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Reorganization of membrane contacts prior to apoptosis in the *Drosophila* retina: the role of the IrreC-rst protein

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SUMMARY

The final step of pattern formation in the developing retina of *Drosophila* is the elimination of excess cells between ommatidia and the differentiation of the remaining cells into secondary and tertiary pigment cells. Temporally and spatially highly regulated expression of the *irregular chiasmC-roughest* protein, an adhesion molecule of the immunoglobulin superfamily known to be involved in axonal pathfinding, is essential for correct sorting of cell-cell contacts in the pupal retina without which the ensuing wave of apoptosis does not occur. *Irregular chiasmC-roughest* accumulates strongly at the borders between primary pigment and interommatidial cells. Mutant and misexpression analysis show that this accumulation of the *irregular chiasmC-roughest* protein is necessary for aligning interommatidial cells in a single row. This reorganisation is a prerequisite for the identification of death candidates. *Irregular chiasmC-roughest* function in retinal development demonstrates the importance of specific cell contacts for assignment of the apoptotic fate.

Key words: *Drosophila*, cell adhesion, apoptosis, targeted misexpression, Notch, retinal development, *irregular chiasmC-roughest*

INTRODUCTION

The compound eyes of insects are highly ordered crystal-like structures. It has been shown in *Drosophila* that cell death plays a major role in their shaping (Wolff and Ready, 1991, 1993). The individual ommatidia are formed by a sequence of inductive interactions between adjacent cells (Ready, 1989; Rubin, 1989; Dickson and Hafen, 1993). R8 photoreceptors form a founder lattice behind the morphogenetic furrow, regularly spaced due to lateral inhibition (Baker et al., 1990). Further cells – the remaining 7 photoreceptors, 4 cone cells and 2 primary pigment cells (1° p.c.) – are sequentially formed by recruitment of undifferentiated surrounding cells. To ensure correct development of all ommatidia, a surplus of cells is initially produced. Thus, after 1° p.c. differentiation, more cells remain between ommatidia than are necessary to form secondary and tertiary pigment cells and mechanosensory bristles. Excess interommatidial cells (IOC) are removed by a short phase of apoptosis, which is disturbed in the two mutants *roughest (rst)* and *echinus (ec;* Wolff and Ready 1991, 1993).

In *rst* mutants, IOC are not constrained to single cell rows between 1° p.c. and cell death subsequently fails. The *ec* mutation appears to affect a later step, since cells are ordered correctly but surplus ones are not eliminated. Phenotypes similar to that of *ec* result from P35-mediated suppression of cell death in the retina (Hay et al., 1994), while the previous process of cell sorting is not affected.

rst mutations map to the same chromosomal location as the *irreC* structural brain mutations, which do not complement their eye phenotype (Boschert et al, 1990). Further analysis of the locus lead to the isolation of the *irreC-rst* gene (Ramos et al., 1993) which encodes a protein of the immunoglobulin superfamily involved in neural recognition processes (Schneider et al., 1995). The rst^{CT} mutation, a partial deletion of its cytoplasmic domain (Ramos et al., 1993), specifically affects cell sorting and apoptosis in the eye but not axonal pathfinding in the brain.

The present paper investigates the role of the IrreC-rst protein in IOC apoptosis and in the reorganization of cellular geometry prior to apoptosis. Dynamically regulated expression with specific subcellular localisation at defined membrane contacts is found during retinal development. Mutant and misexpression analysis show that wild-type expression of IrreC-rst is essential for lining up of IOC in single cell rows prior to the induction of apoptosis. This level of cellular order appears to be a prerequisite for correct assignment of the apoptotic fate to individual cells. The rst^{CT} protein with a truncated cytoplasmic domain is found to differ drastically in subcellular localisation in the retina.

MATERIALS AND METHODS

Fly strains, conditions of culture

All *irreC-rst* strains used are described elsewhere (Boschert et al., 1990; Schneider et al., 1995). *sev-Gal4* flies were a gift from Konrad Basler; elav-Gal4 was obtained from Ernst Hafen, LE07 from Bruce Hay. Gal4 enhancer trap lines screened for eye phenotypes with UAS-

IrreC-rst were obtained from Joachim Urban and Gerhard Technau. *ec* was obtained from the Bloomington stock center. WTB was used as wild-type stock. Flies were raised at 25°C (100% pupal development corresponding to 103 hours) or 20°C (100% pupal development corresponding to 160 hours).

Scanning electron microscopy

Clean heads of 1-day-old flies were collected in 70% ethanol. Heads were vacuum dried and gold coated with a 'SEM UNIT 5100'; preparations were scanned in a 'SEMCO monolab 7' microscope.

Immunofluorescent antibody staining

Immunohistology procedures were essentially as described by Schneider et al. (1995). Briefly, tissue was fixed 30 minutes in 4% paraformaldehyde, blocked with normal goat serum in PBS with 0,1% Triton X-100 and incubated with mAb 24A5.1 (anti-IrreC-rst) in 1:50 dilution. Goat anti-mouse Cy3 (Jackson) was used as the secondary antibody. For staining with c17.9C6 (Anti-Notch), tissue was fixed 15 minutes in 2% paraformaldehyde and permeabilized with 0.1% saponin. Preparations were embedded in Vectashield (Vector) and viewed with a Leica TCS^{4D} confocal microscope.

Construction of UAS-HB3 and transformation

The *Spe*I fragment of the cDNA HB3, containing the entire *irreC-rst* ORF, was inserted into the pUAST vector behind the UAS binding sites for GAL4 (Brand and Perrimon, 1993). The orientation of the recombinant DNA was checked by restriction enzyme digests. Plasmids pUAST-HB3 and helper plasmid pUChsπΔ2-3 were prepared with the Qiagen plasmid kit and resuspended in injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4) at pH 6.8.

w^{1118} embryos aged 0-60 minutes were collected on apple-juice agar plates at 25°C and dechorinated for 4 minutes with fresh 5% NaClO. Together with the helper plasmid, pUChsπΔ2-3 (0.2 mg/ml), pUAST-HB3 (0.6 mg/ml) was injected into embryos for germ-line transformation as described by Spradling and Rubin (1982) and Roberts (1986). Five independent transformants were isolated, all with wild-type eyes. Misexpression experiments yielded identical results with insertions on different chromosomes.

Acridine orange staining

The method has been described by Spreij (1971). The ideal stage for detection of AO stained fragments is 5-10 hours after the onset of interommatidial apoptosis. Retinae were rapidly dissected from p31% retinae in Ringer's solution and stained in 0.1 mg/ml acridine orange for 2 minutes. After a brief wash, retinae were mounted and immediately examined under a Leica CLSM.

RESULTS

IrreC-rst function is necessary for the last cell-fate choice in the development of the *Drosophila* retina – cell death or commitment to the pigment cell fate. We have used a specific antibody to follow IrreC-rst protein expression in wild-type and mutant animals and applied the Gal4/UAS system to intervene in the normal process of eye development by targeted misexpression of the protein.

IrreC-rst is dynamically expressed in a succession of cell types during eye development

IrreC-rst expression in the retina is dynamically regulated throughout development. The protein is localised apically and initially outlines all cell profiles in the morphogenetic furrow (mf, Fig. 1A,B).

