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Drosophila retinal pigment cell death is regulated in a position-dependent manner by a cell memory gene

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ABSTRACT The stereotyped organization of the *Drosophila* compound eye depends on the elimination by apoptosis of about 25% of the inter-ommatidial pigment cell precursors (IOCs) during metamorphosis. This program of cell death is under antagonistic effects of the *Notch* and the EGFR pathways. In addition, uncharacterized positional cues may underlie death versus survival choices among IOCs. Our results provide new genetic evidences that cell death is regulated in a position- dependent manner in the eye. We show that mutations in *Trithorax-like* (*Trl*) and *Iola-like*/*batman* specifically block IOC death during eye morphogenesis. These genes share characteristics of both *Polycomb*-Group and *trithorax*-Group genes, in that they are required for chromatin-mediated repression and activation of *Hox* genes. However, *Trl* function in triggering IOC death is independent from a function in repressing *Hox* gene expression during eye development. Analysis of mosaic ommatidiae containing *Trl* mutant cells revealed that *Trl* function for IOC death is required in cone cells. Strikingly, cell death suppression in *Trl* mutants depends on the position of IOCs. Our results further support a model whereby death of IOCs on the oblique sides of ommatidiae requires *Trl*-dependent reduction of a survival signal, or an increase of a death signal, emanating from cone cells. *Trl* does not have the same effect on horizontal IOCs whose survival seems to involve additional topological constraints.

KEY WORDS: *programmed cell death, Drosophila, apoptosis, Polycomb, Notch, Egf receptor*

Introduction

Morphogenesis makes use of programmed cell death (PCD) as a means to control the precise number of cells that build a structure (McCarthy, 2003). The modalities of the cell death programs are diverse and evolutionary conserved (Baehrecke, 2002), thus allowing us to take avantage of *Drosophila* to decipher the developmental pathways controlling apoptosis (Hay *et al.*, 2004). In *Drosophila*, developmental cell death is in some cases determined by cell lineage, as is observed in the mechanosensory organ (Fichelson and Gho, 2003), or alternatively, by the tight control of cell-cell interactions as is observed during eye morphogenesis. Such interactions allow precise counting of pigment cells by PCD in the retina during metamorphosis which generates the quasi-crystalline organization of the mature compound eye (reviewed in Brachmann and Cagan, 2003). This counting process starts one third of the way through pupal development. At this stage, the retinal epithelium is composed of a loose hexagonal network of photoreceptors arranged in groups of eight cells, each covered by four cone cells that will produce the lens and two primary pigment cells (1°s) that surround the cone cells. Together these cells form a functional unit, the ommatidia. The lattice of this network is composed of three mechanosensory bristle units, as well as undifferentiated epithelial cells hereafter called

Abbreviations used in this paper: 1°, primary pigment cell; 2°, secondary pigment cell; AO, Acridine Orange; CS, Canton-S; IOC, inter-ommatidial cell; GFP, green fluorescent protein; PcG, Polycomb-group; PCD, programmed cell death; p.d., pupal development; trxG, trithorax-group; SEM, scanning electron microscopy; trl, trithorax-like.

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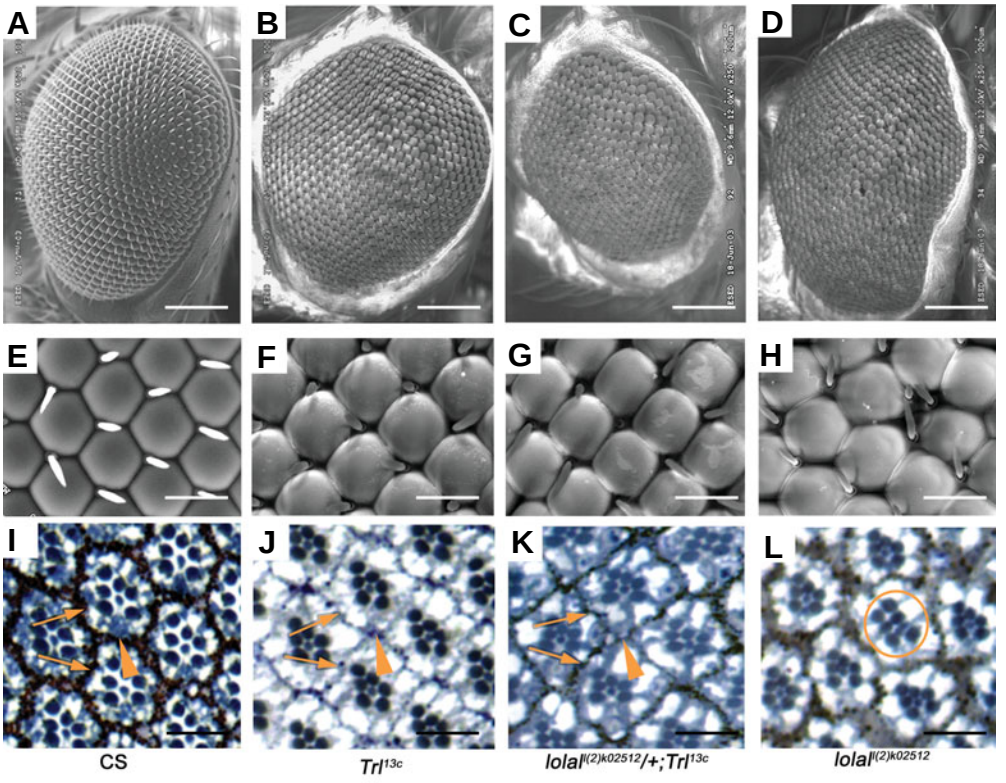

Fig. 1. Adult eye phenotype of *Trl^{13c}* and *lolal$^{l(2)k02512}$* mutants. *Scanning electron micrographs* **(A-H** *and tangential semi-thin sections* **(I-L)** *of adult eyes of* CantonS **(A,E,I)**, *homozygous* Trl^{13c} *escapers* **(B,F,J)**, lolal$^{l(2)k02512}$/+; Trl^{13c} *escapers* **(C,G,K)** *and* lolal$^{l(2)k02512}$ *escapers* **(D,H,L)**. *In* Trl^{13c} *escapers, the two horizontal sides (arrowheads in I,J,K) of each ommatidial hexagon appear shorter than the four oblique sides (arrows in I,J,K). This phenotype is enhanced in* lolal$^{l(2)k02512}$/+; Trl^{13c} *escapers, ommatidial arrangement tending towards a tetragonal rather than hexagonal network* **(K)**. *In* lolal *escapers* **(L)**, *a change in photoreceptor organization from a trapezoidal towards a more symmetrical motif (circled) suggests a change in their the identity of R3/R4 photoreceptors, which is not observed in* Trl^{13c} **(J)**. *The variation in the amount of dark pigment granules in the pigment cell layer observed in semi-thin sections* **(I-L)** *is irrelevant to the phenotypes (see Materials and Methods). Scale bar: 100 μm in (A-D, 20 μm in (E-H), 10μm in (I-L). Anterior to the left and dorsal up.*

interommatidial cells (IOCs). Selection of secondary and tertiary pigment cells (2°s and 3°s, resp.) among a double or triple layer of IOCs requires prior rearrangement of these cells into a monolayer between ommatidiae (Wolff and Ready, 1991). This alignment depends on the presence of an apical DE-cadherin belt in IOCs (Grzeschik and Knust, 2005). This adhesion belt is required for the proper localization of the transmembrane protein Roughest in 1°s at the interface with IOCs (Reiter *et al.*, 1996). Proper localization of Roughest appears to be a prerequisite for subsequent selection of 2°s and 3°s by apoptosis (Brachmann and Cagan, 2003; Reiter *et al.*, 1996). IOCs expressing Hibris, a transmembrane ligand of Roughest (Bao and Cagan, 2005), thus likely tend to maximize contact with 1°s and minimize contact between each other, thereby aligning themselves in a monolayer. Localization of Roughest is under the control of the Notch pathway (Gorski *et al.*, 2000; Grzeschik and Knust, 2005), functioning as a competence factor for death in IOCs (Miller and Cagan, 1998). Notch activation antagonizes EGFR signaling, which is required for the survival of IOCs (Miller and Cagan, 1998). Activation of the EGFR pathway was shown in laser ablation experiments to result from a signal originating from cells in the center of the ommatidiae, *i.e.* cone cells and 1°s (Miller and Cagan, 1998). This life signal antagonizes the proapoptotic RHG gene *hid*, upstream of the proteolytic cascade of caspases (Kurada and White, 1998; Yu *et al.*, 2002).

