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Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis

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The periphery of the fly eye contains a number of concentrically arranged cellular specializations that are induced by Wingless (Wg) signaling from the surrounding head capsule (HC). One of these is the pigment rim (PR), which is a thick layer of pigment cells that lies directly adjacent to the HC and completely circumscribes the rest of the retina. Many of the cells of the PR are derived from presumptive pigment cells that previously surrounded peripheral ommatidia that subsequently died. Here, we describe the Wg-elicited expression of Snail family transcription factors in the eye periphery that directs the ommatidial death and subsequent PR formation. These transcription factors are expressed only in a subset of the ommatidial cells not including the photoreceptors. Yet, the photoreceptors die and, thus, a non-autonomous death signal is released from the Snail-family-expressing cells that direct the death of the photoreceptors. In addition, Wg also elicits a similar peripheral expression of Notum, an enzyme that limits the extent of Wg signaling. Furthermore, we describe a later requirement for Snail family proteins in the 2° and 3° pigment cells throughout the main body of the eye.

KEY WORDS: Wingless, Snail family transcription factors, Fly eye periphery

INTRODUCTION

During the process of morphogenesis, cells can be directed to their appropriate fates by positional information that usually constitutes signals from other cells and tissues. In gradient mechanisms of positional information, a signal diffuses from its source and establishes a graded concentration profile. Cells meter the ambient concentration of the signaling molecule to determine their fates, such that those detecting levels above one concentration threshold become one type, and those above higher thresholds become other types (Wolpert, 1971). By this method, a spatially arrayed gradient can be converted into a spatially arrayed sequence of different cell types. The majority of experiments that have investigated gradient mechanisms have used differential gene transcription to monitor the responses of the cells with little regard to the final cellular states. Here, we examine the organization of the peripheral retina of the fly where a graded signal directs the formation of a spatially arrayed series of distinct morphologically structures. This system thus affords us the opportunity to investigate the process of gradient signaling from the establishment of the signal itself, through the responses of the cells and leading finally to the morphogenetic elaboration of the spatially arrayed structures.

The *Drosophila* compound eye is composed of ~800 subunit ommatidia, each of which contains eight photoreceptors (R1-R8), four lens-secreting cone cells and two primary (1°) pigment cells. A hexagonal array of secondary (2°) and tertiary (3°) cells surround the ommatidia, and mechanosensory bristle cells occupy alternate vertices of the hexagonal array (Ready et al., 1976). At the periphery of the eye lie a number of specializations (Fig. 1A).

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(1) Circumscribing the field of ommatidia and lying adjacent to the head capsule (HC) lies the pigment rim (PR) – a thick band of pigment cells that probably functions to insulate the field of ommatidia from extraneous light rays (Tomlinson, 2003).

(2) Immediately interior to the pigment rim, and only in the dorsal half of the eye lie the ommatidia that detect polarized light – the dorsal rim ommatidia (DRO) (Tomlinson, 2003; Wernet et al., 2003).

(3) The DRO and their ventral counterparts, as well as a number of interior rows of ommatidia are bald – they lack interommatidial bristles. Interior to these three layers of specializations lies the field of standard bristle-bearing ommatidia (Cadigan et al., 2002; Ready et al., 1976; Tomlinson, 2003).

A number of features of the patterning of the periphery have been described, all of which are controlled by Wg secreted from the circumscribing HC.

(1) During the pupal phase the outer-most ommatidia are removed by apoptosis – a complete ring of ommatidia is lost and the most peripheral ommatidia of the adult (including the DRO) were those that previously lay directly interior to this ring (Lin et al., 2004; Wolff and Ready, 1991). The outer ommatidia that die frequently do not contain a full complement of cells (Lin et al., 2004) – photoreceptors, cone cells or 1° pigment cells can be absent. The presumptive 2°/3° pigment cells that surround these dying ommatidia survive and contribute to the PR (Tomlinson, 2003).

(2) The DRO polarized light detectors are specified by the expression of the Homothorax transcription factor (Hth) (Wernet et al., 2003). The exclusive dorsal expression of Iroquis (Iro) transcription factors allows the most peripheral surviving ommatidia to turn on Hth and adopt the DRO fate (Tomlinson, 2003; Wernet et al., 2003).

(3) The bristle groups are derived from sensory organ precursor (SOP) cells that require the Daughterless (Da) transcription factor, and in the peripheral regions repression of Da expression results in the band of bald ommatidia (Cadigan et al., 2002).

In order to further understand the mechanism by which Wg signaling specifies these peripheral cell fates, we looked for potential Wg target genes by screening enhancer trap lines for differential