The first structures emerging from the furrow are rosettes that develop to arcs and then form five-cell preclusters (R8, R2, R5, R3, R4) initially including 'mystery cells' which are later expelled at the anterior end (Wolff and Ready, 1993). IrreC-rst is strongly concentrated at the posterior border of the arcs adjacent to surrounding unpatterned cells (Fig. 1C). However,

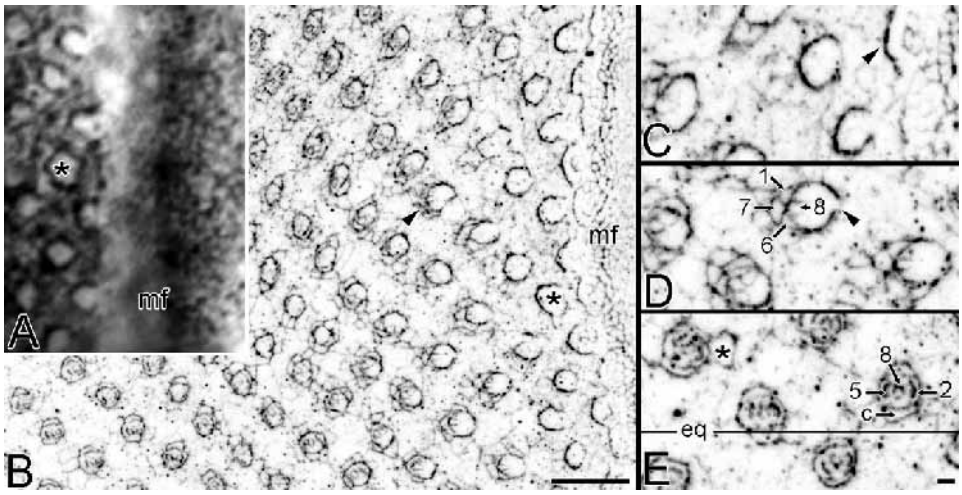

Fig. 1. IrreC-rst expression in the third instar larval eye imaginal disc. (A) Eye disc stained using the Ni-DAB/HRP technique. Immunoreactivity is recognizable in apical membrane domains and cytoplasmically. Forming photoreceptor clusters (*) are initially devoid of immunoreactivity. (B) Confocal view of the apical region of the eye disc. IrreC-rst protein is expressed in the morphogenetic furrow (mf). After forming a thin band with weak staining, protein expression reappears and concentrates at the posterior of arcs and around preclusters (*). Cells directly posterior to the 5-cell preclusters display strong immunoreactivity from the third row onward (arrowhead); R1, R6 and R7 are recruited from this group. All unpatterned cells behind the mf show weak immunoreactivity. (C-E) Enlargements of forming ommatidia and cellular interpretation of IrreC-rst immunoreactivity. The fact that IrreC-rst accumulates preferentially only at some membrane contacts complicates the identification of the cells producing the protein. Considering the cytoplasmic background staining seen in A, it is apparent that the protein accumulated around arcs (C, arrowhead) is not expressed in the arc cells, but is accumulated from cells posterior of it. In the 5-cell precluster (D), it is not expressed by the anterior R3 and R4 cells, but accumulates around the entire cluster from outside. Gaps may be due to mystery cells which are expelled from the precluster at this stage (arrowhead). Staining appears on different membrane contacts of R8, R2, R5 (C,D). R1, R6 and R7 transiently express IrreC-rst (D). Expression in these cells disappears during cone cell formation (E), while R2, R5 and R8 as well as the forming cone cells retain expression. eq, equator of eye disc. *In the posterior region of the disc, cells contacting the anterior and posterior cone cells show elevated irreC-rst immunoreactivity. Scale bars (A,B) 10 μm, (C-E) 1 μm. For assignment of cell types in ommatidial preclusters, refer to Wolff and Ready (1993).

cytoplasmic staining is strongest between the arcs and preclusters; the interior of clusters emerging from the furrow is not stained (see Fig. 1A). Thus, IrreC-rst protein produced in undifferentiated cells selectively accumulates at the border abutting differentiating cells.

Within the 5-cell precluster, staining is present where membrane contacts between R8, R2 and R5 cells occur (Fig. 1D). The future R1, R6 and R7 cells that are recruited from the posterior of the cluster show strongest expression at this stage. No immunoreactivity is detectable in the anterior part of the cluster, containing R3 and R4. Cone cells form in the sixth to seventh row behind the furrow; they also express IrreC-rst (Fig. 1E), while membrane contacts among R1, R6 and R7 lose immunoreactivity. R2, R8 and R5 retain expression and show specific accumulation of the protein at different membrane contacts (Fig. 1E).

During early pupal development (Fig. 2A, p12%), all membrane contacts among unpatterned cells between ommatidia stain equally strongly. Membranes of developing 1° p.c. show a slightly higher level of expression. After the pair of 1° p.c. has surrounded the cone cells, the ommatidial lattice compacts (Fig. 2B, p16%). The protein now accumulates at the borders between 1° p.c. and IOC. This border, previously straight, develops an involuted contour (Fig. 2B,C).

The interommatidial cells – distributed randomly at first – reorganise into chains lying end to end between ommatidia from approx. p16% to p21% (Fig. 2, compare cell positioning in B, C and D). Preferential accumulation of the protein at the IOC/1° p.c. border is retained throughout this stage. At approx. p23% (Fig. 2E), during IOC apoptosis, the pattern of IrreC-rst expression shows a striking change: it disappears from all borders among IOC, and is only detectable at the borders of these cells with 1° p.c. In the primaries it retreats from the border with cone cells but initially remains present at the two contact sites between themselves (visible until p33% as two opposed bars in each ommatidium, Fig. 2F). After elimination of surplus cells, bristle cells are recognizable by their weaker staining (Fig. 2F).

Even after downregulation of IrreC-rst in 1° p.c., borders among IOC remain almost devoid of staining (p35%, Fig. 2G), indicating the presence of a factor that forces accumulation of IrreC-rst at the border with 1° p.c.: in situ hybridizations at this stage show strong interommatidial *irreC-rst* mRNA expression and no detectable levels of mRNA in the primaries (Ramos et al., 1993). Thus, there is no protein made in 1° p.c. that can contribute to the strong staining seen at their outer border.

IrreC-rst expression is retained until ca. p60% and shows a specific pattern of accumulation around the bristle cell complex in late stages (Fig. 2H,I).

IrreC-rst expression and sorting of IOC are not affected by the *echinus* mutation