The mechanisms underlying the selection of IOCs which will die, while other IOCs will be spared, remains elusive. Recent real-time studies showed that cell death is non random and that the position of an IOC affects its fate (Monserrate and Baker Brachmann, 2006). Positional clues may come from the bristle cells, around which a “death zone” is located. In this zone, a reduced response to EGFR activation would be responsible for more frequent cell death than in cells located further away from the bristles. There also seems to be two positions where IOCs are protected from death, notably when IOCs are in contact with four 1°s from two adjacent ommatidiae, (position of the horizontal 2° pigment cell), or with three 1°s at the vertex between three ommatidiae (position of the 3°s).

Up to now, most of the information concerning the mechanisms selecting secondary and tertiary pigment cells involves cell-cell interactions. Little is known about transcriptional regulation involved in this process. A transcription factor, Klumpfuss, is differentially expressed in IOCs during pupal stages when selection occurs. Loss of function of *klumpfuss* specifically in IOCs is associated with extra pigment cells (Rusconi *et al.*, 2004). Genetic and molecular analysis indicates that Klumpfuss represses EGFR signaling in IOCs (Wildonger *et al.*, 2005). Its expression is under the direct regulation of the transcription factor Lozenge, which also acts in cone cells to activate *argos* directly, an antagonist of EGFR signaling (Wildonger *et al.*, 2005). Among more general transcriptional regulators, several *Polycomb* and *trithorax* Group genes (*Pc*G and *trx*G, resp.) were identified in a mosaic genetic screen as affecting eye development, independently of their effect on the expression of homeotic genes. However, they seem to act at a more upstream level than the selection of pigment cells (Janody *et al.*, 2004).

Two genes that share characteristics with both *Pc*G and *trx*G genes, *Trithorax-like* (Farkas *et al.,* 1994) (*Trl*) and *lolal* (also named *batman*, Faucheux *et al.*, 2001; Faucheux *et al.*, 2003; Mishra *et al.*, 2003) were previously reported as displaying rough eyes in mutant escapers (Farkas *et al.*, 1994; Faucheux *et al.*, 2003) This phenotype could be reversed by introduction of trans-

genic copies of these two genes, thereby indicating that both genes are involved in eye morphogenesis (Faucheux *et al.*, 2003). *Trl* and *lola* are known to be involved in the maintenance of cell positional identity through the regulation of *Hox* genes. *Trl* encodes the GAGA factor that binds to d(GA) repeats found in many Polycomb Response Elements, composite cis-regulatory sequences required for the maintenance of *Hox* gene expression (Mihaly *et al.*, 1998) and that of many other potential PcG/TrxG targets (Negre *et al.*, 2006; Ringrose *et al.*, 2003). LOLAL encodes a direct partner of TRL, and binds to the same sites as TRL on larval polytene chromosomes (Faucheux *et al.*, 2003). Many of these binding sites are not shared with other PcG and TrxG proteins (Faucheux *et al.*, 2003), suggesting that TRL and LOLAL may control many developmental processes independently of other *Pc*G and *trx*G genes. Here we show that the two transcriptional regulators TRL and LOLAL are required together for cell death in the pupal retina.

Results

Trithorax-like *mutations affect retinal pigment cell number*

The eye of wild type flies is composed of functional units called ommatidiae, arranged in a regular hexagonal network (Figure 1A). In contrast, the eye surface of Trl^{13c} escaper flies appears rough (Farkas *et al.*, 1994), as shown on Figure 1B. Although ommatidiae remain arranged in parallel rows in Trl^{13c} (Figure 1,