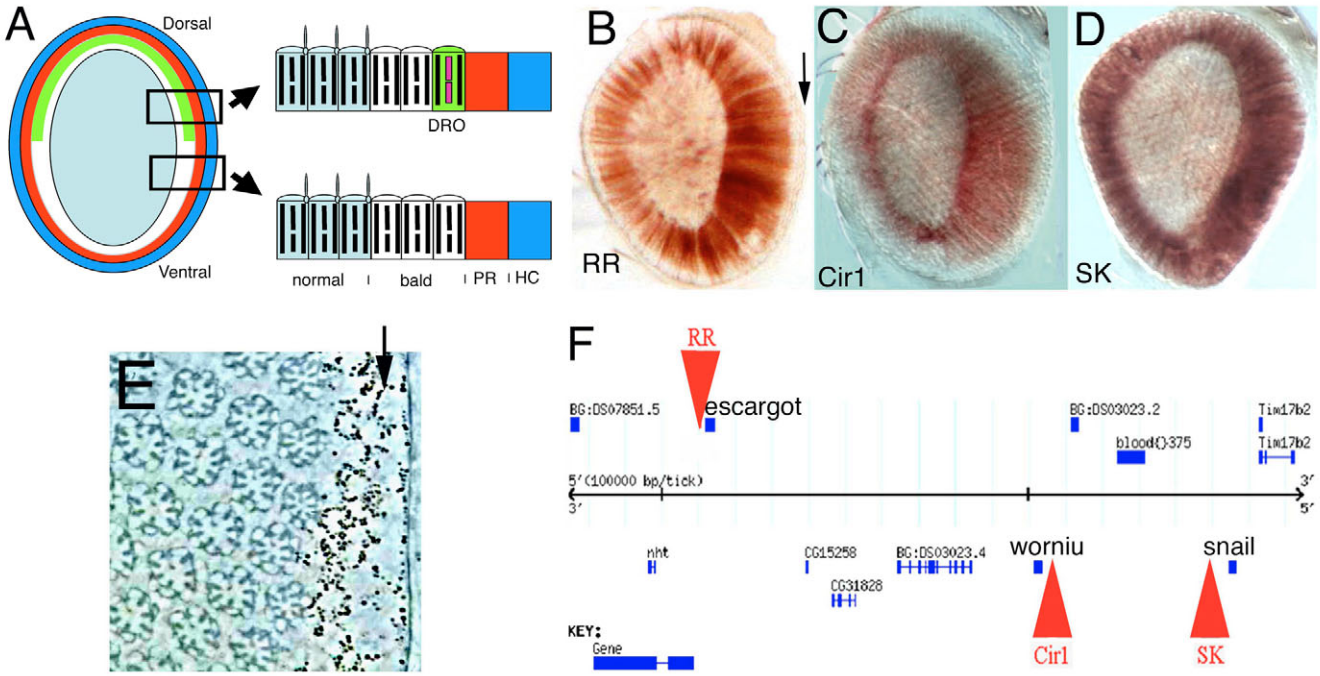

Fig. 1. Enhancer trap lines showing marginal pigmentation in the fly eye. (**A**) Schematic depiction of the peripheral specializations of the eye. Residing immediately adjacent to the head capsule (blue) is the pigment rim (red) that completely circumscribes the eye. The dorsal rim ommatidia (green) lie next to the pigment rim only in the dorsal margin of the eye. The dorsal rim ommatidia and their ventral corresponding ommatidia, together with a number of interior rows of ommatidia (white), are devoid of bristles. The central field of ommatidia (light blue) bears bristles. Shown within the dorsal and ventral boxed areas are the photoreceptor-bearing ommatidia. The outer photoreceptors extend throughout the entire ommatidium, whereas the inner photoreceptor R7 and R8 each occupies half of the ommatidium (black bars). Inner photoreceptors of the dorsal rim ommatidia have enlarged rhabdomeres (pink bars) when compared with those of normal ommatidia. (**B-D**) Whole-mount views of the adult eyes of RR (B), Cir1 (C) and SK (D) showing marginal pigmentation pattern. The pigmentation deceptively appears to extend well into the body of the eye. This results from the cupped shape of the retina. Compare arrow in B with arrow in E. (**E**) Section through the anterior region of the eye of Rim Red showing pigment expression only in the pigment rim. The arrow indicates the pigment in the PR, and the corresponding position is indicated in B above. (**F**) Genomic map of the Snail region. The red arrowheads indicate the positions of the three P-element insertions.

expression patterns in the eye periphery. Four Wg target genes were identified: three encoded transcription factors of the Snail family; the fourth was *notum* (also known as *wingful*) that encodes a member of the α/β-hydrolase superfamily that regulates Wg signaling by modifying *Drosophila* glypicans such as Dally (Division abnormally delayed) and Dally-like (Gerlitz and Basler, 2002; Giraldez et al., 2002; Han et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004).

Here, we identify Snail family genes as transcriptional targets of Wg in the eye periphery that are required for the removal of the peripheral ommatidia and subsequent PR determination. Furthermore, we show that *notum*, a known Wg target gene, functions to modulate Wg signaling itself. In addition, we identify a later role for *escargot* in the specification of 2° and 3° pigment cells throughout the eye.

MATERIALS AND METHODS

Drosophila genetics

All crosses and staging were performed at 25°C. Pupal development was expressed as hours after puparium formation (APF) where white pre-pupae were defined as 0 hours APF. Stocks used were: *Actin>CD2>Gal4, UAS-GFP* (Johnston and Sanders, 2003), *UAS-arm** (Zecca et al., 1996), *Actin>y+>wg* (Struhl and Basler, 1993) and *Notum-Gal4* (S168) (Gerlitz and Basler, 2002), *wg-lacZ, UAS-wg* and *UAS-lacZ* (Bloomington Stock Center). The $dsh^{[v26]}$ and arr^{2} clones were induced as previously described (Wehrli and Tomlinson, 1998); the esg^{G66B} *FRT40* and esg^{G66B} sna^{1} *FRT40* chromosomes were from Shigeo Hayashi (Fuse et al., 1996); the $notum^{3}$ *FRT80B* chromosome was described previously (Giraldez et al., 2002).

The enhancer trap screen

An enhancer trap screen was conducted in which flies containing mobilized P-elements carrying Gal4 with a minimal hsp70 promoter were screened in a *w[-]; UAS-w[+]* background for peripheral pigmentation in the adult eyes. Inverse PCR using P-element primers was used to identify genomic insertion sites.

Constructs

The two *UAS-worniu*^{RNAi} transgenes were made from PCR-generated fragments (nucleotides 842-1430 and 1701-2176 of the cDNA) inserted in opposite orientations into a pUAST-RNAi intron vector (Lee and Carthew, 2003). Phenotypes were examined at 30°C.

Immunostaining

Standard immunostaining was performed. Primary antibodies: rat anti-Escargot (1:250) was from Xiaohang Yang; mouse anti-Cut (1:100), mouse anti-Wg (1:50), mouse anti-Armadillo (1:10) and Rat anti-Elav (1:100) were from the University of Iowa Hybridoma Bank; mouse anti-Coracle (1:500) was from Richard Fehon; mouse anti-Worniu antibody was from Cai et al. (Cai et al., 2001); mouse anti-Snail (1:500) was a gift from Pierre Chambon; rabbit anti-βGal (1:2000) was purchased from Cappel; Cy3- and Cy5-conjugated secondary antibodies were from Jackson Immunochemicals (West Grove, PA).

Standard histology and X-Gal staining

Histological preparation of adult fly eye and X-Gal staining of pupal eye discs were performed as described previously (Tomlinson, 2003).

RESULTS

Identification of differential peripheral gene transcription

To identify potential target genes of peripheral Wg signaling, we examined enhancer trap lines that showed differential pigmentation in the marginal regions of the eye (see Materials and methods). One line (RR, Fig. 1B,E) was generated in a screen we performed (see Materials and methods), another (SK, Fig. 1D) was a gift from Sui-kwong Chan, and Cir1 (Fig. 1C) has been published by Tang and Sun (Tang and Sun, 2002). Analysis of the genomic sequences flanking the insertion sites showed that each corresponded to a different member of the Snail gene complex (Fig. 1F); Cir1 was inserted ~500 bp upstream of *worniu* (*wor*) (Tang and Sun, 2002); RR was ~400 bp upstream of the *escargot* (*esg*) gene; SK was ~6 kb downstream of *snail* (*sna*).