If IrreC-rst is responsible for organising IOC in single cell

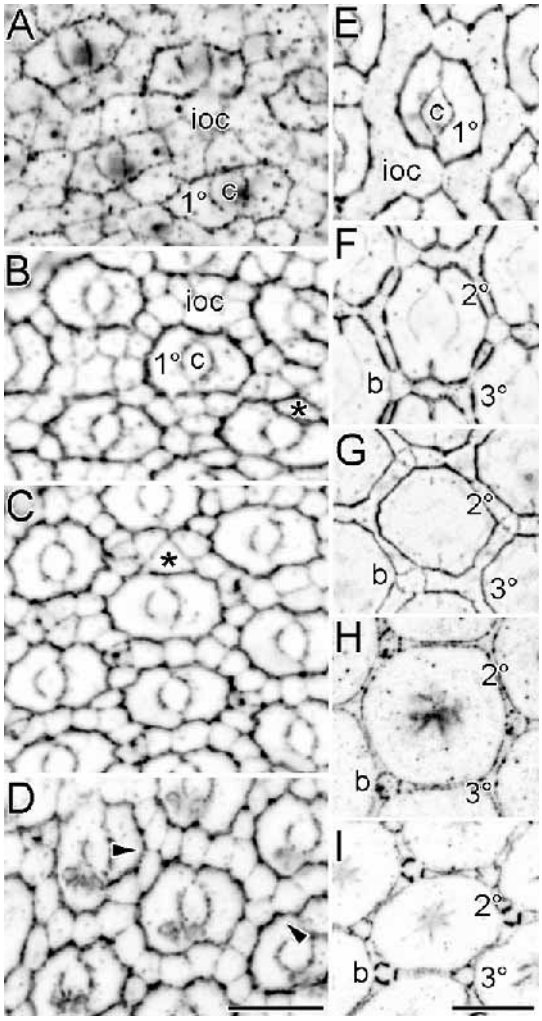

Fig. 2. IrreC-rst expression and sorting of interommatidial cells. (A) p12%: formation of primary pigment cells (1°) and beginning closure around the cone cell quartet (c). Interommatidial cells (ioc) are distributed randomly, borders between cells are straight, and distribution of IrreC-rst immunoreactivity is roughly equal among all stained membrane domains. (B) p16%, before cell sorting. 1° p.c. formation is nearly completed, only a few ommatidia still have more than two cells contacting the cone cell quartet (*). IrreC-rst staining highlights the border of IOC and 1° p.c. There are typically large numbers of excess IOC between neighbouring ommatidia. (C) p18%: cell sorting in process. In the center and lower part of the figure, IOC have become constrained into chains of cells lying end to end. Near the top, a cell marked * is connecting to the 1° p.c. of the neighbouring ommatidium. (D) p21%: completion of cell sorting. The ommatidium at the center makes contact with 15 IOC, two vertices are occupied by three IOC (arrowheads), and there are often two IOC between two vertices. These surplus cells are subsequently removed by apoptosis. (E) p23%: cell death stage. Borders of 1°p.c. and IOC in this and the preceding stages are involuted. Immunoreactivity disappears entirely from the borders of IOC among themselves and is visible only in a fine vesicular pattern within IOC. (F) p28%: specification of interommatidial cell fates is complete after the apoptotic phases: The ommatidium contacts only 12 IOC, all vertices are occupied by one cell each, there is only one 2° pigment cell between vertices. b, bristle; 2°, secondary; 3°, tertiary pigment cell. Note conspicuous bars of immunoreactivity at contact sites of 1° p.c. (G) p35%: even after downregulation of expression in 1° p.c., IrreC-rst protein does not appear at borders of 2° with 3° pigment cells, although it is expressed in both at this stage (Ramos et al., 1993). (H,I) At later stages, p44% and p56%, borders among IOC become immunoreactive and conspicuous staining of rhabdomeres appears. (I) There is strong protein accumulation at contacts of bristles and 2° p.c., leading to a characteristic circle broken by three gaps (bristle contacts to nonexpressing 1° p.c.). Scale bars 10 μm; bar in D applies to (A-H).

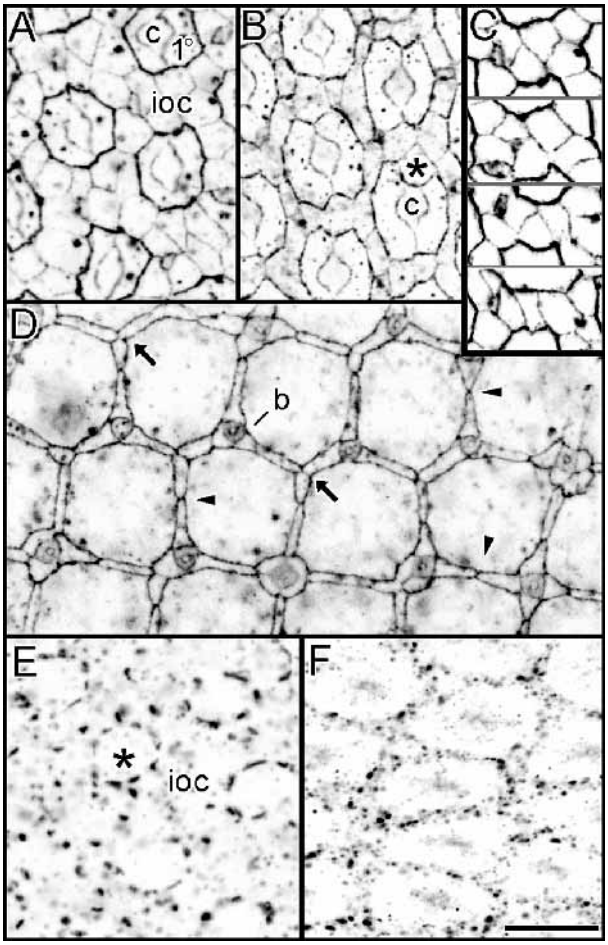

Fig. 3. IrreC-rst expression in mutants affecting retinal cell death – *ec* and *rst*. (A-D) Developmental series of *ec*. (A) p16%. IrreC-rst distribution and retinal geometry are similar to wild type (Fig. 2B). (B) p24%: at this stage, cell death would normally occur in the wild type. IOC have been sorted to chains lying end to end but surplus ones are not eliminated, often leaving more than one between vertices and three occupying a vertex. (*), failure of 1° p.c. differentiation, an IOC is still contacting the cone cells. (C) Cell sorting process in *ec*. The sections are taken from p16% to p18% retinae. Cell sorting proceeds normally and is accompanied by accumulation of IrreC-rst at the IOC/1° p.c. border.
(D) Representative *ec* retina at p42% (see Fig. 2H for wild type). 2° p.c. are very often doubled end to end (arrowheads), and those vertices where 3° p.c. should develop are occupied by three cells (arrows). Side to side doublings are rare. (E) rst^{CT} retina at p21%. Subcellular localisation of the mutant protein is altered. No contiguous expression of IrreC-rst protein at the IOC/1° p.c. border is visible (see Fig. 2D for wild type). Instead, the protein accumulates in discrete plaques and intracellular vesicles. Distances between ommatidia (*) are greater than in the wild type. (F) p36%: expression is localized almost exclusively in vesicular bodies (comparable stage of wild-type: Fig. 2G). Scale bars, 10 μm.

rows, this process should not be disturbed in the *ec* mutant. mAb 24A5.1 staining shows that IrreC-rst protein accumulates at the IOC/1° p.c. border in early pupal *ec* retinae (Fig. 3A,B). Cell sorting proceeds normally (summarized in Fig. 3C) but apoptosis does not occur, leaving many surplus IOC in later pupal stages (Fig. 3D). Characteristically, two putative secondary pigment cells lie end to end, and three candidate tertiary pigment cells occupy one vertex.

This demonstrates that sorting of IOC and apoptosis of IOC are dependent on separate genetic functions.

Subcellular localisation of IrreC-rst protein is altered in the rst^{CT} mutant

The rst^{CT} mutation truncates the intracellular domain of the IrreC-rst protein (Ramos et al., 1993) and causes a severe eye phenotype (Wolff and Ready, 1991), but no axonal pathfinding defects in the optic lobe. This suggests a specific function of the intracellular domain in apoptosis during retinal development (Ramos et al., 1993; Steller and Grether, 1994).

Immunostaining of rst^{CT} retinae reveals a drastic alteration of subcellular protein localisation (Fig. 3A,B). No homogenous staining of the apical membranes is found; instead, the protein collects in small patches along those membrane domains where it accumulates in the wild type. Conspicuous vesicular bodies are found in the cytoplasm (Fig. 3). Such immunoreactive vesicles are also present in smaller numbers in wild-type and *echinus* retinae (e.g. Figs 2A,B, 3A,B). Protein distribution in the optic lobe is indistinguishable from the wild type (data not shown).