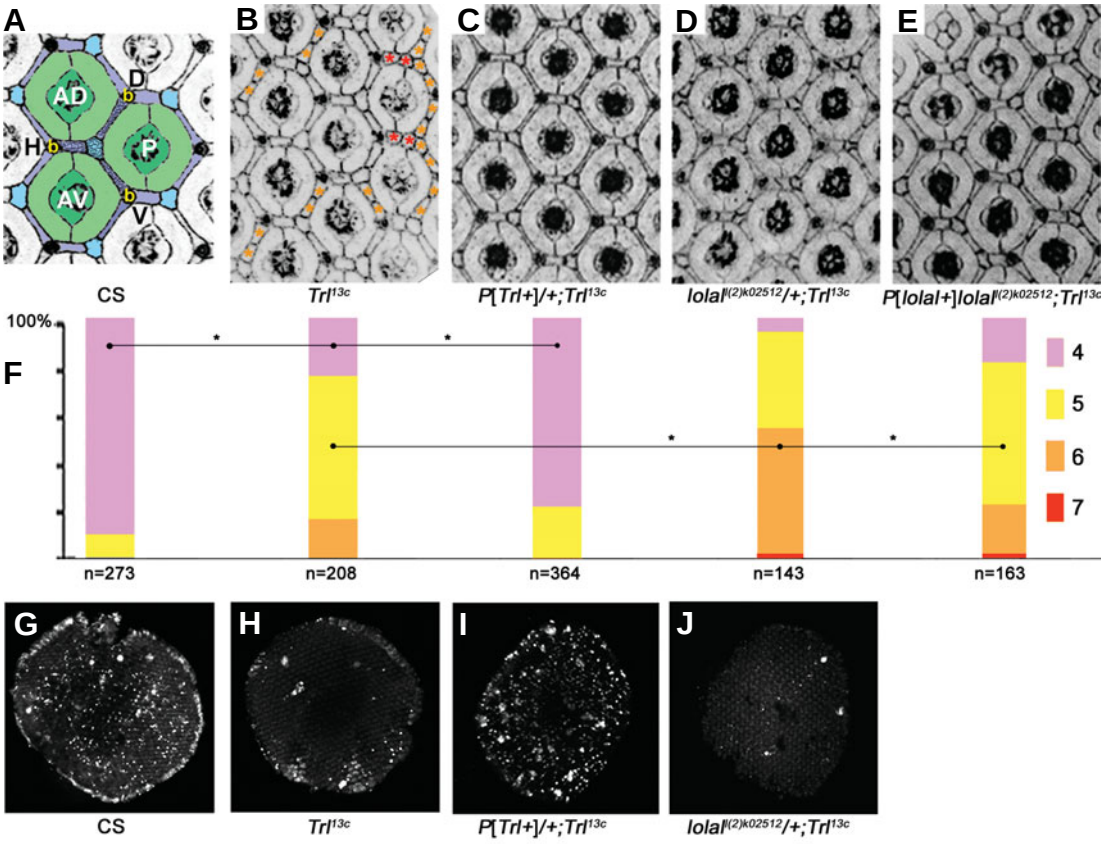

Fig. 2 . Cell count and cell death in Trl^{13c} pupal retina. (A-E) *Apical views of pupal retina after the peak of cell death (31% pupal development, anterior to the left) stained with anti-Armadillo antibody to outline cell membranes (black). Genotypes are indicated under each panel* **(A-E)** *and above the corresponding quantitative diagram* **(F)**. *Rescue experiments with transgenic copies of* Trl *(P[Trl+])* **(C)** *and* lola *(P[lola+])* **(E)** *are described in Materials and Methods.* **(A)** *Stereotypical organization of a wild type retina (CS). Ommatidiae are composed of 4 cone cells (dark green), located above photoreceptor cells (out of focus) and surrounded by two primary pigment cells (light green). Each ommatidia is surrounded by a monolayer of secondary pigment cells (2°, violet). These 2°s constitute the branches of an hexagonal network, in which the nodes are occupied alternatively by a bristle (b) and by a tertiary pigment cell (3°, blue). All these cells that fill the space between ommatidial units are generically called inter-ommatidial cells (IOCs). For quantitative analysis, we grouped IOCs in a "triskele" unit (shaded) that includes a total of four IOCs (three 2° plus one 3°) between three consecutive bristles in the wild type (CS). Triskeles are subdivided into three branches. The position of the bristle on the horizontal branch (H) defines the anterior pole, as well as the dorsal oblique (D) and ventral oblique (V) branches. Three ommatidiae (colored) are adjacent to each triskele in positions anterior-dorsal (AD), anterior-ventral (AV) and posterior (P). In* **(B)**, *orange stars indicate the presence of additional IOCs on oblique branches, whereas red stars indicate the presence of additional IOCs on horizontal branches.* **(F)** *Distribution of the number of IOCs per triskele (4, 5, 6, or 7). Comparisons were done for each pair of genotypes using the Khi-squared test. P-values inferior to 10^{-7} are indicated by a horizontal bar for each pair analyzed. Note that in cases where bristles were missing or displaced, the corresponding triskeles were not considered.* **(G-J)** *Acridine Orange (AO) staining of the retina at 31% pupal development (50 h p.d. at 20ºC).* **(G)** *Maximum levels of AO staining are detected at this stage in wild-type control retina (CS).* Trl^{13c} **(H)** *and* $lola^{2512}/+;Trl^{13c}$ **(J)** *pupal retina at the same stage show reduced AO staining.* P[Trl+]/+; Trl^{13c} *retina* **(I)** *show levels of AO staining similar to that of* CS.

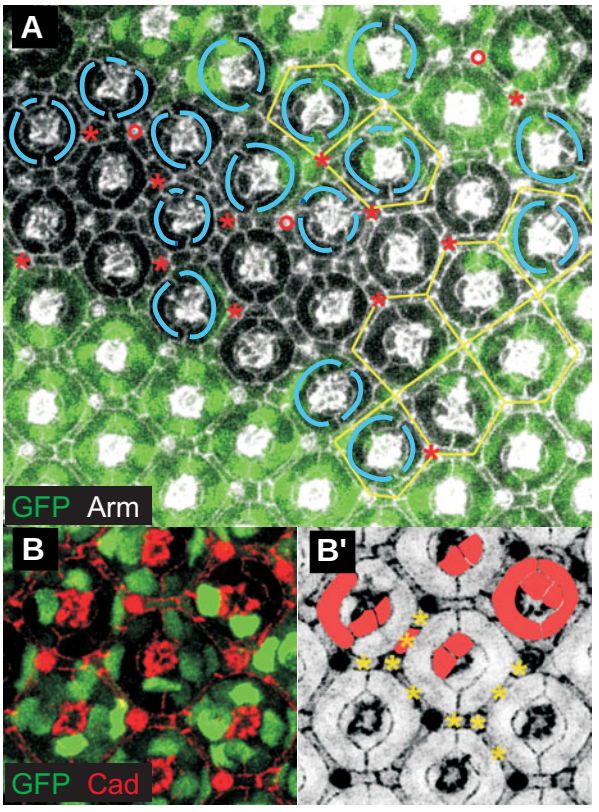

Fig. 3. Phenotype of *Trl$^{81.1}$* clones in 37 h p.d. mosaic retina. *Cell shape is revealed using anti-Armadillo (Arm); white in (A), or anti-Cadherin (Cad); red in (B), black in (B') and mutant cells are identified by the lack of GFP expression.* **(A)** Trl$^{81.1}$ *clones exhibit various defects in their organisation. Within the clone, bristles are often absent (red stars) or misplaced (red circles). Mutant and mosaic ommatidiae also present defects in the alignment, number and size of 1°s (blue lines), associated with misplacement of the junction between 1°s. Regardless of bristle and 1° defects,* Trl$^{81.1}$ *clones exhibit supernumerary inter-ommatidial cells (IOCs). In this field, mosaic ommatidiae located at the clone border present a pentagonal structure (yellow lines), and organize astrally around bristles.* **(B,B')** *Mosaic* Trl$^{81.1}$ *ommatidiae with few mutant cells. At this focal plane, GFP is detected in surface cell nuclei* **(B)**. Trl$^{81.1}$ *mutant cells are identified by the absence of GFP; false colored in red in* **(B')**. *Presence of extra IOCs in a triskele branch correlates with that of mutant cones in an adjacent ommatidia, irrespective of the IOC genotype. In this field, two triskeles containing supernumerary IOCs are identified by the presence of 5 or more IOCs (yellow stars) between three bristles. Such triskeles with extra IOCs are referred to as "T5s".*

compare E,F), they form a network that differs from that observed in the wild type. Whereas each side of the hexagon is equivalent in size in the wild type when observed on semi-thin sections (Figure 1I), in *Trl^{13c}* the two horizontal sides of each ommatidial hexagon, parallel to the equator (arrowhead in Figure 1J), appear shorter than the four oblique sides (arrows in Figure 1J). Nonetheless, photoreceptor groups contain the same number of rhabdomeres arranged in a trapezoidal motif as that observed in the wild type (compare Figure 1 I,J). The rare *lola$^{(2)k02512}$* mutant escapers also display rough eyes (Figure 1D). However, this phenotype differs from that of *Trl^{13c}* escapers in that alignment of ommatidiae is perturbed (Figure 1 D,H,L), ommatidiae are sometimes missing (not shown) or misoriented, and photoreceptors may be affected in their identity (circled in Figure 1L). Whereas heterozygous *lola$^{(2)k02512}$* flies have wild type eyes (data not shown), the heterozygous *lola$^{(2)k02512}$* context, however, enhances the phenotype of *Trl^{13c}* homozygotes, ommatidial arrangement tending towards a tetragonal rather than hexagonal network (Figure 1K), with repositioning of bristles at each corner between four ommatidiae (Figure 1G). Sections of the retina from *lola$^{(2)k02512}$/+; Trl^{13c}* flies did not reveal alteration of photoreceptor position or identity as is observed in *lola$^{(2)k02512}$* homozygotes (Figure 1 K,L), thereby indicating that *lola* function in photoreceptor development may not depend on its interaction with *Trl*. In contrast, the enhancement of *Trl* phenotype by a loss of a single dose of *lola*, without any alteration of photoreceptors, suggests that *lola* and *Trl* cooperate in the arrangement of the regular hexagonal ommatidial network.

The structure of the mature retina is established after the first third of pupal development (p.d.). Cell types can be identified by their shape and position in the pupal retina using membrane-associated markers such as E-cadherin or Armadillo (Figure 2A). In an IOC unit defined by the triskele motif drawn between three consecutive bristles (b in Figure 2A), four pigment cells (three 2°s plus one 3°, outlined in blue in Figure 2A) are counted in almost all the cases (Figure 2 A,F) in a wild type mature retina (37 h p.d.). In contrast, five or more cells are found in a large majority of *Trl^{13c}* triskeles at the same stage (76%, marked by stars in Figure 2B). These cells are usually aligned in a single layer separating adjacent ommatidiae, as is observed in the wild type retina at this stage. This phenotype is reversed to wild type in the presence of at *Trl+* transgene (material and methods, Figure 2 A,C,F), indicating that mutation of *Trl* is indeed responsible for the presence of supernumerary IOCs in *Trl^{13c}* escapers. When one dose of *lola* is removed in a *Trl^{13c}* mutant, the frequency of triskeles containing 5 or more IOCs increases from 76% to 95% (Figure 2 D,F). When one copy of *lola+* is added to these *lola$^{(2)k02512}$/+; Trl^{13c} / Trl^{13c}* flies, this frequency falls to 75%, similar to that of *Trl^{13c}* (Figure 2 E,F). Therefore, we conclude that *lola* function is also required in the developmental process that leads to the correct number of pigment cells.