Characterization of the expression patterns of the Snail family protein in the eye periphery

sna, *esg* and *wor* are considered to be functionally redundant members of the *Drosophila* Snail gene family that encode zinc-finger transcription factors (Ashraf et al., 1999; Boulay et al., 1987; Whiteley et al., 1992). To examine the expression profiles of the encoded proteins, we performed immunostaining in larval and pupal eyes. Esg protein was not detected in the larval eye disc (data not shown) and first appeared at ~32 hours after puparium formation (APF) in the most peripheral regions of the eye (Fig. 2A). At this stage, the peripheral ommatidia destined to be removed by apoptosis were still present, and Esg staining was found in the HC [Fig. 2D – we identify the HC at this stage by its expression of Hth (Dominguez and Casares, 2005; Kenyon et al., 2003)]; in the early PR (the thin layer of pigment cells that circumscribes the retina at this stage, Fig. 2A); in the peripheral interommatidia pigment cells (these are the cells that will join the PR after their ommatidia die, Fig. 2A); and in the cone cells of the peripheral ommatidia (Fig. 2C). Staining was not observed in the photoreceptors (Fig. 2A″) or the 1° pigment cells of the peripheral ommatidia (data not shown). We were unable to detect either Wor or Sna protein with the available antibodies but suspected that this was due to ineffective detection of the proteins rather than to their absence from the tissues. As Cir1 is a Gal4 insertion in the *wor* locus (Fig. 1F), transcriptional activity of the gene was monitored using *UAS-lacZ*. Here, *lacZ* activity was found to correspond with the expression of Esg; being absent form the larval eye disc, and turning on at a third of the way through pupal life in the pattern described above for Esg (Fig. 2B). There was no transcriptional reporter present in SK (the *sna* transposon insertion; Fig. 1F) with which pupal transcription could be monitored. However, given that all three Snail family gene insertions show the same expression pattern in the adults (which probably results from perdurance of pupal transcripts), we infer that *sna* may also show the same expression profile as *esg* and *wor*.

Characterization of peripheral Wg expression

Lin et al. documented that Wg signaling from the HC elicits Wg expression itself (~32 hours APF) in the ommatidia that are destined to die (Lin et al., 2004). Our analysis indicates that this secondary Wg expression is restricted to the cone cells of these ommatidia, not the photoreceptors. We stained both *wg-lacZ* and α-Wg, and counterstained with markers for cone cells and photoreceptors (Fig. 2E,F). Thus, at this stage Sna family proteins are co-expressed with Wg in the HC and in the cone cells but are additionally expressed in the 2° and 3° pigment cells surrounding the peripheral ommatidia (Fig. 2G).

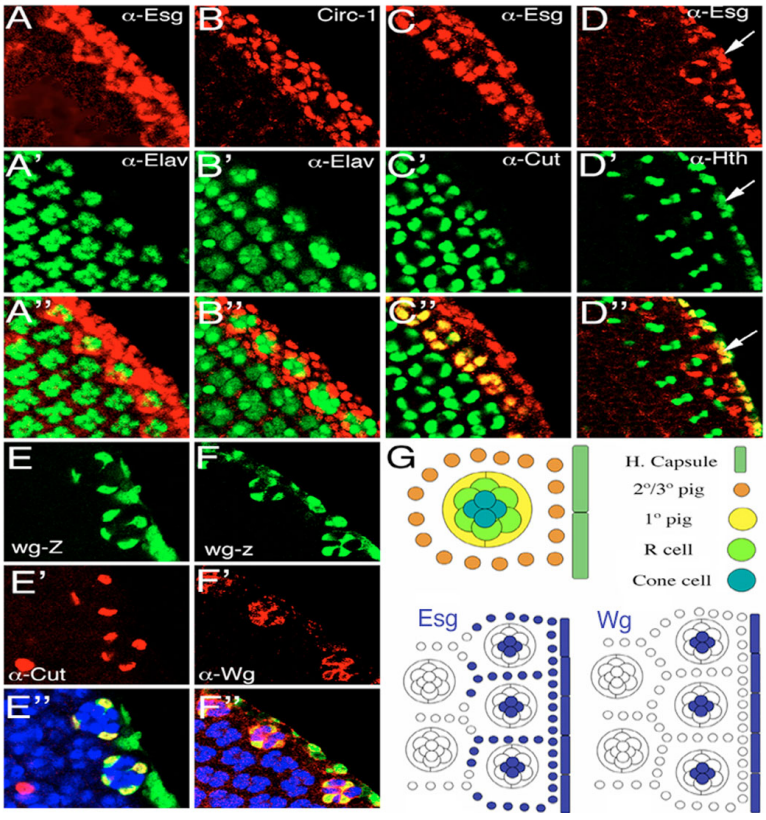

Fig. 2. Expression patterns of Escargot, Worniu and Wingless in the peripheral eye. (**A-F**) All micrographs are flat mounted pupal retinas of ~32 hours APF. (A-A″) Pupal eye stained for Escargot (red) and Elav (green). Escargot is expressed in the HC, PR and peripheral 2°/3° pigment cells. (B-B″) *Cir1-Gal4>UAS-lacZ* eyes stained for lacZ (red) and Elav (green). Cir1 and Escargot show the same expression profile. (C-C″) Escargot (red) and Cut (green, specifically labels cone cells) are co-expressed in the marginal cone cells. (D-D″) Escargot (red) and Homothorax (green) co-expressed in the head capsule (arrow). Homothorax is also expressed in the central photoreceptors of the outer ommatidia (those that die) and an inner ring that will eventually form the DRO. (E-E″) *wg-lacZ* eyes stained for lacZ (green), Cut (red) and Elav (blue) showing Wg expression in the perimeter cone cells. (F-F″) *wg-lacZ* eyes stained for lacZ (green), Wg (red) and Elav (blue) showing the absence of both Wg expression in the perimeter photoreceptor cells. (**G**) Summary of the expression patterns of Esg and Wg. Top panel: a key to the cell types depicted below. Bottom panels: Schematic summary of Wg and Escargot expression in the eye periphery at ~32 hours APF. Escargot is expressed in the HC, peripheral 2°/3° pigment and cone cells. Wg is expressed in the HC and peripheral cone cells.