Sequestering of the mutant rst^{CT} protein in vesicular bodies occurs in all other epithelial tissues of imaginal discs expressing IrreC-rst (antennal, wing, haltere and leg discs; not shown). This indicates that the rst^{CT} mutation selectively affects IrreC-rst protein localization in epithelial but not in neural tissue.

These vesicles are probably the result of endocytosis from the membrane and not stages of synthesis and transport to the membrane. There is no indication from mRNA in situs (Ramos et al., 1993) or western blotting (Schneider et al., 1995) that expression in rst^{CT} is regionally increased and so it is not obvious why more vesicles are observed in the mutant if they represent stages of synthesis. Also, the large size of these vesicles, easily detectable by light microscopy, is untypical of Golgi vesicles in non-secretory cells.

sevenless enhancer driven misexpression of IrreC-rst disturbs cell sorting and suppresses apoptosis in the eye imaginal disc

Analysis of mutant phenotypes demonstrates the necessity of intact IrreC-rst protein for apoptosis of interommatidial cells. Study of the expression pattern reveals a specific accumulation of the protein at the border between primary pigment and interommatidial cells, a contact zone where cellular geometry is rearranged immediately before apoptotsis.

We have attempted further functional analysis of IrreC-rst by specifically altering the expression pattern, using the Gal4/UAS system (Brand and Perrimon, 1993). If IrreC-rst acts strictly as a receptor for a cell death inducing signal, then extending its expression to additional cell types could yield two possible results. Either additional cell death could occur, if the targeted cells receive the proper signal, and if the apoptotic program is not otherwise inhibited in them, or, no additional cell death would be expected if the targeted cells do not have contact with the signal, or the apoptotic program is inhibited by the differentiational state of the cells.

Transformants expressing Gal4 under control of the *sevenless* enhancer were used to target misexpression of IrreC-rst to the retina in UAS-HB3 transformants. The *sevenless*

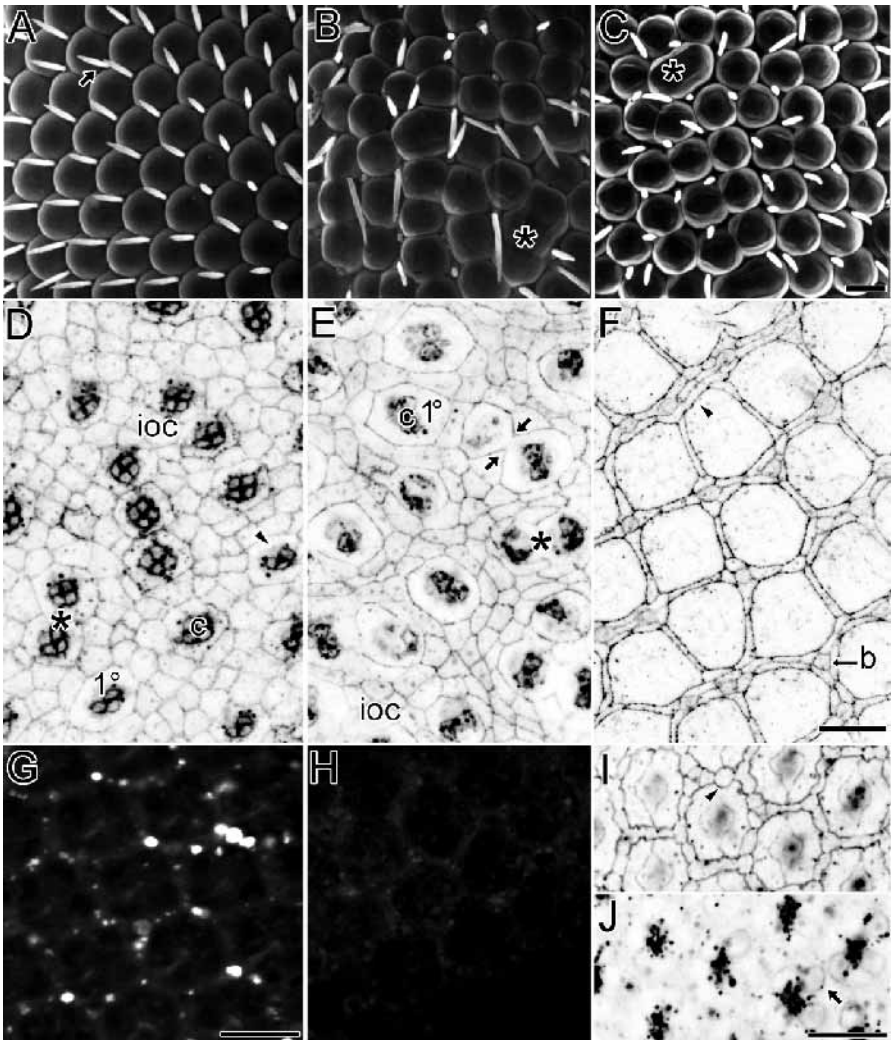

Fig. 4. Sorting of cell contacts, specific accumulation of IrreC-rst protein, and cell death are abolished in *sev-Gal4/UAS-HB3* retinae. (A) Scanning electron micrograph of *sev-Gal4/sev-Gal4* adult eye surface with wild-type phenotype. The ommatidial lattice is highly regular and only occasionally disturbed by dislocated bristles (arrow). (B) *sev-Gal4/UAS-HB3.* The eye surface is extremely rough, with frequent ommatidial fusions (*) and randomized bristle distribution. The misexpression phenotype is similar in severity to the null mutant, *rst vt*, shown in C. *UAS-HB3/UAS-HB3* develops a wild-type retina (not shown). (D) *sev-Gal4/UAS-HB3* retina at p22%. IrreC-rst misexpression in the cone cell quartet (c) is obvious. Expression at the borders of 1° p.c. with IOC is equal as among IOC, i.e. the normal accumulation of IrreC-rst at the IOC/1° p.c. border is abolished (compare Fig. 2D,E). IOC and 1° p.c. are not targeted by misexpression in *sev-Gal4/UAS-HB3*. No sorting of IOC is apparent. Arrangement of ommatidia appears random, with clustering in some regions and sparse distribution in others. 1° p.c. of some ommatidia contact each other, giving rise to ommatidial fusions (*) seen in B. Fused ommatidia do not share primaries. Note that borders of 1° p.c. with IOC are not involuted as in comparable stages of the wild type (e.g. Fig. 2D,E). Otherwise, morphology of primaries is normal, they have completely enclosed the cone cell quartet. Cone cells are normal as well; occasional apparent lack of one cone cell (arrowhead) is caused by selective downregulation of *sev-Gal4* regulated IrreC-rst expression (see Tomlinson et al., 1987, for specifics of *sev* regulation). (E) IrreC-rst expression in *sev-Gal4/UAS-HB3* at p35% (compare to Fig. 2G). Neither cell sorting nor reduction of cell number have taken place. The phenomenon of lacking IrreC-rst expression at borders among IOC does not appear, i.e the factor accumulating IrreC-rst around 1°p.c appears to be neutralized. Failure of IOC to fully separate ommatidia is marked with arrows, complete ommatidial fusion with (*). Selective downregulation of the *sev-Gal4* element has continued; only one or two cone cells per ommatidium still show expression. (F) IrreC-rst staining of p42% *sev-Gal4/UAS-HB3* retina. *sev-Gal4*-driven expression has been completely downregulated. Staining of interommatidial cells is due to expression of the endogenous *irreC-rst* gene and can be used to diagnose cellular arrangements in comparison to the *ec* mutant (Fig. 3D) and wild type (Fig. 2H). Many surplus cells are present; they are typically arranged side by side between neighbouring ommatidia, and not end-to end as in *echinus*. This indicates that cell sorting as well as cell reduction have failed. (G,H) Acridine orange stainings of p31% wild type (G) and *sev-Gal4/UAS-HB3* (H) retinae. No apoptotic cell fragments are visible in the retinae of the transformants, demonstrating the inhibition of cell death. (I,H) IrreC-rst misexpression in photoreceptors is not responsible for the *sev-Gal4/UAS-HB3* phenotype. *elav-Gal4/UAS-HB3* as a control targeting misexpression to all photoreceptors developed no retinal defects. Apical IrreC-rst expression and arrangement of cells are normal at p22% (I). Surplus cells lie end to end but not all have been eliminated (arrowhead: example of surplus 2° p.c.). In a plane of section 2 μm deeper (J), additionally expressed IrreC-rst protein is seen to concentrate in the rhabdomere precursors; it is only weakly detectable on the outer membranes of photoreceptor cells (arrow). Scale bars, 10 μm.