Since amorphic *Trl* alleles are recessive lethal, homozygous mutant clones for the *Trl$^{81.1}$* amorphic allele (Greenberg and Schedl, 2001) were generated (materials and methods, Figure 3). Mutant clones generated during early second instar appear equivalent in size to wild type twin clones (data not shown), indicating that the complete loss of *Trl* function does not significantly affect cell viability under these conditions. The loss of *Trl* in large clones does not lead to a loss of ommatidiae (Figure 3A). Therefore, the function of *Trl* is not required for cell viability or growth in the eye disc. A major effect of the loss of *Trl* function in *Trl$^{81.1}$* clones is the presence of numerous supernumerary IOCs in the mature retina (Figure 3A, triskeles marked with stars in Figure 3B'), as is observed in *Trl^{13c}* escapers, thereby indicating that this phenotype is not allele specific. In addition, bristles are often missing or displaced (stars and circles, respectively, in Figure 3A), the shape of 1°s of a single ommatidia is often asymmetrical (blue bars on Figure 3A), and in some cases additional 1°s are present. The frequent lack of bristles in the eye is compatible with the *Trl* loss of function phenotype described in other regions such as thoracic segments (Bejarano and Busturia,

2004). The $Trl^{81.1}$ phenotype in the retina thus includes phenotypes not detected in Trl^{13c}. The difference in severity between the two alleles is expected from previous work in which the two alleles were compared, in particular for their lethality (Faucheux *et al.*, 2003; Greenberg and Schedl, 2001). In mosaic regions in which *Trl* mutant cells and heterozygous or wild type cells are intermingled, the main defect is the presence of additional IOCs, often in the absence of any defect in the number or correct position of 1°s and bristles (Figure 3 B,B'). Based on the study of Trl^{13c} mutant escapers and $Trl^{81.1}$ mutant clones, in particular mosaic ones, we thus conclude that *Trl* function is required in the eye for the presence of the correct number of IOCs.

In order to address whether cell death was affected in Trl^{13c} mutants, Acridine Orange (AO) staining of Trl^{13c} mutant pupal retina was compared to that of wild type. At the stage when a peak of PCD is observed in IOCs in wild type individuals (31% p.d.) (Wolff and Ready, 1991; Yu *et al.*, 2002), AO labeling was always strongly reduced in Trl^{13c} mutants (Figure 2 G,H respectively). This reduction is not compensated for by an extended cell death period as shown by the absence of detectable AO labeling at 40% p.d., which corresponds to the end of the peak of cell death in the wild type retina, nor by earlier cell death before 30% p.d (data not shown). The presence of one copy of the Trl^{+} transgene (see material and methods) allows restoration of the wild type AO staining pattern in Trl^{13c} homozygotes (Figure 2I), thereby indicating that *Trl* function is required for apoptosis in pupal

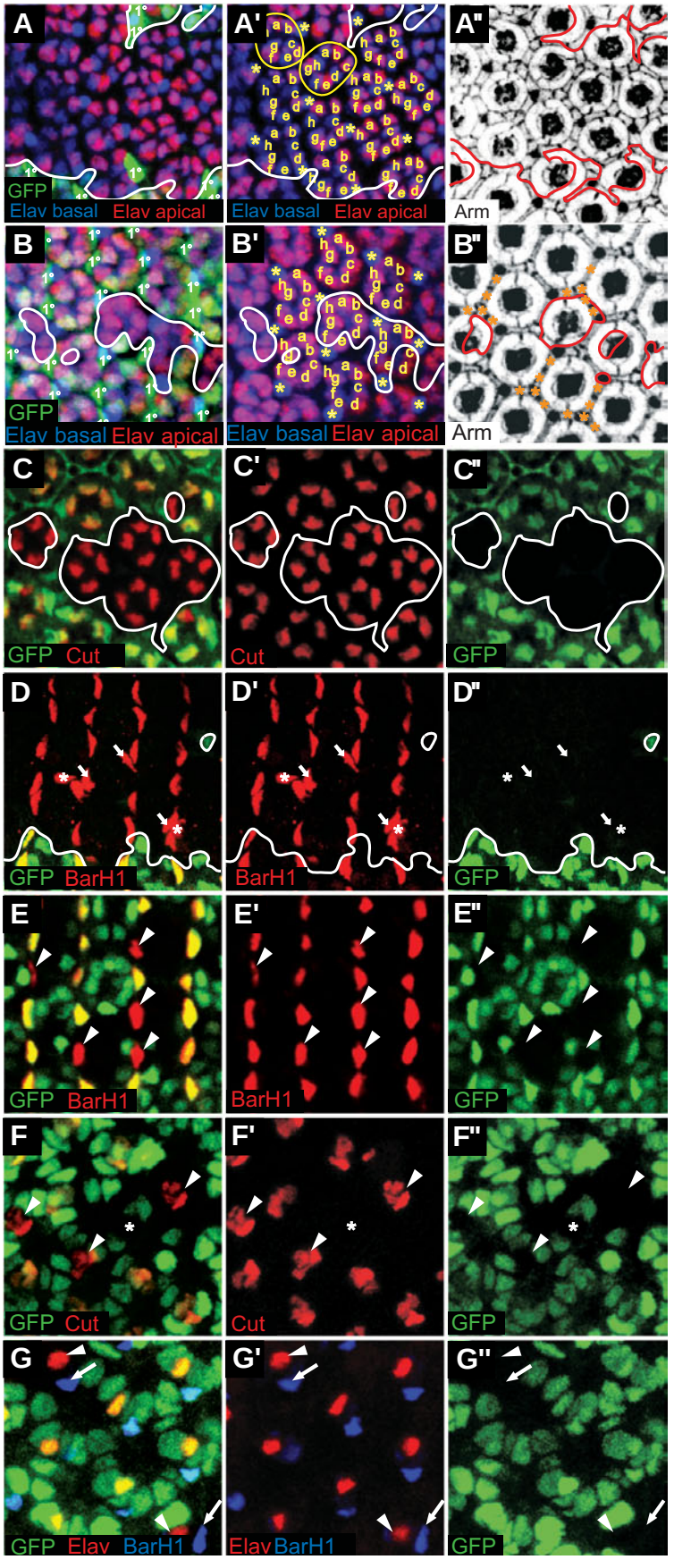

Fig. 4. Expression of retinal differentiation markers in $Trl^{81.1}$ clones. *Mutant cells are identified by the lack of GFP expression at 37 h p.d.* **(A-B")** *or 42h p.d.* **(C-G")**. **(A-B")** *Anti-Elav reveals neuronal nuclei* **(A,A', B,B')** *and anti-Armadillo outlines membranes; black in* **(A",B")**. *Elav signal was numerically separated between the blue chanel for basal sections and the red channel for apical sections, so that Elav-positive nuclei could be identified depending on their position* **(A,A', B,B')**. *The 8 photoreceptor nuclei are marked by yellow letters, basal signals corresponding to bristle group neuronal nuclei are indicated by stars* **(A', B')** *and 1°s nuclei expressing GFP are marked; 1° in* **(A,B)**. *Limits of clones are outlined (white line at the level of photoreceptor nuclei in A-A' and B–B', red line at the level of apical Armadillo signal in A'', B'').* **(A-A")** *In large clones the number of photoreceptor nuclei is not significantly affected in* Trl *ommatidiae, but their arrangement is sometimes irregular (see for example the two groups of photoreceptor nuclei surrounded by a yellow line in A').* **(B-B")** *In mosaic ommatidiae, the presence of supernumerary IOCs (stars in B'') is not associated to alteration of the number and the arrangement of photoreceptor nuclei when compared to neighboring GFP-positive ommatidiae.* **(C-C")** *Cone cell nuclei are visualized with anti-Cut staining in apical sections (red). Limits of clones are outlined (white). Number and arrangement of cone cell nuclei is not disturbed in* Trl *mutant clones.* **(D-E")** *Anti-BarH1 identifies 1°s nuclei in apical sections (red). In large* Trl *clones* **(D-D")**, *some 1°s nuclei are misplaced (arrows) and additional 1°s are sometimes present (stars) whereas in the case of mosaic ommatidiae* **(E-E")**, *number and arrangement of 1°s nuclei is not affected.* **(F-G")** *Anti-Cut identifies the four nuclei of each bristle group in basal sections (red in F-F''), among which one is stained by anti-Elav (red in G-G'') and another is labeled with anti-BarH1 (blue in G-G"). Mutant bristles cells are marked by arrows and arrowheads. A star marks the position where a bristle is missing* **(F-F")**.