Wg signaling regulates snail family gene expression

To determine whether Wg signaling induced the peripheral Snail family expressions, Wg signaling was ectopically activated in the main body of the retina and the effects were assayed in both adult and pupal eyes. The arrow in Fig. 3A indicates the ectopic expression of *RR-esg* in an adult eye in cells in which Wg was ectopically expressed (*SK-sna* and *Cir1-wor* behaved similarly). In pupal retinas, ectopic expression of Wg and constitutively activated Armadillo (ΔArm) (Zecca et al., 1996) induced both ectopic *Cir1-wor* transcriptional activity (Fig. 3B) and Esg protein (Fig. 3C). The ΔArm induces Esg expression non-autonomously (Fig. 3C), which results from concomitant ectopic Wg expression (data not shown). Thus, ectopic activation of the Wg pathway in the main body of the eye induces the expressions that Wg normally controls at the periphery – namely the induction of Snail family genes and *wg* itself.

The consequences of removing Wg signaling from the periphery were examined in clones mutant for two obligate Wg transducers – *dishevelled* (*dsh*) and *arrow* (*arr*). In both *dsh* and *arr* clones, there was a dramatic reduction or loss of Esg expression in all the peripheral cell types (HC, PR and the cone cells and 2°/3° pigment cells of the peripheral ommatidia) (Fig. 3D,E). At this stage, all these cell types appeared to be in place, but failed to express the Snail family genes when Wg signaling was abrogated – for example Fig. 3E″ shows Cut-expressing cone cells (in blue) that would normally express Esg. Abolishment of Wg signaling was previously shown to remove peripheral Wg expression (Lin et al., 2004). Collectively, the results of manipulation of Wg signaling are consistent with the notion that Wg signaling induces the peripheral expression of Snail family and *wg* gene expressions.

Wg signaling and the regulation of peripheral ommatidial cell death

The expression of Wg and Snail family proteins in the border regions prefigures the subsequent removal of the peripheral ommatidia. Earlier analyses of the apoptosis of these peripheral ommatidia examined only the death of the photoreceptors (Hay et al., 1994; Lin et al., 2004; Wolff and Ready, 1991). As the cone cells express Wg and Snail family genes in these ommatidia, we examined the sequence of apoptosis and found that the cone cells died first (at ~36 hours APF), some four hours before the death of the photoreceptors. To demonstrate that cone cell removal occurred by the apoptotic mechanism described for the photoreceptor, peripheral *H99* homozygous clones [mutant for the three pro-apoptotic genes – *hid*, *reaper* and *grim* (White et al., 1996; Wing et al., 2002)] were induced, resulting in the survival of the peripheral cone cells (Fig. 4B). Furthermore, in clones mutant for *dsh* or *arr*, the peripheral ommatidia survived as evidenced by ectopic peripheral persistence of Cut-expressing cells (the cone cells; Fig. 4A), Elav-expressing cells (the photoreceptors; Fig. 4D″, arrowhead in Fig. 4D′), and Bar-expressing cells (the 1° pigment cells; Fig. 4E). As these ommatidia did not die, they did not release their associated 2° and 3° pigment cells to join the PR. As a consequence, the PR in the *dsh* or *arr* clones was significantly reduced in relation to the neighboring wild-type tissue in which the ommatidia died (arrowhead versus arrow in Fig. 4D′).

Snail family regulation of peripheral cell fates

When Wg is ectopically expressed at high levels in the main body of the eye, it induces the death of the ommatidia leaving a small eye containing only pigment cells (Fig. 4C) (Tomlinson, 2003). When Esg (Fig. 4C) and Sna (data not shown) were similarly expressed, small pigmented eyes resulted. Thus, overexpression of Esg and Sna phenocopy Wg overexpression in the eye, suggesting that they mediate at least some of the Wg signaling that occurs at the periphery.

The roles of the Sna group proteins in mediating the peripheral Wg signal were now examined in loss-of-function clones. Here, the experiments were compromised by redundancy of gene function in the Snail complex (Ashraf et al., 1999; Ashraf and Ip, 2001; Cai et al., 2001). Examination of peripheral *esg* clones in pupal discs showed only infrequent effects on the peripheral structures. *esg, sna* double mutant clones showed a modest increase in these effects, but not until we made triple mutant clones [by simultaneously introducing two different *wor* RNAi constructs (see Materials and methods) in the *esg*, *sna* clones] did substantial effects occur; the survival of peripheral ommatidia (Fig. 4F,G) and concomitant reduction of the PR (Fig. 4H). Although clear and robust effects occurred in the triple mutant clones, not all clones showed these effects. This probably resulted from the incomplete removal of *wor* gene function by the RNAi technique. No effects were observed on aspects of Wg peripheral signaling other than peripheral ommatidial death and PR formation. For example the expression of Hth in the DRO precursors remained unaffected (Fig. 4I), as did the patterning of the bald ommatidia (data not shown).