enhancer is active in a subset of photoreceptors and the cone cells. Activity in cone cells persists into the pupal stage (Tomlinson et al., 1987). Animals homozygous either for *sev-Gal4* (Fig. 4A) or UAS-HB3 have wild-type retinae.

When IrreC-rst is misexpressed in *sev-Gal4/UAS-HB3* transformants a strong rough eye phenotype results (Fig. 4B). The general regularity of the ommatidial lattice is severely disturbed. Neighbouring ommatidia occasionally fuse, forming a single lens. Semithin sectioning reveals the number of photoreceptor neurons is normal (not shown); the distribution of mechanosensory bristles is randomized, but their number is not significantly influenced. Therefore, the disruption of the ordered lattice in misexpressing retinae does not reflect an effect on neural differentiation. The outside appearance of *sev-Gal4/UAS-HB3* is similar to that of the null mutant for the IrreC-rst gene, *rst-vt* (Fig. 4C).

Distribution of IrreC-rst immunoreactivity in interommatidial cells, which are not targeted by the misexpression, is strongly altered in *sev-Gal4/UAS-HB3*. While in the wild type the border between 1° p.c. and IOC is highlighted by strong

immunoreactivity (Fig. 2C,D), there is no longer any preferential accumulation of the protein at this border in *sev-Gal4/UAS-HB3* (Fig. 4D). Instead, apical membrane domains of IOC and 1° p.c. show equal intensity of staining. The cone cells – the only apical cell type targeted by misexpression in *sev-Gal4/UAS-HB3* – show the expected strong immunoreactivity.

In *sev-Gal4/UAS-HB3*, IOC/1° p.c. borders are not involuted, spacing of ommatidia is irregular and no sorting of IOC into chains between primaries is apparent (compare Fig. 4D with Fig. 2D). This suggests that the accumulation of IrreC-rst protein at the border of primary pigment and interommatidial cells is responsible for the proper sorting of cell contacts, and that this accumulation can be influenced by the presence of IrreC-rst in cone cells. No effects of IrreC-rst misexpression on the differentiation of primary pigment and cone cells were apparent.

The *sev-Gal4/UAS-HB3* phenotype is not due to an induction of cell death in populations misexpressing the IrreC-rst protein, but arises as a result of cell death inhibition in interommatidial cells. Acridine orange staining of *sev-Gal4/UAS-HB3* pupal retinae reveals few or no apoptotic fragments between the ommatidia (Fig. 4G,H: comparison of wild type and *sev-Gal4/UAS-HB3*), and many surplus IOC are visible in later pupal stages (Fig. 4E; compare with Fig. 2G). mAb 24A5.1 staining of retinae at p42% (Fig. 4F), when *sev-Gal4*-driven expression is no longer present but IOC still express the normal *irreC-rst* gene, shows that several 2° p.c. lie between ommatidia and are aligned side by side as in *rst*, not end to end as in *ec* (Fig. 3D). Thus, the primary effect of misexpression is on the sorting of cells. Preferential accumulation of IrreC-rst protein at the IOC/1° p.c. border appears to be required for this sorting process.

An additional effect seen in *sev-Gal4/UAS-HB3* is the frequent fusion of ommatidia (Fig. 4D, E - direct contact of 1° p.c., leading to formation of a common lens in the adult as seen in Fig. 4B). Contiguous chains of up to four ommatidia were observed. Apparently, interommatidial cells have a decreased tendency to interact with 1° p.c. and to insert themselves between them to maximize the contact area, or alternately 1° p.c. have an increased affinity for each other.

The observed effects are also found in control stainings with IOC markers independent of IrreC-rst (Fig. 5). Staining with mAb C17.9C6 against the Notch protein (Fig. 5A-C, E-G), which is expressed in IOC during much of pupal development (Fehon et al., 1991), shows the same effects on positioning and number of IOC. The LE07 enhancer trap line can be used to visualize nuclei of 2°/3° p.c. (Fig. 5D,H); X-gal staining of *sev-Gal4/UAS-HB3/LE07* reveals large numbers of chaotically arranged nuclei.

The *sev* enhancer targets expression to photoreceptors as well as cone cells, beginning in the third instar larva. Which of these cell types causes the suppression of apoptosis by IrreC-rst misexpression? As a control, we used an *elav-Gal4* construct (Luo et al., 1994) to drive IrreC-rst expression. The *elav* promoter is active in all neurons (Robinow and White, 1988), e.g. in photoreceptors, but not in cone cells.

No retinal defects result; cell sorting proceeds normally, and the subcellular localization of the IrreC-rst protein in IOC is normal (Fig. 4F). Misexpressed IrreC-rst protein in photoreceptors is transported almost exclusively to the precursor structures of the rhabdomeres (Fig. 4G), which correspond to the apical domain of photoreceptors (Wolff and Ready, 1993).

This control experiment shows that IrreC-rst misexpression during retinal development does not generally influence the differentiation process of targeted cells or their neighbours. Therefore, expression in cone cells must be responsible for the *sev-Gal4/UAS-HB3* phenotype.

By screening of Gal4 enhancer trap lines for appearance of rough eyes in the F_1 with *UAS-HB3*, another line (*MZ1369*) was identified which also caused severe eye roughness and survival of supernumerary interommatidial cells. In the pupal retina, it targets expression to the cone cell quartet. Protein distribution in the *MZ1369/UAS-HB3* retina is similarly altered as in *sev-Gal4/UAS-HB3* (not shown). Misexpressing cone cells and their immediate neighbours (1° p.c. and photoreceptors) develop normally. No Gal4 lines, which target expression only to photoreceptor cells, produced any eye phenotype with *UAS-HB3*. The intriguing conclusion is that misexpression of IrreC-rst in cone cells can exert a cell-death suppressing effect on interommatidial cells across the primary pigment cells, by way of disturbing the rearrangement of cellular geometry prior to apoptosis.