retina. Removing one wild type copy of *lola* in an otherwise Trl^{13c} homozygous context slightly reduces AO staining when compared to that in Trl^{13c} individuals (Figure 2J).

Taken together, these results suggest that *Trl* function is required for reducing the number of secondary and tertiary pigment cells in the retina through the activation of programmed cell death at the first third of the pupal stage.

Cell identity is maintained in Trl *mutant clones*

Trl and *lola* are both known for their function in maintaining positional indentity along the anterior-posterior axis, as defined by the expression of Hox genes. However, Hox protein expression from both the Antennapedia (Scr, Antp) and the Bithorax (Ubx, Abd-B) complex was found to be unaffected by the loss of *Trl* function in the eye disc (data not shown). We next asked whether the phenotypes observed in $Trl^{\beta 1.1}$ mutant clones correlate with any alteration in the determination of specific ommatidial cell types, as defined by the expression of specific markers. In the mature retina, ommatidiae included in $Trl^{\beta 1.1}$ mutant clones contain the expected number of eight photoreceptors that are positive for the neuronal marker Elav (Figure 4 A-B''), four cone cells positive for Cut (Figure 4 C-C''), and two 1°s positive for BarH1 (Figure 4 D-E''). Therefore, early steps of eye morphogenesis that lead to the determination of these cell types are not affected by the loss of *Trl* function. In the case of large $Trl^{\beta 1.1}$ clones, photoreceptor nuclei arrangement is less regular than in wild type regions (Figure 4 A-A'), mutant 1°s can be misaligned and sometimes additional 1°s are present (Figure 4 D-D'', stars and arrows, respectively), when compared to neighboring heterozygous or wild type ommatidiae. However, cone defects, which are good sensors of early developmental defects, were rarely found. Importantly, induction of small $Trl^{\beta 1.1}$ clones included in mosaic ommatidiae leads to survival of extra IOCs in a context devoid of alteration in the number and arrangement of photoreceptors, cones, and 1°s (Figure 4 B-B'' and E-E'' and Table 1). Therefore, alteration of cell death of IOCs due to loss of function of *Trl* can be uncoupled from alterations in photoreceptor and 1°s arrangement that can be observed in large clones.

In some cases, mainly in the center of large mutant clones, a few bristles groups were absent (star in Figure 3A and Figure 4 F-F''), or misplaced (circled in Figure 3A), indicating that a function of *Trl* in the specification of eye bristle groups cannot be excluded. However, the identity of mutant bristle cells, when present, appears unaffected since they always contain the expected group of four Cut expressing cells (arrowhead in Figure 4 F-F''), including an Elav positive cell (arrowheads in Figure 4 G-F'') adjacent to a BarH1 positive cell (arrows in Figure 4 G-G'').

In conclusion, the number of IOCs is specifically affected in both Trl^{13c} and $Trl^{\beta 1.1}$ mutant contexts, and this phenotype can be dissociated from defects in cell determination of neurons, bristles, cones and 1°s cells. Therefore, *Trl* plays a specific function in the control of developmental apoptosis of supernumerary IOCs during pupal stages.

Trl *function is required in cone cells for reducing the number of pigment cells*

During retinal morphogenesis, *Trl* and *lola* are both expressed in the nuclei of all cell types of the retina (Figure 5 and supplementary data). In order to identify which of the various ommatidial cell types require the function of *Trl* for the proper counting of IOCs, $Trl^{\beta 1.1}$ mosaic experiments were analyzed. Mosaic ommatidiae with missing or misplaced bristles were excluded from this analysis. For triskeles containing extra IOCs (hereafter called T5s for triskeles containing five cells or more), the genotype of each IOC and bristle cells as well as that of cone and 1°s composing adjacent ommatidiae was determined (Table provided as a supplementary file).

The frequency of T5s containing at least one $Trl^{\beta 1.1}$ mutant IOC (43/83) is similar to that of T5s containing only wild type or heterozygous IOCs (40/83) (see examples on Figure 3B'). Therefore, a $Trl^{\beta 1.1}$ mutant IOC genotype is not a prerequisite for the presence of additional pigment cells, indicating that at least part of *Trl* function in triggering apoptosis of IOCs is non cell-autonomous.

Analysis of the genotype of cells composing ommatidiae surrounding T5s revealed that almost every T5 (93%, n=83) is adjacent to at least one $Trl^{\beta 1.1}$ mutant cone cell. In cases where a single mutant cell is found among those composing the mosaic T5 and adjacent ommatidiae (6/83), this cell is always a cone cell. Most strikingly, when branches containing supernumerary IOCs are analyzed separately, at least one mutant cone cell is almost always (91%, 79/87 branches) located in the ommatidia directly adjacent to the branch of the triskele where supernumerary cells were found (Figure 3 B,B'). The mutant cone cell is however not necessarily the closest cone cell to the branch with additional IOCs. In addition, in the five cases where more than one arm of

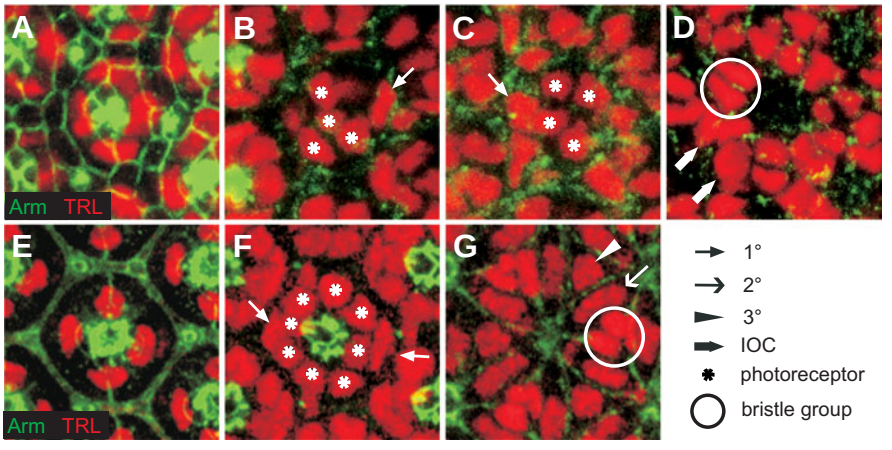

Fig. 5. Expression of *Trl* in the retina during pupariation. (A-D) *24 h p. d. and* **(E-G)** *42 h p. d.. Cell borders are outlined with anti-Armadillo (green). The expression of TRL (red) is detected in nuclei. For each stage, z-axis projections were performed at the apical* **(A,E)**, *medial* **(B,C,F)** *and basal* **(D,G)** *levels of the ommatidia, in order to identify all ommatidial cell types. The most apical nuclei are those of the four cone cell nuclei* **(A,E)**. *Medial projections allow to count the expected 8 photoreceptor nuclei (stars in B,C,F) as well as that of the two 1°s (arrows in B,C,F). Bristle group cells (circled) and numerous nuclei from IOCs are visible (thick arrow), yet forming an irregular pattern, at the basal level at 24h p. d. At 42 h p. d., when supernumerary IOCs have been eliminated, the remaining 2° (arrows) and 3° pigment cells (arrow heads), occupying stereotypical basal positions, all express TRL* **(G)**.

the triskele contains additional cells, mutant cone cells are usually present in at least two ommatidiae (4/5). Together, these results suggest that the presence of at least one *Tr/* mutant cone cell is a prerequisite for triggering additional IOCs in adjacent ommatidiae, irrespective of the IOC genotype. Therefore, *Tr/*function is necessary in cone cells in order to promote the elimination of supernumerary IOCs.

