but, as demonstrated by P-MAPK levels, this is unlikely to be owing to a lower level of dEgfr signaling. Thus, if the death zone is defined by dEgfr signaling, this may be owing to a qualitative change in cytoplasmic P-MAPK activity. A more likely possibility is that dEgfr signaling (dampened by Notch) merely poises the cell to allow a third signal to induce PCD specifically in the death zone. In support of this idea is the finding that cells in the death zone regions are more likely than cells in other regions to die in response to a brief interruption in dEgfr signaling.

Localization of apoptosis in the lattice to death zone regions brings additional complexity to the problem of PCD regulation. Clearly, the signal that regulates this final cell fate must be localized. The most likely candidate cell to produce the regulating signal is the primary pigment cell. However, each primary pigment cell contacts numerous lattice cells; some that will survive and some that will die. Thus, we suggest a model in which the dorsal and ventral regions of each primary pigment cell localize a death-inducing factor. Evidence for such a subcellular domain has been seen in primary pigment cells (CBB, unpublished results). The contact regions at which the two primary pigment cells meet must either block this signal or localize a strong survival signal.

Placed in the context of other examples in which the architecture of a tissue is shaped through selective PCD, the *Drosophila* retina is a unique model in that it can be manipulated genetically, microscopically and development can be visualized in the living animal. The determination that apoptosis is spatially regulated in the pupal eye directs future experiments designed to identify how the death zone is created.

Materials and Methods

***Drosophila* strains.** Stocks used were: *UAS-gap43GFP* on II (gift of P. Garrity, MIT), P[*sev-wg*]/*TM3* (gift of K. Cadigan, University of Michigan), *UAS-sSpi* (gift of B.Z. Shilo, Israel) and *hs-N^ECN* (gift of S. Artavanis-Tsakonas, Yale University). *AyGal4 UAS-GFP.S65T (Actin5C>y+>Gal4, UAS-GFP)*, *hs-Gal4*, *UAS-DN-Egfr*, P{*GawB*}*LL54* (lattice cell Gal4), *hs-FLP*[12] on X were all obtained from the Bloomington Stock Center.

Live visualization of pupal retinae. Pupae were picked as white prepupae and aged for 22–24 h at 25°C. The pupal case covering the eye was removed and the pupa was laid on its side on a mounting pad of normal *Drosophila* media containing an extra 2% agar and covered with a coverslip. The mounted pupa was placed on the stage of a Zeiss Axiovert 200 and kept humidified and at 23–25°C during the experiment. Imaging was performed with an oil-immersion ×40 objective using a Xenon bulb and a GFP filter. For each time point, a Z-series was imaged. The Z-series was required because of the dome shape of the eye and because the focal plane changes as the volume of the pupa increases developmentally. Images were captured with a Quantix CCD camera (Photometrics Ltd) and ImagePro Plus Software (ImagePro) and were processed through one round of sharpening and one or two rounds of low pass filter prior to analysis using Metamorph Software (Universal Imaging). In most cases, a region from each focal plane in which the apical membranes were best viewed was cut out. The cut regions were flattened into one image. All cell counting was performed by hand. Live imaging of the pupal eye had no effect on the viability of the pupa nor did it lead to any visible change in the adult eye.

Immunohistochemistry of pupal retinae. Pupae were aged at 25°C. Retinae were dissected into PBS and fixed in 4% paraformaldehyde/PBS and permeabilized in PBS/0.2% Triton X-100. Primary antibodies were: anti-armadillo N27A1 (1 : 10, Developmental Studies Hybridoma Bank, DSHB), anti-betagalactosidase 40-1a (1 : 10, DSHB), anti-cut (1 : 10, DSHB), anti-E(spl) (mAB 323 1 : 2, Gift from S. Bray), anti-dpERK (1 : 200, Sigma) and anti-CC3 (1 : 200, Cell Signaling Technologies). For anti-dpERK immunostaining, retinae were dissected into PBS containing 1 μM okadaic acid, incubated for 5 min and fixed in PBS/4% paraformaldehyde for 30–45 min. Secondary antibodies were Alexa-conjugated (Molecular Probes). TUNEL labeling was as described,[21] except that FITC-dUTP was incorporated (Molecular Probes).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)