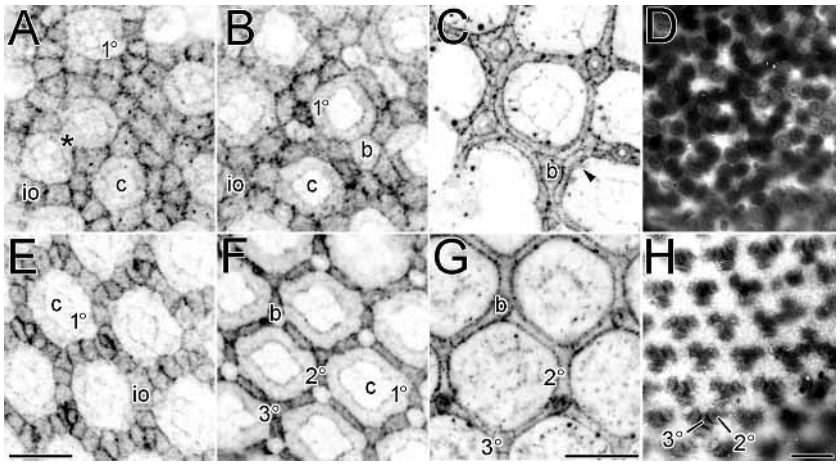

Fig. 5. Control stainings of *sev-Gal4/UAS-HB3* (A-D) and wild-type (E-H) with IOC markers independent of IrreC-rst. Juxtaposed stages in (A-C) and (E-G) are p21%, p28% and p44%, stained with mAb C17.9C6 against Notch protein, which is expressed in IOC throughout much of pupal development (see Fehon et al., 1991). This control confirms the lack of cell sorting at p21% (A: numerous IOC side by side; E: sorting in single cell rows), the lack of cell reduction at p28% (many surplus cells in B, while the final cell number has been reached in F), and the *rst*-like side by side arrangement of surplus cells at p44%. During the second half of pupal life, cell contacts and cell fate choices are not redefined. *, ommatidial fusion. (D,H) Xgal staining of enhancer trap LE07 on *sev-Gal4/UAS-HB3* (D) and wild-type (H) background. This enhancer trap line targets *lacZ* expression to 2°/3° p.c. (see Hay et al., 1994). Stained nuclei in (D) are chaotically arranged and more numerous than in (H), where they are ordered in a regular lattice. Scale bars, 10 μm; bar in E applies also to A-C,F.

IrreC-rst distribution and rearrangement of interommatidial cells is affected in *facet* mutants

Since the accumulation of IrreC-rst at the border of primary pigment cells with their undifferentiated neighbours appears to play a decisive role for cell sorting and apoptosis, we investigated the consequences of defects in 1° p.c. differentiation on subcellular IrreC-rst protein targeting. For this, we used the *facet strawberry* (*fa^{swb}*) mutant. The *facet* mutations are a complementing group of *Notch* alleles; most are intronic insertions of transposable elements specifically influencing retinal development (Markopoulou and Artavanis-Tsakonas, 1989, 1991). *Notch* function is necessary for various cell decisions in the developing retina (Cagan and Ready 1989a). *fa^{swb}* is a small deletion 5′ of *Notch* (Grimwade et al., 1985). A characteristic of this mutant is a variegating effect on the differentiation of primary pigment cells: in some ommatidia, none develop, some ommatidia develop one, in others one of two cells is stunted; some ommatidia develop two 1° p.c. with wild-type shape (Wolff and Ready 1991). *fa^{swb}* therefore provides a differentiational mosaic which can be used to study IrreC-rst protein localisation.

Staining of *fa^{swb}* retinae with mAb 24A5.1 shows that IrreC-rst accumulation at the outer border of interommatidial cells correlates with the formation of normally shaped 1° p.c. (Fig. 6, p30%, compare with Fig. 2F,G). In the vicinity of ommatidia with peripheral IrreC-rst accumulation, staining of membranes between neighbouring interommatidial cells is very weak or not detectable. This indicates that the observed IrreC-rst accumulation at the outer border is a result of relocalization of the protein in interommatidial cells (Fig. 6, around ommatidia marked with +).

Sorting of cell contacts into a tight lattice only proceeds when 1° p.c. of several immediately neighbouring ommatidia differentiate normally (as indicated by IrreC-rst accumulation at their outer circumference; Fig. 6B). In *fa^{swb}* specimens with larger fields of correctly developed 1° p.c., tightening of the lattice proceeds, and IrreC-rst protein distribution resembles that shown in Fig. 2G for wild type.

Within 1° p.c., immunoreactive vesicular bodies appear in correlation with peripheral IrreC-rst accumulation (arrow in Fig. 6). Such vesicular bodies are found throughout development in the wild type as well and may be associated with ligand-receptor interaction and endocytosis of the ligand-receptor complex, as it has been described for sevenless/boss (Krämer et al., 1991) and Notch/Delta interactions (Fehon et al., 1990, Kooh et al., 1993).

In the complete absence of 1° p.c. (the *facet^{glossy}* mutant, Fig. 6C,D) all interommatidial cells appear similar in shape to secondary pigment cells and are not sorted.