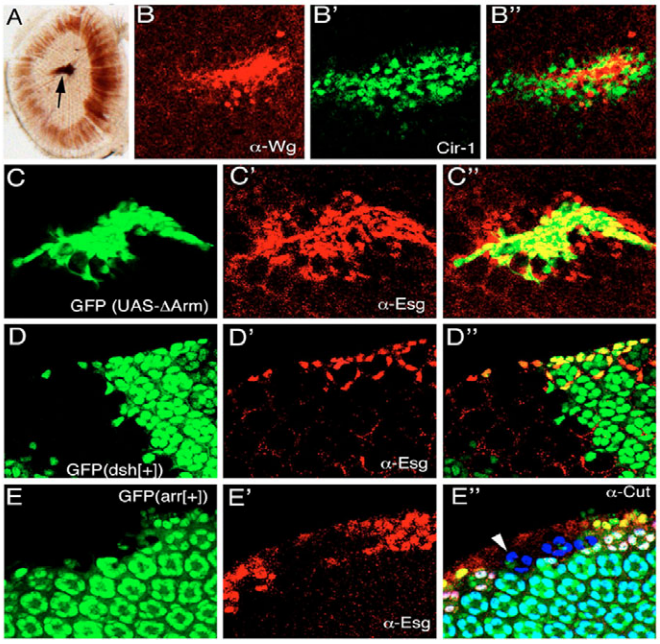

Fig. 3. Wg signaling regulates the expression of Snail family factors. (**A**) A clone of *actin-wg* in adult RR eye induces ectopic expression of the transcriptional reporter (arrow). (**B-B″**) A clone of *actin-wg* (red) in Cir1 pupal eye (~32 hours APF) showing ectopic expression of the transcriptional reporter (green). (**C-C″**) An *actin>Gal4*; *UAS-ΔArm* clone in pupal retina (~32 hours APF) marked by GFP (green) shows ectopic Escargot (red) non-autonomously. (**D-D″**) A large *dsh[V26]* clone in the pupal eye periphery (~32 hours APF) marked black by the absence of GFP. Peripheral Esg (red) is almost entirely abolished. The faint lattice of staining is the incipient Esg expression in the main retina 2°/3° pigment cells – this is not Wg dependent. (**E-E″**) An *arr[2]* clone in the pupal eye edge (~32 hours APF) marked black by the absence of GFP. Escargot expression (red) is lost in the clone. (E″) A merge of the two with an additional stain for Cut (blue). The arrowhead indicates a cone cell that would normally express Escargot but fails to do so in the clone.

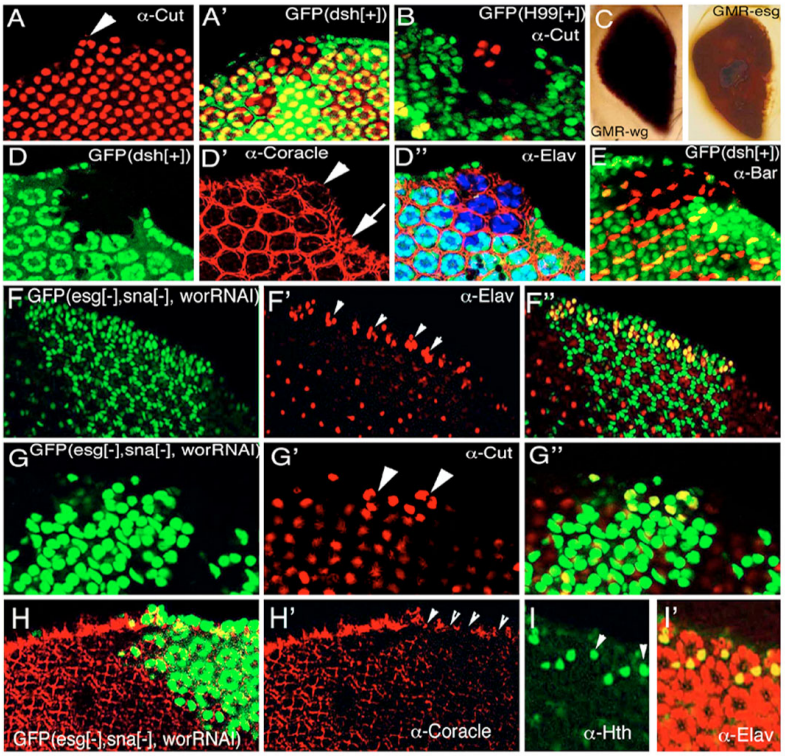

Fig. 4. Snail transcription factors mediate the effects of Wg signaling in peripheral eye development. With the exception of C, all micrographs are flat mounted pupal retinas of ~40 hours APF. (**A-A'**) A *dsh[V26]* clone at the eye margin marked black by absence of GFP showing ectopic cone cells stained with Cut (red, arrowhead). (**B**) A *Df(3L)H99* clone in the eye margin marked black by the absence of GFP showing ectopic cone cells (red). The cone cells from more interior ommatidia are not visible at this focal plane. (**C**) Whole-mount views of adult eyes uniformly ectopically expressing Wg and Escargot under the GMR promoter. High level Wg and Escargot result in small heavily pigmented eyes. (**D-D"**) A *dsh[V26]* clone at the eye margin marked black by absence of GFP and showing retinal protrusion caused by the inappropriate survival of the peripheral ommatidia stained for Elav (blue). The PR, highlighted by coracle staining (red) is significantly reduced in the clone (arrowhead) compared with the adjacent wild-type patch (arrow). (**E**) A *dsh[V26]* clone at the eye margin marked black by absence of GFP showing ectopic primary pigment cells stained with BarH1 (red). (**F**) A clone doubly mutant for *esg* and *sna*, and overexpressing two different *worniu* RNAi constructs (triple mutant clone) in the eye margin marked by GFP (green). (**F'**,**F"**) Ectopic photoreceptor cells stained for Elav (arrowheads, red) are present in the clone. The photoreceptors of the more internal ommatidia are not visible at this focal plane. (**G**) A triple mutant clone marked by GFP (green) at the eye margin. (**G'**,**G"**) Ectopic cone cells (red) are present in the clone (arrowheads). Cone cells of interior ommatidia are not fully visible at this focal plane. (**H**,**H'**) A triple mutant clone in the eye margin marked by GFP (green) showing a significant reduction of the pigment rim (red, arrowheads). (**I**,**I'**) A higher magnification view within a triple mutant clone (clone marker not shown) showing no effect on homothorax expression (green) in both ectopic (arrowheads) and standard DRO ommatidia.

Expression and function of Notum at the retinal margin

Another enhancer trap line (JW) given to us by Jill Willdonger carried a *lacZ* reporter that showed peripheral eye expression (not shown). This was an insertion ~250 bp upstream of the *notum* gene, that encodes an α/β-hydrolase that functions in the wing to restrict Wg diffusion by modifying heparin sulfate proteoglycans such as Dally and Dally-like (Gerlitz and Basler, 2002; Giraldez et al., 2002). Using a n*otum-Gal4* line–S168-Gal4 (Gerlitz and Basler, 2002), expression from the locus was found to begin ~32 hours APF in the HC, in the pigment cells of the PR and the peripheral interommatidial pigment lattice, and in the cone cells of the peripheral ommatidia (Fig. 5A,B,F). This expression was similar to that of Esg (See Fig. 2G), except that the Notum expression did not extend as far into the interommatidial pigment cells (Fig. 5F).

To investigate the role of Notum in peripheral patterning, *notum* clones were examined in pupal eyes, and modest but reproducible effects were observed. There was an elevation of Wg protein level in *notum* clones (arrowhead in Fig. 5E), which correlated with a precocious induction of Esg expression (arrowheads in Fig. 5C′). There was also an increase in the number of peripheral ommatidia that underwent apoptosis (Fig. 5D) and a corresponding expansion of the PR (not shown). These phenotypes are consistent with Notum functioning to restrict the potency and range of action of the Wg signal.