Next we wanted to address whether *Tr/*function is also necessary in IOCs for the control of PCD. If this was the case, the presence of mutant IOCs on one branch of the triskele is expected to increase the probability of finding additional IOCs on this same branch when compared to branches with no mutant IOC, in situations where neighboring cells are not mutant for *Tr/*. However, when at least one mutant IOC is found on one branch, it is always associated with at least 3 mutant cells of other cell types, and in all but one case, these cells are cone cells located in an adjacent ommatidia. In addition, the frequency of mutant IOCs in ommatidial unit. Taken together these results suggest that the function of *Tr/* is region-specific and predominantly required for the elimination of oblique rather than horizontal IOCs.

We found that when oblique cells are of the wild type or heterozygous genotype, there is 49% chances to find extra IOCs on the dorsal branch (30/61, Table 1) whereas this frequency drops down to 30% on the ventral branch (17/56). Analysis of the genotype of cone cells in neighboring ommatidiae shows that this significant difference ($P < 5\ 10^{-4}$) does not correlate with a bias in the distribution of mutant cone cells on the dorsal versus the ventral ommatidiae, adjacent to the branch considered (see data in supplementary table). This result suggests that oblique cells are not equivalent between dorsal and ventral branches in their response/dependency to a *Tr/*-dependent signal from cone cells.

Our analysis also revealed that when supernumerary IOCs are present on horizontal branches, 1° pigment cells are usually misaligned. In the wild type, the single horizontal 2° pigment cell

TABLE 1

GENOTYPE AND NUMBER OF IOCS PER BRANCH IN TRISKELES CONTAINING EXTRA IOCS (T5S) FROM *TRL$^{81.1}$* MOSAIC OMMATIDIAE AT 37H P.D.

IOCs genotype[1]		**+**					***Trl^{-}***					
Branch phenotype[2]		**normal**		**supernumerary**		***Total***	**normal**		**supernumerary**		***Total***	***n*[5]**
T[3]		135	74%	48	26%	*183*	28	42%	38	58%	*66*	249
	H[3]	65	98%	1	2%	*66*	10	59%	7	41%	*17*	83
Regional distribution	D[4]	31	51%	30	49%	*61*	5	23%	17	77%	*22*	83
	V[4]	39	70%	17	30%	56	13	48%	14	52%	27	83

A total of 83 T5s were analyzed (data are available as a supplementary file). Each triskele is constituted of three branches. Since four among 83 mosaic triskeles analyzed contained extra IOCs on two branches, the total number of branches with supernumerary IOCs is 87. The last column indicates the total number of branches analyzed. [1]The genotype of IOCs (as defined by GFP expression levels) found on each branch was determined, and branches placed in two categories, "*Trl*-" when at least one IOC had no GFP expression (*Tr$^{81.1}$* mutant IOC), and "+" (corresponding to the *Tr$^{81.1}$*/+ and +/+ genotypes) otherwise. [2]IOCs were counted on each branch, allowing distinction of two phenotypes, "normal" corresponding to the wild type number, "supernumerary" indicating the presence of supernumerary IOCs on this arm. Percentages indicate the frequency of branches with or without additional IOCs within each genotypic category. [3]Results are presented as the number of branches in each case, irrespective of the position (T), or according to the horizontal (H), the oblique dorsal (D) or the oblique ventral (V) position in the triskele (see Figure 2). The distribution of mutant *Tr$^{81.1}$* cells on the H branch versus oblique branches (D+V) is equivalent (P>0.4). The H and oblique (D+V) distributions of supernumerary IOCs are different ($P < 10^{-7}$). [4]The frequency of supernumerary IOCs increases significantly from 2% to 41% ($P<2\ 10^{-3}$) on the horizontal (H) branch and from 39% to 70% on the oblique (D + V) branches ($P<3\ 10^{-4}$) in the presence of at least one mutant IOC. However, this result could not be interpreted in terms of cell autonomous function of *Tr/*, as discussed in the text. [5]*n* is the total number of branches analyzed.

a triskele unit increases linearly with that of mutant neighboring ommatidial cells (Supplementary data). As a consequence, the effect from IOCs genotype cannot be dissociated from that of the environment. Therefore, we could not address whether the genotype of IOCs infers on the number of additional pigment cells.

In conclusion, the mosaic analysis indicates that a wild type function of *Tr/* in cone cells is a pre-requisite for the control of the correct number of IOCs.

Analysis of Trl *function reveals separate mechanisms for the control of cell death in oblique and horizontal IOCs*

Strikingly, in *Tr^{l3c}* escapers, 87% of additional IOCs (n=190) are located on the oblique branches of the triskele. In *Tr$^{81.1}$* mosaic retina, we also found that in most of the triskeles that contain additional cells (i.e. T5s), these cells were located on the oblique branches (90%, 78/87, Table 1), whereas only few T5s had additional cells on the horizontal branch (9.2%, 8/87, Table 1). The distribution of mutant cells between oblique and horizontal branches for IOCs and bristle cells (see footnotes in Table 1 and supplementary file), or between each of the three sectors, dorsal, ventral, and posterior for cone and 1° cells, is not statistically different. Therefore, the differential effect of *Tr/* mutation on the presence of oblique versus horizontal supernumerary IOCs is not due to a bias in the distribution of mutant cells composing the contacts, dorsally and ventrally, the junctions formed by two 1°s, and these junctions are aligned between ommatidiae on the dorsal-ventral axis (Figure 2A). In contrast, this cellular organization is modified in 80% of the cases where extra IOCs are found in *Tr^{l3c}* escapers (20/25, Figure 2B) and 75% (6/8, data not shown) in mosaic *Tr$^{81.1}$* ommatidiae. In these cases, two horizontal IOCs are present, each contacting only one junction between 1°s either dorsally or ventrally, and 1°s are not correctly aligned on the dorsal-ventral axis (Figure 2B and data not shown). In *Tr$^{81.1}$* mosaic ommatidiae, the same is observed independently of the genotype of the 1°s. Such a correlation suggests that mechanisms involved in the regulation of PCD in IOCs by *Tr/* differ between horizontal and oblique positions.