DISCUSSION

Apoptosis is a ubiquitous phenomenon in multicellular

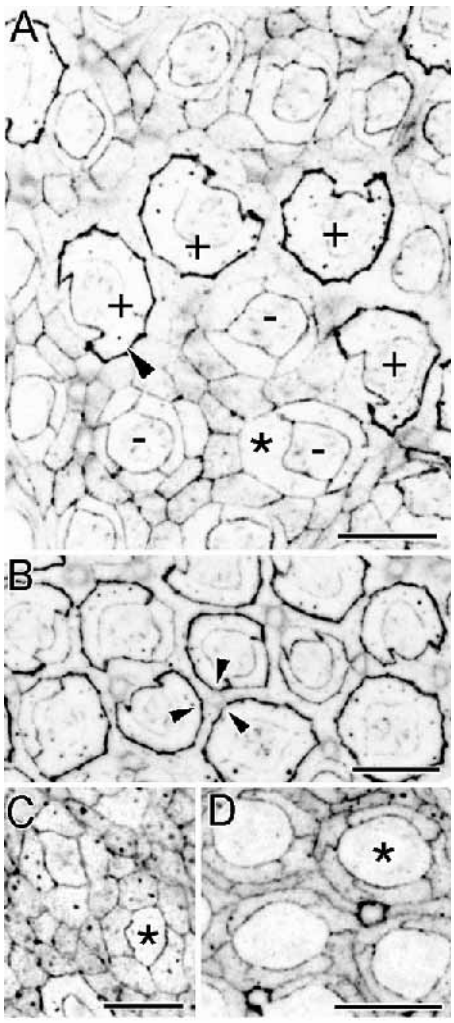

Fig. 6. Influence of 1° p.c. differentiation on subcellular localization of IrreC-rst protein in neighbouring IOC. (A) Retina of a male *fa^{swb}* fly at p36% (comparable stage in wild type, Fig. 2G). Several ommatidia with single, hyperexpanded 1° pigment cells (+) have formed in this field. These cells show strong IrreC-rst immunoreactivity at their outer borders with IOC while the membranes of interommatidials contacting them show only very weak staining. In the vicinity of ommatidia whose cone cell quartet is not surrounded by strongly immunoreactive cells (–), all membrane domains of neighbouring IOC stain equally strongly. The ommatidium marked (*) is surrounded by two large cells which have occluded other cells from contact with the cone cells and therefore fulfill the morphological requirements for 1° p.c. They do not show a peripheral accumulation of IrreC-rst, however. This suggests that the factor responsible for accumulation results from a distinct step in 1° p.c. differentiation. Punctate immunostaining within cells (arrowhead) and involuted contours of IOC/1° p.c. borders occur in correlation with peripheral protein accumulation. In regions without correctly developed 1° p.c., distribution of IOC and localisation of IrreC-rst protein is remarkably similar to that in *sev-Gal4/UAS-HB3* (Fig. 4E). (B) Retina of a *fa^{swb}* female at p30%. The *fa^{swb}* phenotype is sexually dimorphic and expressed only weakly in females. A large number of neighbouring ommatidia has formed at least one 1° p.c.; +, marks an ommatidium with 2 wild-type 1° p.c. Interommatidial spaces are more constricted than in A, borders among interommatidial cells are not traceable. The overall pattern is more similar to the wild-type (Fig. 2G), especially at vertices between three ommatidia with 1° p.c. (arrowheads). (C) p23% *fa^{g-2}* retina. 1° p.c. do not differentiate. Cone cell quartets (*) are surrounded by 4 to 5 cells which do not differ from other IOC in shape or IrreC-rst expression. (D) p48% *fa^{g-2}* retina. No sorting of cells occurs; all putative pigment cells appear similar to 2° p.c. IrreC-rst staining accumulates at borders of IOC with bristles, similar to the wild-type (Fig. 2I). Scale bars, 10 μm.

organisms (Tomei and Cope 1991, Raff 1992). Organisms such as *Caenorhabditis elegans* (Ellis and Horvitz 1986, Hengartner et al., 1992) and *Drosophila melanogaster* (White et al., 1994) offer themselves for a genetic dissection of cell death programs and their inductive events. It has been shown by Wolff and Ready (1991) that the wild-type *rst* genetic function in *Drosophila* is necessary for certain cell deaths during eye development. The accumulated knowledge of eye imaginal disc development (Ready, 1989; Rubin, 1989; Wolff and Ready, 1993) and the cloning of the *irreC-rst* gene (Ramos et al., 1993) enabled us to investigate this phenomenon in more detail.

Cellular geometry and apoptosis in the *Drosophila* retina

Ommatidial formation is characterized by an accretive mode of growth. As the last cells to develop have common borders with several ommatidia, such an accretive process cannot be continued to complete retinal development. Corrective measures are necessary to integrate the individually formed ommatidial elements into a regular lattice. Such measures are provided by the initial overproduction of cells and the wave of cell death that eliminates surplus interommatidial cells. To produce a regular lattice, generalised cell death is not sufficient; individual cells that do not fit into the pattern must be identified and specifically eliminated.

This is made possible by a reorganization of cell contacts, constraining interommatidial cells into chains lying end to end. In a following step, surplus cells are eliminated. These steps are disturbed in *roughest* and *echinus* mutants (Wolff and Ready, 1991, see also Figs 3 and 7). *rst* disrupts the initial step of contact reorganization so that surplus cells lie side by side. *ec* allows the reorganization but cell death does not ensue. Thus, interommatidial apoptosis relies on at least two distinct, sequentially acting genetic functions prior to activation of the general apoptosis machinery in an individual cell. Both *irreC-rst* and *echinus* are not general cell death genes but are specifically required for weeding out unnecessary interommatidials. The second aspect of retinal development mediated by apoptosis – elimination of perimeter clusters – occurs normally in the absence of both genes (Wolff and Ready, 1991). Suppression of the apoptosis machinery by retinal expression of the baculovirus P35 protein (Hay et al., 1994) or of *Drosophila* IAP1/IAP2 (Hay et al., 1995) does not interfere with cell sorting and causes an interommatidial phenotype similar to *ec*, but additionally rescues perimeter clusters.

Is IrreC-rst a receptor for a cell death signal?

Previous work has suggested that IrreC-rst might act as a receptor for a cell death signal, since it is expressed in the population of cells that is decimated and disruption of its intracellular domain blocks apoptosis (Ramos et al., 1993; Steller and Grether, 1994).

Our results yield no evidence for IrreC-rst as a receptor for a cell death inducing signal. Misexpression of IrreC-rst in cone cells inhibits death of interommatidial cells and their preceding rearrangement; no extra cell deaths occur either in cone cells or their neighbours (Figs 4, 5).

However, if the role of *irreC-rst* is primarily that of selectively sorting cell contacts in the developing retina, it is necessary to explain why a mutation of the intracellular domain completely blocks its function. The mutant protein concentrates in intracellular compartments and is not contiguously expressed in the cell membrane (Fig. 3E,F). It is therefore available neither for adhesional nor for signal-transducing processes. Possibly, the truncation of the intracellular domain alters the way IrreC-rst interacts with other proteins, thus increasing its rate of endocytosis.

A model for sorting of cell contacts by IrreC-rst interactions

How does the process of cell sorting in the pupal retina proceed to shift a side by side arrangement of IOC with usually one contact to 1° p.c. to an end-to-end arrangement with two or three contacts? We propose a model in which undifferentiated cells explore cell contacts and tend to sustain any contact made to a primary pigment cell. Contacts to other interommatidial cells should not be especially attractive. It is obvious from morphological studies that unpatterned cells do not retain the same contacts throughout all of development (Cagan and Ready, 1989b). Given that different cell contacts are explored and that 1° p.c. provide an attractive border, cells would rearrange to achieve a greater number of contacts to primary pigment cells. We propose that this attractive border is provided by adhesional interactions between IOC and 1°p.c which are mediated by IrreC-rst. An additional mechanism that can resolve side by side doublings of IOC is the extension of the IOC/1° p.c. contact zone into interommatidial spaces (see Fig. 7C).

The ommatidial fusions found in *sev-Gal4/UAS-HB3* and *irreC-rst* mutants (Fig. 4B,C) are also explainable by loss of an adhesional interaction at the IOC/1° p.c. border. In the absence of an attractive border, IOC would not be prevented from giving up contacts to 1° p.c.

IrreC-rst protein distribution requires a specific ligand

The IrreC-rst protein mediates homophilic adhesion in transfected S2 cells and selectively accumulates at the membrane contacts of expressing cells (Schneider et al., 1995). A model based on this mode of binding, however, fails to explain the protein distribution seen in the retina. The arcs formed around assembling ommatidial clusters, for instance, are formed by several cells producing IrreC-rst (Fig. 1B,E). The protein accumulates only at their border with the photoreceptor cluster, not at borders among themselves, while initially the photoreceptor cluster shows no immunoreactivity. Similarly, homophilic adhesion cannot explain the dynamics of protein localization during pupal development. At a stage when interommatidial cells strongly express *irreC-rst* mRNA (Ramos et al., 1993), the protein does not accumulate at their common border, rather it accumulates at the border with primary pigment cells (Fig. 2G). This accumulation persists beyond the downregulation of IrreC-rst in 1° p.c., suggesting that a heterophilic interaction is the underlying mechanism. Expression of a putative IrreC-rst ligand would be expected on 1° p.c. IrreC-rst protein distribution in the $facet^{strawberry}$ mutant points in the same direction: individual 1°p.c. that differentiate correctly in this mutant accumulate IrreC-rst and create a ‘sink’ of immunoreactivity in surrounding IOC, indicating the presence of a binding activity.