Expression and role of Escargot in late eye development

Following its peripheral expression at ~32 hours APF, Esg becomes expressed specifically in all the 2° and 3° interommatidial pigment cells throughout the retina at ~40 hours APF (Fig. 6A). *Cir1-wor* showed an identical expression (data not shown), but we were unable to monitor Sna expression. Although clones of *dsh* or *arr* remove peripheral Esg expression (Fig. 3D,E), in the main body of the retina Esg expression was unaffected (Fig. 6B). Thus, the earlier peripheral expression of the Snail group genes appears to be controlled by Wg, whereas the later central expression is not.

The precursors of the 2° and 3° pigment cells are a disordered group of retinal cells that lie between the developing ommatidia. An apoptotic cell pruning mechanism converts the array into a precise hexagonal lattice by ~36 hours APF (Cagan and Ready, 1989; Miller and Cagan, 1998), and by ~44 hours APF, the previously relaxed apical profiles of the 2° and 3° pigment cells become constrained into a necklace-like array (Fu and Noll, 1997; Wolff and Ready, 1991). Expression of Esg (~40 hours APF) occurred in all the 2° and 3° pigment cells after their apoptosis but before their apical constriction.

In *esg* mutant clones, the 2° and 3° pigment cell lattice formed correctly, but the cells failed to undergo apical restriction (Fig. 6C). Rhodamine phalloidin staining of the clones revealed no obvious defects in the actin cytoskeleton or cellular morphology throughout the depth of the cells (data not shown). There was no increase in severity of this phenotype in *esg*, *sna* or *esg*, *sna*, *UAS-wor*^{RNAI} clones, suggesting that Esg is the sole effector of the maturation of the 2° and 3° cells. This contrasts with the organization of the periphery, where all three proteins of the Snail complex appear to function redundantly. *esg* and *esg*, *sna* clones were examined in adult eye and a dramatic loss of 2° and 3° pigment cells was observed (although some still survived), resulting in gaps between ommatidia (arrowheads in Fig. 6D). All the other retinal cells appeared unaffected, and thus Esg appears to be specifically required for the survival and/or maturation of the 2° and 3° pigment cells.

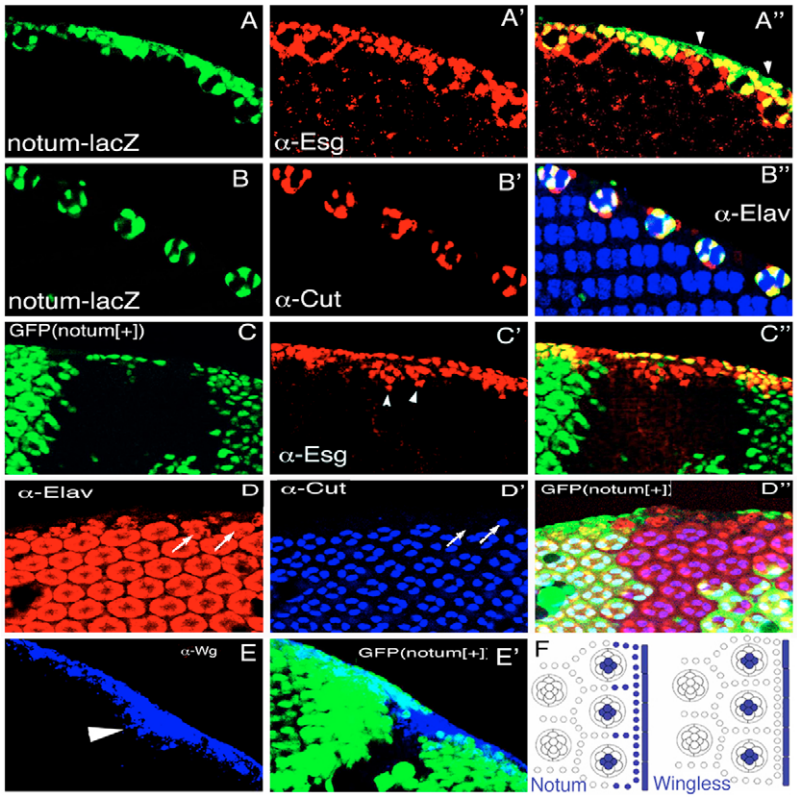

Fig. 5. The expression and function of Notum in the eye periphery. (**A-A″**) A *notum-Gal4*, *UAS-lacZ* pupal retina (~34 hours APF) doubly stained for *lacZ* (green) and Escargot (red). Notum and Escargot expression coincides in the HC (arrowheads), PR and some 2°/3° pigment cells. (**B-B″**) A *notum-Gal4*, *UAS-lacZ* pupal retina (~32 hours APF) stained for *lacZ* (green), Cut (red) and Elav (blue). Notum expression is detected only in the cone (green) and not photoreceptor cells (blue). The more interior cone cells (B′) are not visible at this focal plane. (**C-C″**) A *notum* clone in the pupal eye (~32 hours APF) marked black by the absence of GFP (green) showing precocious expression of Escargot (red) in the cone cells of peripheral ommatidia (arrowheads). (**D**,**D′**) Patterning defects in *notum* clones (absence of green in D″) of ~40 hours APF retinas. At this stage, the ommatidial death is in process, and degenerate cone cells (blue) and photoreceptors (red) can be observed in the second interior row of ommatidia that would not normally be affected (arrows). (**E**,**E′**) A *notum* clone in the pupal eye (~32 hours APF) marked black by the absence of GFP (green) showing an expansion of the Wg protein expression domain (blue, arrowhead). (**F**) Schematic summary of Notum expression in the marginal eye structures when compared with Wg expression (see also a summary of Escargot expression in Fig. 2G).

DISCUSSION

At the periphery of the developing fly retina, Wg emanating from the surrounding HC organizes a series of circumferentially arranged morphological specializations. We are working to understand how different levels of Wg signaling can direct the formation of the different specializations. Here, we have described the role of Snail group proteins and their specific role in mediating one aspect of the Wg patterning mechanism: the death of the peripheral ommatidia and the formation of the PR. The manipulations of Snail group proteins affected only the peripheral death mechanism; for example, no aberrations occurred in the specification of the DRO or bald ommatidia. However, the RNAi construct probably only reduces rather than removes *wor* function, and residual activity of this Snail family protein may hide any effects on these other aspects of peripheral patterning.