Discussion

Cell death and cell memory genes

Polycomb and *trithorax* group genes are mainly known for their role in epigenetic maintenance of homeotic gene expression in *Drosophila*. Among PcG and TrxG proteins, TRL is one of the few that has the capacity to bind DNA directly, potentially acting as an anchor and/or a docking site for PcG/TrxG complexes. *Tr/* encodes the GAGA factor, which is also thought to establish a chromatin state compatible with transcription possibly by replac-

ing nucleosomes (Hagstrom *et al.*, 1997; Lehmann, 2004). However, TRL is not sufficient to define an on/off transcriptional state which requires other maintenance proteins of the PcG/TrxG (Bejarano and Busturia, 2004). Here we focused on the role of *Trl* in the morphogenesis of the eye, a developmental process that does not involve homeotic genes of the *Antennapedia* and *Bithorax* complexes. Despite the early discovery of the rough eye phenotype in rare escapers of the hypomorphic allele Trl^{13c} (Farkas *et al.*, 1994), nothing was known about the cellular basis for this phenotype. Since induction of amorphic Trl^{R85} clones was later shown to reduce the size of the eye in a *Minute* mutant background (Bejarano and Busturia, 2004), *Trl* may control growth, which may be a possible explanation of the rough eye phenotype in Trl^{13c} escapers. However, we could not detect major growth differences between null $Trl^{\beta1.1}$ clones and wild type twin clones in mosaic retina, thus suggesting that mechanisms other than those affecting cell growth are responsible for the rough eye phenotype. Indeed, our results demonstrate that the rough eye phenotype of *Trl* mutants results from a specific requirement for *Trl* function in developmental cell death of interommatidial cells. Other *trx*G and *Pc*G genes involved in eye development were previously identified in a mosaic genetic screen (*Pc*, *E(z), trx, kto, skd and brm*) (Janody *et al.*, 2004). Loss of function of these genes leads to diverse defects including the loss of photoreceptors, which distinguishes these genes from *Trl*. In addition, derepression of *Ubx* was observed in *Pc* and *E(z)* mutant clones, and *Ubx* ectopic expression in the eye could at least partially phenocopy the loss of function of *Pc* and *E(z)*, suggesting that homeotic gene regulation plays a part in the eye phenotype of these two *Pc*G mutants. In contrast, we and others (Bejarano and Busturia, 2004) did not detect misexpression of homeotic genes from the *Antennapedia* and *Bithorax* complexes in the eye in *Trl* null clones. It is thus likely that the block in IOC death in *Trl* mosaic retina does not result from a loss in cell identity as defined by the restricted expression of *Hox* gene.

In genetic assays conducted previously, no dominant interactions were detectable between *Trl* and *Pc* or *ph* for the eye phenotype (our unpublished results), whereas a genetic interaction in *Hox* gene regulation was found between *Trl* and a second member of the *Pc*G / *trx*G, *lola*/ (Faucheux *et al.*, 2003; Mishra *et al.*, 2003). Since interactions have not been systematically tested between *Trl* and other *Pc*G / *trx*G genes for the eye phenotype, it cannot be excluded that other members of these groups control cell death in the eye. Comparison of polytene chromosome binding sites between TRL and other PcG proteins suggests that many *Trl* functions do not require interaction with other *Pc*G member (Faucheux *et al.*, 2003).

Several reports indicate a requirement for *Pc*G genes for cell survival in Drosophila (Bello *et al.*, 2007; Saget *et al.*, 1998; Smouse *et al.*, 1988) as well as in mammals (Liu *et al.*, 2006; Miyazaki *et al.*, 2005), rather than for cell death. One exception is *lola* (Paige Bass *et al.*, 2007), first characterized as an axonal guidance gene (Giniger *et al.*, 1994), and more recently as coding an epigenetic factor of the Polycomb group (Ferres-Marco *et al.*, 2006). The function of *lola* appears to be required in the ovary for chromatin condensation during cell death. Since LOLA, TRL and LOLAL share a common BTB/POZ protein interaction domain, and since TRL has already been shown to control chromosome structure (Bhat *et al.*, 1996), the three proteins may thus potentially function as partners at the level of chromatin structure in programmed cell death. One can hypothesize that the lack of *Trl* function would impair cell death due to its requirement for accomplishment of the cell death program at the step of chromatin condensation. However, this hypothesis is not compatible with our results demonstrating a function of *Trl* in cone cells for promoting programmed cell death of IOCs (see below). Therefore, we propose that the two BTB/POZ-containing, epigenetic factors, *Trl* and *lola*, may not have identical functions in the control of cell death.

Multiple functions of Trl *in the precise counting of secondary and tertiary pigment cells*

During early pupal stages, before cell death normally occurs in the wild type, the number of IOCs in Trl^{13c} escapers and in $Trl^{\beta1.1}$ amorphic clones (3 days after clone induction) does not appear different from wild type, indicating that *Trl* mutant cells are not significantly affected in their proliferation in the eye disc. Later, when developmental cell death is at its climax in the wild type, AO staining is almost absent when *Trl* function is reduced, and subsequently, in the mature retina, extra pigment cells are found. Strikingly, defects in the number of IOCs is often found in the absence of any morphological defects in the overall structure of the retina in escapers, indicating that *Trl* plays a specific role in the selection of IOCs through cell death. This is confirmed by our finding that expression of retinal cell type-specific markers is not affected in null $Trl^{\beta1.1}$ mosaic ommatidiae. However, this does not exclude additional roles of *Trl* in early eye morphogenesis since photoreceptors and 1°s cells are sometimes displaced, extra 1°s are counted, and bristles are often missing in large clones. Since these defects are rarely observed in mosaic ommatidiae, it is likely that these early functions of *Trl* are at least partly compensated for by neighboring wild type cells, suggesting that they are non cell-autonomous.

In order to characterize the function of *Trl* specifying the correct number of secondary and tertiary pigment cell at the end of metamorphosis, we focused our analysis on ommatidiae that do not show defects concerning other cell types in both hypomorphic Trl^{13c} escapers and mosaic $Trl^{\beta1.1}$ context. We showed that extra IOCs were always found associated to mutant cone cells, thereby suggesting that *Trl* function may be to control a signal emanating from cone cells, either by preventing the production of a survival signal, or by allowing the production of a death signal. Strikingly, the suppression of IOC cell death by the loss of function of *Trl* preferentially occurs on the oblique, rather than the horizontal faces of the ommatidia. Little is known about the nature of signals acting in a position-dependent manner for the removal of supernumerary IOCs. However, non equivalence between the oblique versus the horizontal face was also revealed when the EGFR pathway was temporary blocked before the onset of cell death, at 23.5 h p.d (Monserrate and Baker Brachmann, 2006). In that case, the effect was the death of almost all horizontal IOCs, whereas oblique IOC number was normal. Removing *Trl* function thus appears to have the opposite effect when compared to the temporary blockage of the EGFR pathway, since it does not affect cells on the horizontal face and prevents death on the oblique face. Therefore, *Trl* function might be to reduce EGFR activation specifically when cell death starts in the normal retina, allowing PCD of supernumerary oblique cells.

Strikingly, dorsal oblique IOCs appear to be more dependent upon *Trl* function than ventral IOCs. The eye epithelium is a polarized field in which cells are regularly oriented. The regular organization of cells on each side of the equator, oriented in opposite direction, is referred to as dorsal / ventral planar polarity. This polarity depends on several factors including Wingless, Notch and Frizzled (for review, see McNeill, 2002), and JAK/STAT (Zeidler *et al.*, 2000). One central factor of dorsal identity is the *Iroquois* complex that encodes transcription factors expressed in the dorsal half of the eye (McNeill, 2002). In addition, Wingless imposes scalar coordinates specifying peripheral cell fates (Tomlinson, 2003). None of these factors have yet been described as specifying strictly dorsal/ventral polarity of ommatidiae, rather they control the equatorial/polar axis in a mirror image across the midline. Up to now, to our knowledge, there is no report on the genetic control of a dorsal / ventral axis in ommatidiae. Our results suggest either that dorsal and ventral IOCs differ in their positional identity thus responding differentially to signals likely emanating from cone cells, or that a *Trl*-dependent signal produced in cone cells spreads directionally along the dorsal/ventral axis. In both cases, this result favors the hypothesis that ommatidiae may be intrinsically polarized along the dorsal/ventral axis by yet unknown factors.