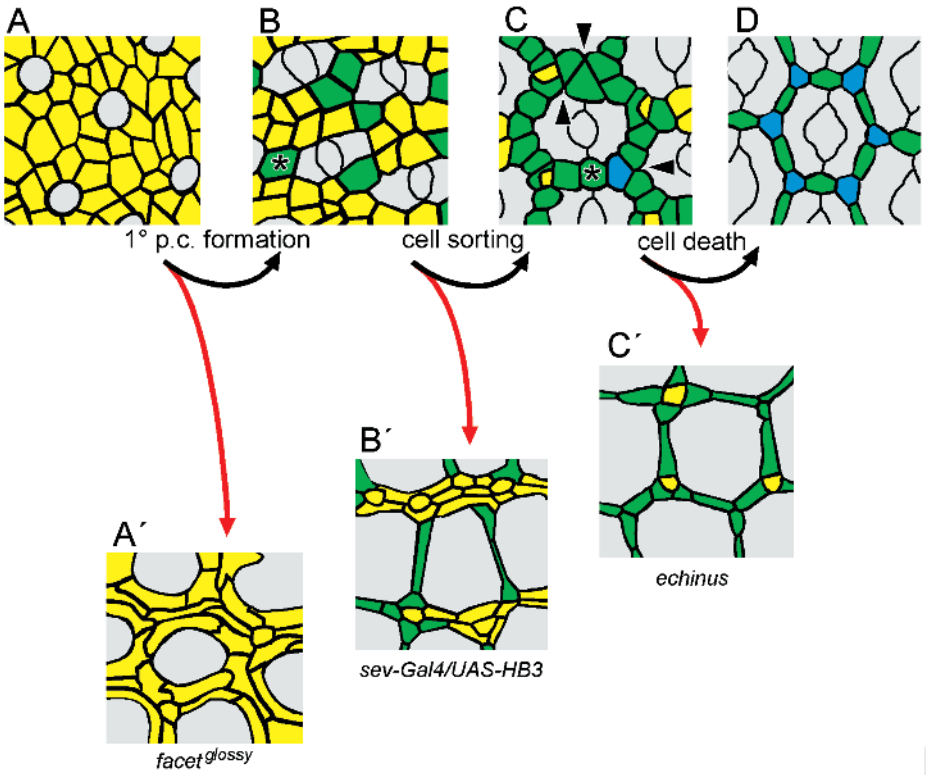

Fig. 7. Cellular geometry and apoptosis in wild-type and mutant retinae. (A-D) Sequence of development in the wild-type retina from pupation to p24%. (A′-C′), midpupal retinae of mutants and transformants that arrest development at different stages. Interommatidial cells (IOC) are color-coded by number of contacts to ommatidia: yellow, contact to no more than one ommatidium; green, contact to two ommatidia; blue, contact to three ommatidia. (A) At pupation, cone cells have formed at the posterior end of the retina. Ommatidia are never separated by less than two undifferentiated cells. (B) At approx. p12%, primary pigment cells (1° p.c.) close around the cone cells and displace remaining cells into the interommatidial spaces. Cells that have contact with two ommatidia at this stage (*) survive as oblique 2° p.c. (C) From p16% to p21%, cells are rearranged, giving all IOC contact with at least two ommatidia. During this stage, the IOC/1° p.c. borders develop a characteristic involuted contour. Extension of the IOC/1° p.c. contact zone into the interommatidial spaces may help to resolve side by side doublings by 'unzipping' IOC. At the end of the cell sorting process, future fates of some IOC are predictable from their position, e.g. cells contacting four different 1° p.c. from two ommatiida are destined to become horizontal 2° p.c. (*). Arrowheads mark cells making new contacts with 1° p.c. (D) After completion of cell sorting, surplus IOC are eliminated by apoptosis. (A′) The *facet glossy* mutant does not form 1° p.c. An immature cellular geometry similar to that found at pupation is conserved into the adult. Ommatidia are separated by two or more IOC in all directions. (B′) In *sev-Gal4/UAS-HB3*, 1° p.c. develop normally but cell sorting does not occur. Only a few IOC form contacts with two ommatidia; cellular geometry is similar to that of the wild type at ca. p12%. *rst* mutants show a similar pattern (see Wolff and Ready, 1991). Both lack the specific accumulation of IrreC-rst protein at the IOC/1° p.c. border. (C′) In *echinus*, 1° p.c. formation and cell sorting proceed normally in most parts of the retina, and IrreC-rst protein accumulates at the IOC/1° p.c. border. However, surplus cells are not eliminated after arrangement in end-to-end chains. Cellular geometry at approx. p21% is conserved into the adult. Cell sorting and cell death are distinct steps associated with different genetic functions. Cell death depends on previous cell sorting, and proper formation of 1° p.c is a prerequisite for both processes.

How does IrreC-rst misexpression in cone cells inhibit interommatidial apoptosis?

The absence of any retinal defect in *elav-Gal4/UAS-HB3* flies (Fig. 4H,I) demonstrates that additional expression of IrreC-rst protein in photoreceptor neurons does not disturb interommatidial cell sorting or apoptosis. *sev-Gal4/UAS-HB3* misexpression - targeted to cone cells and photoreceptors, but not interommatidial or primary pigment cells – must therefore act on the apical rearrangement of cell contacts through the cone cells. It is necessary to explain how additional IrreC-rst expression in the cone cell quartet can affect the interommatidial cells across the intervening primary pigment cells.

Defects in the differentiation of 1° p.c. disturb subsequent steps of retinal pattern formation, as seen in the *facet*^{glossy} mutant (Fig. 6C,D; Cagan and Ready 1989a). Resulting phenotypes can be superficially similar to *rst*, but developmental analysis of *sev-Gal4/UAS-HB3* shows that 1° p.c. develop morphologically normally and close around cone cells (e.g. Fig. 4D). We therefore conclude that misexpression of IrreC-rst in cone cells does not inhibit the formation of 1° p.c.

A diffusible isoform derived from misexpressed IrreC-rst protein could saturate binding sites on putative interacting molecules. This isoform would have to arise from posttranslational proteolytic modifications as the transcript from the misexpressed cDNA cannot be spliced to yield a soluble form. As yet, there are no data that support the existence of such a form.

Membrane bound IrreC-rst in cone cells could saturate ligands present on 1° p.c., so that the ligand is underrepresented at the IOC/1° p.c. boundary. As a consequence, the interommatidial cells would not maximize their membrane contacts with 1° p.c.

What algorithm selects the cells to kill?

Our results show that IrreC-rst plays an essential role in the rearrangement of interommatidial cells prior to apoptosis. It remains an intriguing question whether it plays an additional role in the induction of the death program. The observation that misexpression does not induce additional cell death does not exclude the possibility that IrreC-rst plays a role in the induction of apoptosis, since a ligand necessary for activating IrreC-rst in this context could possibly be saturated in the presence of misexpressed protein.

How do interommatidial cells integrate the signals they receive from neighbouring differentiated cells? Interommatidial cells must be capable of determining how many different primary pigment cells they make contact with; a purely quan-

titative signal proportional to the surface area of contact is not sufficient. As cells contacting anterior as well as posterior primary pigment cells are generally determined for survival and the 2° p.c. fate, differing molecular markers on the anterior and posterior 1° p.c. could be involved.

The nature of the molecular mechanism finally targeting individual surplus cells for elimination still remains elusive. The membrane contact reorganization mediated by IrreC-rst expression appears to be a prerequisite for it. The *ec* mutant shows that there is an additional step between cell sorting and activation of the general apoptosis program: interommatidial apoptosis is suppressed in this mutant, but cell death is not generally inhibited. *ec* may play a role in identifying the surplus cells in the lattice ordered by IrreC-rst and activating the cell death program in them.

The present study demonstrates that cell contacts are of equal importance in specifying the apoptotic fate as in earlier cell fate decisions of retinal development: apoptosis can be completely suppressed when the proper sorting of cell contacts is disturbed.

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