Direct regulation of Snail family proteins by Wnt/Wg pathway

We have demonstrated that Wg signaling regulates the expression of the Snail family genes, and have identified a number of TCF-binding sites in the region of the three Snail genes, which is consistent with, but not proof of a direct regulation by the Wg transduction pathway (data not shown). In mammalian systems, it had been shown that Snail transcription is elicited by the inhibition of glycogen synthase kinase-3 (GSK-3) (Bachelder et al., 2005) which represses Snail expression by inhibiting the transcriptional activity of NFκB on the Snail promoter (Bachelder et al., 2005; Barbera et al., 2004). In addition, GSK-3 can phosphorylate Snail at two consensus motifs, one for protein degradation (site I) and the other for subcellular localization (site II) (Zhou et al., 2004). Thus, in mammalian systems, Wnt signaling regulates Snail gene activity both at the level of the transcript and the protein.

Snail group protein expression and the apoptosis signal

The apoptotic removal of the most peripheral ring of developing ommatidia releases the surviving surrounding pigment cells to join and thicken the PR. Ectopic expression of Snail family proteins mimics the ommatidial death that is engendered by Wg expression, and loss of these proteins prevents the normal Wg-dependent removal of the peripheral ommatidia and consequently disrupts the PR. The Snail family transcription factors thus appear to direct the death of the peripheral ommatidia and development of the PR. However, within the peripheral ommatidia these proteins are expressed only in the cone cells – they are absent from the photoreceptors (R cells) and the 1° pigment cells. They are also present in the pigment cells surrounding the ommatidia. This expression profile raises a number of points.

(1) As the Snail family proteins are transcription factors, then the death signal is probably under their transcription control, but the molecular nature of the signal remains unknown.

(2) As the R cells and 1° pigment cells are directed to apoptosis by the expression of Snail family proteins in other cells, then there is non-autonomous death induction. We envisage the non-autonomous initiation of death in two possible forms. In the first model, the Snail-expressing cells sequester a survival factor that is thereby denied to other cells. Given that the cone cells express the Snail proteins but still die, this seems unlikely. The second model is that there is a factor released by Snail-expressing cells that directs the death of the ommatidial cells. The cells expressing the death factor may be the peripheral cone cells, the surrounding pigment cells or both. We favor the second model and the remainder of this discussion assumes this to be correct with appropriate reservation.

(3) The pigment cells surrounding the peripheral ommatidia are impervious to the death signal. One possibility is that the death signal is presented exclusively by the peripheral cone cells and only to the cells of the ommatidia (including themselves, and R cells and

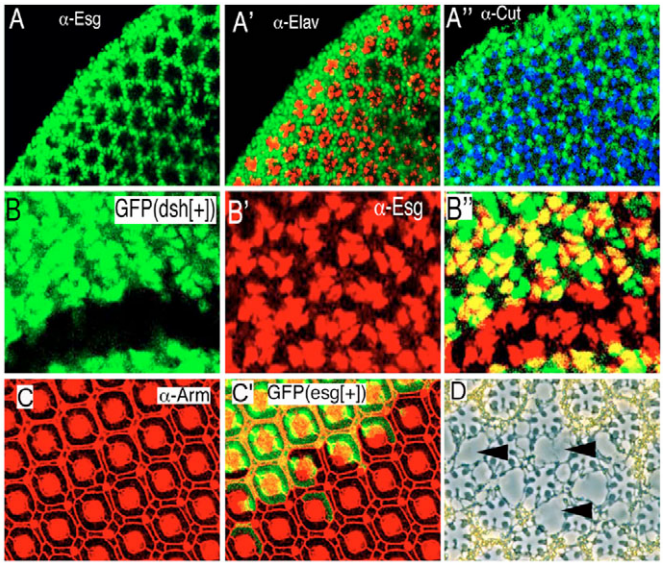

Fig. 6. Expression and function of Esg in late-stage differentiation of interommatidial pigment cells. (**A-C'**) Flat mounted pupal retinas of ~42 hour APF. (A,A') Wild-type pupal retina stained for Escargot (green), Elav (red) and Cut (blue), showing the selective expression of Escargot in the pigment cells surrounding the ommatidia. (B-B'') Pupal retina containing a *dsh[V26]* clone marked by the absence of GFP (green) stained for Escargot (red). Escargot expression is not perturbed in the interior interommatidial pigment cells. (C,C') Pupal retina containing a clone mutant for *escargot* (marked by the absence of green) is stained for Armadillo (red) that outlines the cellular profiles. The mutant pigment cells fail to undergo apical constriction. (**D**) Section through an adult eye in which *escargot* clones have been induced (marked by the lack of pigmentation). Interommatidial pigment cells are absent or defective, causing vacuolar structures (arrowheads) and ommatidial fusion (not shown).

1° cells) – not to the surrounding pigment cells. The cone cells die before the R cells (we have not examined the time of death of the 1° cells), and if the cone cells were the source of the death signal then they would probably receive the signal first. Alternatively, the pigment cells may release the death signal (secreted by themselves or the cone cells) but are programmed not to respond.

(4) Only the cone cells of the peripheral ommatidia express Snail family proteins (and Wg and Notum) in response to Wg signaling from the HC – the R cells and 1° cells do not. This probably represents a predisposition of the cone cells to respond to the Wg signal resulting from the selective expression of cone cell specific factors; Cut, for example, is a homeodomain transcription factor restricted to the cone cells at this stage.

Our finding that Snail transcription factors promote death in *Drosophila* eye periphery is in contrast to their anti-apoptotic roles in other systems. For example in *C. elegans*, the Snail-like CES-1 (cell death specification) protein blocks death of the NSM sister cells during embryogenesis (Thellmann et al., 2003). In vertebrates, Slug (Snail2) is aberrantly upregulated by the E2F-HLF oncoprotein in some leukemias, leading to increased cell survival (Inoue et al., 2002; Inukai et al., 1999). Mammalian Snail has also been shown to confer resistance to cell death induced by the withdrawal of survival factors in cell cultures (Vega et al., 2004). However, in the fly eye we describe a non-autonomous effect of Snail transcription family members in apoptosis, which suggests that a different molecular pathway is regulated from those of the autonomous examples above.