Programmed cell death of horizontal IOCs is less dependent on *Trl* function than that of oblique IOC, and may involve a topological constraint. In wild type, the surviving horizontal IOC always occupies a central position with stereotyped contacts with its neighbors. It has been proposed that these central IOCs may survive because they contact the junctions of two ventral and two dorsal pigment cells (Monserrate and Baker Brachmann, 2006). There are very few cases in *Trl* escapers as well as in *Trl* mosaic clones where supernumerary cells were found on the horizontal face, but in most of these, each of the two remaining horizontal IOCs contacted only one of the two junctions between primary pigment cells, either dorsally or ventrally, and these junctions were not aligned along the dorsal/ventral axis. Strikingly, we also observed that in the wild type mature retina the occasional presence of additional horizontal IOCs also correlates with dorsal/ventral misalignment of 1°s junctions. This correlation might be the consequence of a topological constraint, such as the existence of a contact-mediated survival signal at the junction of two 1°s. If one 1°s junction is sufficient for the survival of horizontal IOCs, it would support the idea that contact between two adjacent primary pigment cells creates a specific signal that counteracts cell death in IOCs (Monserrate and Baker Brachmann, 2006). Alternatively, the observed correlation might be a topological consequence on the alignment of 1°s due to the presence of two surviving horizontal cells.

Control of apoptosis in IOCs appears thus to involve localized signals that modulate position-dependent death of IOCs. Data presented here bring additional complexity to the question of apoptosis regulation since they suggest that the signals responsible for the death of IOCs according to their position on an oblique or horizontal face are differentially controlled: a first signal regulated by *Trl* and emanating from cone cells would induce oblique cell death (or block their survival), and a second signal independent of *Trl* would induce horizontal cell death (or block their survival). In addition, analysis of *Trl* function reveals that dorsal and ventral IOC cell death may be modulated through different pathways. Future experiments looking for TRL targets in cone cells should help determine how the fate of IOCs is differentially determined through this process of developmentally regulated PCD.

Materials and Methods

Fly stocks, genetics and rescue experiments

Flies were grown on standard corn-agar medium at 25°C except when otherwise mentioned. All the markers and stocks used in this study are referred to in Flybase (http://flybase.bio.indiana.edu/). Most of the stocks were provided by the Bloomington or Umea stock centers, and other providers are referred to below. *Trl* alleles and transgenes were kindly provided by A. Greenberg. Trl^{13c} is a hypomorphic *P* insertion mutant exhibiting a reduced viability (Farkas *et al.*, 1994). $Trl^{81.1}$ is an EMS induced null allele (Greenberg and Schedl, 2001). Trl^{R67} is a presumed null allele generated by the imprecise excision of the Trl^{13c} *P*-element. *P*[*Trl+*] refers to the $hsp83{:}GAGA_{519}$ transgene, which allows ubiquitous expression of one of the two isoforms of the GAGA factor, the product of the *Trl* gene. One copy of this transgene was previously shown to be sufficient to rescue the rough eye phenotype of Trl^{13c} homozyotes. Since Trl^{13c} homozygous flies carrying the *P*[*Trl+*] are indistinguishable from wild type flies (Faucheux *et al.*, 2003), the *Trl* mutation, rather than a second site mutation, is responsible for the rough eye phenotype in Trl^{13c} escapers. *P*[*lolal+*] refers to a transgenic wild type copy of *lolal* (synonymous to *batman*), namely *pBB10,* which was previously shown to restore viability and a wild type eye phenotype to $lolal^{(2)k02512}$ homozygous flies as well as to reverse the enhancement of the *Trl* rough eye phenotype found in $lolal^{(2)k02512}/+;\ Trl^{13c}$ to that observed in Trl^{13c} flies (Faucheux *et al.*, 2003).

$Trl^{81.1}$ *mosaic analysis in the pupal retina and statistics*

Clones of homozygous $Trl^{81.1}$ mutant cells were obtained by FLP/FRT mitotic recombination (Xu and Rubin, 1993) using *hs-FLP*. Flies of the *yw P*[*hsp70*-FLP122]/*w*; *P*[UbiGFP] *P*[FRT(w^{hs})2A] / $Trl^{81.1}$ *P*[FRT(w^{hs})2A] or *yw P*[*hsp70*-FLP122] / *Y*; *P*[UbiGFP] *P*[FRT(w^{hs})2A] / $Trl^{81.1}$ *P*[FRT(w^{hs})2A] genotype were reared at 25°C for 48-60 hours after egg laying, heat shocked (1 h at 38°C), grown at 25 °C until pupariation, and staged during the first hour of pupariation. Pupal retinas were dissected at 25, 30, 37 or 42 hours after puparium formation at 25°C, corresponding to 24, 29, 36 and 41 % of pupal development, respectively. For statistical analysis of phenotypes, distributions were tested for conformity to the appropriate hypothesis or for homogeneity using the Chi-2 test. When this test was not applicable (high numbers, low frequencies) data were compared assuming a binomial distribution. *P* values are given in the text or in Table 1 footnotes.

Electron microscopy and eye section

For scanning electron microscopy, adult *Drosophila* were stored in 70% EtOH at room temperature. Scanning electron microscopy using a S-3000 N HITACHI electron microscope (vacuum pressure 90Pa, 12kV) was performed at the Institut de Biologie des Plantes, Univ. Paris Sud, Orsay. Semi-thin sections were prepared and stained as described (Tomlinson and Ready, 1987), and images were collected using a Leica DMR microscope using a 40X objective. Differences in the aspect of the pigment cell layer result from the use of a w^{1118} mutant background for the analysis of Trl^{13c} mutants (no pigment granules), whereas $lolal^{(2)k02512}$ carries a *miniwhite* transgene. Wild type *CantonS* have a maximum level of pigment.

Acridine Orange and antibody staining

For assaying AO staining in *Trl* escapers, pupae were grown at 20°C for 50 hours, which corresponds to 31 % p.d. at this temperature, and dissected in 0,1M phosphate buffer, and stained in AO diluted at 100 ng/

ml in 0.1M phosphate buffer for 3 minutes. After a brief wash, retinas were mounted and immediately examined in phosphate buffer under confocal laser-scanning microscopy (Nikon ph1/0.3DL 60/1.7).

The following primary antibodies were used: rat anti-DE-cadherin DCAD2, mouse anti-Armadillo N2 7A1, mouse anti-SCR 6H4.1, mouse anti-ANTP 4C3, mouse anti-ABD-B 1A2E9, rat anti-ELAV 7E8A10 and mouse anti-CUT 2B10 (all from Developmental Studies Hybridoma Bank), rabbit anti-GFP A11122 (Molecular Probes), mouse anti-GFP (Roche), mouse anti-UBX FP.3.38 (White and Wilcox, 1984), rabbit anti-BarH1 (Hayashi *et al.*, 1998), rabbit anti-GAGA (a kind gift from D Gilmour, Leibovitch *et al.*, 2002)) and anti-LOLAL (Faucheux *et al.*, 2003). Dissection of retina from staged pupae was performed in 0.1M phosphate buffer. Retinas were fixed (20 min., room temperature) in 30 mM Pipes (pH 7.4), 160 mM KCl, 40 mM NaCl, 4 mM Na_3EGTA, 1 mM spermidine, 0.4 mM spermine, 0.2% BSA, 0.1% Triton, 3.7% formaldehyde and washed in 1X PBS, 0.3% Triton. Retinas were then blocked in 1X PBS, 0.3% Triton, 1% BSA. Antibody labeling was performed according to standard procedures. Antibodies were diluted (1/400 except for anti-BarH1: 1/100 and anti-CUT: 1/200) in 1X PBS, 0.3% Triton, 1% BSA. Secondary antibodies (Alexa 488, 594 and 647 conjugates, Molecular Probes) were diluted 1/500. Confocal laser-scanning microscopy was performed with an inverted Nikon Diaphot 300 microscope equipped with an argon-krypton ILT laser using a 60X Nikon Plan Apo DM/ph4†160/0.17 oil immersion objective. Images were recorded in the slow-scan mode at 30% laser output, digitalized with the Biorad Laser Sharp 3.2 software and further processed using ImageJ 1.34S (NIH), and Adobe Photoshop CS2.

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