Wg signaling, the death of the peripheral ommatidia and the formation of the pigment rim

The death of the peripheral ommatidia appears to serve two functions – it removes these degenerate optical units (Lin et al., 2004) and it supplies cells for the PR that optically insulate the entire eye (Tomlinson, 2003). With regard to the PR, there are two sources of cells. First there is the thin layer of pigment cells that circumscribes the entire pupal eye and second there are the later cells, originally associated with the moribund ommatidia, that eventually incorporate into the existing PR to thicken it. Both aspects of PR formation appear to be under Wg signaling control. During the larval phase, the Hedgehog (Hh) morphogenetic wave sweeps the presumptive retina, triggering the ommatidial differentiation process (Heberlein et al., 1993; Ma et al., 1993). However, Wg is expressed in the flanking HC which inhibits the inductive mechanism (Treisman and Rubin, 1995). Thus, the larval retinal tissue directly adjacent to the HC does not undergo ommatidial differentiation (Fig. 7A). The 2° and 3° pigment cell fate appears to

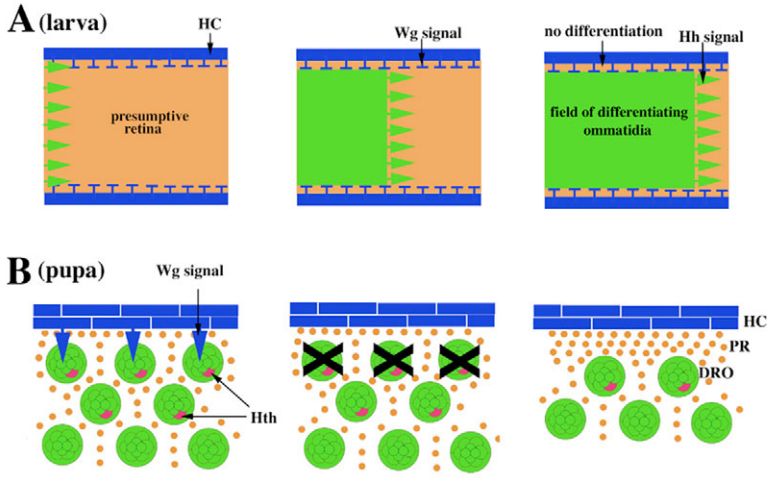

Fig. 7. Schematic description of how the pigment rim is formed. (**A**) Upper panel shows the presumptive retina at three stages of the third instar larva. Towards the left, the early retina is flanked by the Wg-secreting presumptive head capsule (HC, blue) and the Hh wave is incipient (green arrows). In the middle and to the right; as the wave sweeps the retina, the antagonistic Wg signal (blue t-stops) prevents ommatidia from differentiating close to the HC. (**B**) Lower panel shows a high power view close to the HC during the pupal phase. Left: the strip immediately adjacent to the HC is occupied by presumptive pigment cells because ommatidial differentiation was inhibited there. The outer two rows of ommatidia express Hth, thereby specifying them as DRO, but the most peripheral row also receives the Wg signal (blue arrows) that indirectly causes their apoptosis. Middle: the peripheral ommatidia die. Right: the pigment cells that surrounded the dying ommatidia now join the peripheral pigment cells to form the PR, and the most peripheral ommatidia are now the surviving DRO units.

be the ground state of the retinal tissue (Campos-Ortega and Gateff, 1976), and thus the cells directly adjacent to the HC are destined to the pigment cell fate. Later in the pupa, Wg signaling triggers the death of the peripheral ommatidia and releases their pigment cells to join the PR and increase its thickness (Fig. 7B).

The co-expression of Wg and Notum in the peripheral cone cells

The expression of both Wg and Notum (its antagonist) by the cone cells of the peripheral ommatidia is interesting. It may suggest that high levels of Wg expression are required in the peripheral cone cells, but that the diffusion of this cone-cell derived Wg needs to be tightly contained. For example, in the model above where the death signal is provided by the peripheral cone cells, high levels of Wg may be needed to trigger sufficient levels of the apoptotic signal but any diffusion of the high levels of Wg would disturb other aspects of the peripheral patterning.

In the absence of Notum, the effects of Wg signaling spread approximately one more ommatidial row into the eye periphery. This relatively mild phenotype suggests that there could be redundant mechanisms restricting the movement of Wg gradient at the eye margin. In *Drosophila* wing disc, the Wg receptor *Drosophila* Frizzled2 (Fz2) stabilizes Wg and allows it to reach cells far from its site of synthesis. Wg signaling represses Fz2 expression, creating a gradient of decreasing Wg stability towards the D/V boundary (Cadigan et al., 1998). This might also be the case in the eye periphery, where Wg signaling, in addition to activating Notum, might also represses Fz2 to limit the extent of Wg diffusion.

Escargot regulates the maturation of 2° and 3° pigment cells in the main retina

Snail family gene expression in the 2° and 3° pigment cells appears to be under two different control mechanisms; in the peripheral regions it is activated by Wg signaling, but in the main body of the eye it is not. Furthermore, the genes of the Snail complex appear functionally redundant in the periphery but not in the main body of the eye. Here, the phenotypes of *esg* clones are as strong as those of the mutations in all three genes. This may be explained by differential regulation of the gene promoters in the two positions. For example, in the main body of the eye, Esg expression in the 2° and 3° pigment cells may activate expression of the two other genes, but in the periphery, Wg signaling directly activates each of the genes, with no cross-regulation between them. The majority of studies on the specification of the main body 2° and 3° pigment cells have focused on the mechanism of weeding out the surplus inter-ommatidial cells which occurs between 18 hours and 36 hours APF (Bao and Cagan, 2005), but little is known about their subsequent maturation. Our data showed that Esg is expressed in the interommatidial pigment cells after the cell pruning mechanism, but before any sign of morphological differentiation. In the *esg* mutants, the 2° and 3° pigment cells do not undergo correct apical constriction, indicating that these cells are either developmentally delayed compared with their wild-type counterparts or are blocked in their maturation. If the cells are simply developmentally delayed, they should mature over time, but *esg* mutant clones in the adult eye show degenerate or lost 2° and 3° pigment cells (Fig. 6D). Thus, Esg appears required for the appropriate maturation/survival of the 2° and 3° pigment cells. What happens to the *esg* mutant pigment cells after the point when they fail to undergo apical restriction (whether they delaminate or die/degenerate in place) remains to be investigated.

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