IrreC/rst-mediated cell sorting during *Drosophila* pupal eye development depends on proper localisation of DE-cadherin

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Summary

Remodelling of tissues depends on the coordinated regulation of multiple cellular processes, such as cell-cell communication, differential cell adhesion and programmed cell death. During pupal development, interommatidial cells (IOCs) of the *Drosophila* eye initially form two or three cell rows between individual ommatidia, but then rearrange into a single row of cells. The surplus cells are eliminated by programmed cell death, and the definitive hexagonal array of cells is formed, which is the basis for the regular pattern of ommatidia visible in the adult eye. Here, we show that this cell-sorting process depends on the presence of a continuous belt of the homophilic cell adhesion protein DE-cadherin at the apical end of the IOCs. Elimination of this adhesion belt by mutations in *shotgun*, which encodes DE-cadherin, or its disruption by overexpression of DE-cadherin, the intracellular domain of Crumbs, or by a dominant version of the monomeric GTPase Rho1 prevents localisation of the transmembrane protein IrreC-rst to the border between primary pigment cells and IOCs. As a consequence, the IOCs are not properly sorted and supernumerary cells survive. During the sorting process, Notch-mediated signalling in IOCs acts downstream of DE-cadherin to restrict IrreC-rst to this border. The data are discussed in relation to the roles of selective cell adhesion and cell signalling during tissue reorganisation.

Key words: Zonula adherens, *crumbs*, *Notch*, *RhoA* (Rho1), Retinal development

Introduction

Morphogenesis during animal development involves not only the specification of particular cell fates and differentiation of specific cell types, but the organisation of cells into a defined three-dimensional structure. This process requires cell-cell communication and the formation of adhesive contacts between cells. A given pattern may be further refined by the elimination of surplus cells via programmed cell death (reviewed by Brachmann and Cagan, 2003; Freeman, 1997; Vaux and Korsmeyer, 1999).

The *Drosophila* compound eye is ideally suited for the dissection of the genetic and cell biological requirements that govern pattern formation in a complex system. The adult eye is composed of about 750 individual units, called ommatidia, that are arranged in a highly stereotypic pattern (Fig. 1A). Each ommatidium is composed of eight photoreceptor cells, four cone cells and two primary pigment cells. Ommatidia are separated from each other by secondary and tertiary pigment cells, and the number and arrangement of these defines the precise honeycomb-like arrangement of the ommatidia. Mechanosensory bristles are formed at alternating vertices of the hexagonal array (Fig. 1A,A′).

The adult compound eye develops from a single-layered epithelium, the eye imaginal disc. During the third larval instar, the eight photoreceptor cells and four lens-secreting cone cells are specified by a stereotypic sequence of inductive interactions (Freeman, 1997; Tomlinson, 1985). In the course of early pupal stages, the clusters of photoreceptor cells come to lie below the apical surface of the epithelium and are gradually covered by the four cone cells. The latter recruit the two primary pigment cells, which serves a sheath for the cone cell quartet. At this stage, the non-specified cells of the epithelium form the so-called interommatidial lattice. As pupal development proceeds, two-thirds of these interommatidial cells (IOCs) become specified as secondary or tertiary pigment cells, while the remaining one-third is eliminated by programmed cell death (Cagan and Ready, 1989). Preventing apoptosis during this stage by mutation of the gene *echinus* or persistent expression of the baculovirus inhibitor of apoptosis (p35) leads to the development of a rough-eye phenotype (Cagan and Ready, 1989; Hay et al., 1994; Wolff and Ready, 1991).

Programmed cell death during pupal development of the retina depends, as in other developmental systems, on the activation of the caspase cascade (reviewed by Hay, 2000; Song and Steller, 1999). However, the upstream events that initiate apoptosis and decide which cells of the interommatidial lattice survive and which die are poorly understood. The regulation of cell number by programmed cell death depends on at least two processes. First, signalling between neighbouring cells is crucial for the determination of cell fate, including the decision to live or die. Second, formation of correct adhesive structures between cells is an important aspect of cell fate specification and pattern formation. Two major

signalling cascades, mediated by the EGF and Notch receptors respectively, control cell fate specification and apoptosis in the *Drosophila* retina during pupal development. The EGF receptor/Ras pathway is required throughout eye development for the recruitment of cells into the developing ommatidia, which in turn prevents them from undergoing apoptosis (Freeman, 1996). Overexpression of an activated form of the EGF receptor allows all the cells in the developing eye to survive (Miller and Cagan, 1998). Expression analysis suggests that the signalling centre, which secretes the ligand Spitz, is localised in the cone cells and/or primary pigment cells. Ablation of these cells leads to increased programmed cell death (Miller and Cagan, 1998). The second signalling pathway involved, the Notch pathway, antagonises EGF receptor signalling. Activation of Notch-mediated signals between interommatidial precursor cells is required to remove excess cells. Thus, loss of *Notch* function in the IOCs results in the survival of surplus lattice cells, and this even occurs when the source of the survival signal has been eliminated by ablation of the primary pigment cells (Miller and Cagan, 1998).

Formation of adhesive contacts between neighbouring cells is the second important prerequisite for pattern formation. Cells within a given tissue can be sorted by selective adhesion, and the contacts they make may determine which signals they receive, and hence their future behaviour (reviewed by McNeill, 2000; Tepass et al., 2002). In the developing pupal retina, the IOCs are initially arranged in double or triple rows between the forming ommatidia. These cells then rearrange to form a single row of cells, aligned head-to-tail. Only after this reorganisation is complete are the surplus cells eliminated, suggesting that this cell-sorting process is a prerequisite for programmed cell death (Brachmann and Cagan, 2003; Reiter et al., 1996; Wolff and Ready, 1991). The importance of correct cell sorting during eye development is manifested by the retinal phenotype of flies that are mutant for *irregular chiasm C-roughest* (*irreC-rst*). Here, sorting of the IOCs into single rows does not occur, leaving the cells in double and triple rows (Reiter et al., 1996). In the wild-type retina, IrreC-rst accumulates at the interface between primary pigment cells and IOCs, and loss of IrreC-rst or its ubiquitous expression on all membranes prevents the end-to-end alignment of cells and the subsequent removal of supernumerary cells (Reiter et al., 1996). So far, only two genes have been reported to affect the distribution of IrreC-rst, *Notch* and *Delta*. Reducing the function of either during the crucial period causes a redistribution of IrreC-rst throughout the apical membranes, and prevents sorting and programmed cell death (Gorski et al., 2000).

As mentioned above, the recruitment and reorganisation of cells during eye development takes place in a single-layered epithelium. In spite of the morphogenetic changes that occur as development advances, such as the spreading of the cone cells over the photoreceptor cell cluster early in pupation or the sheathing of the cone cells by the primary pigment cells, the basic features of the epithelium – cell-cell adhesion and apicobasal polarity – are largely maintained. It is therefore tempting to assume that disruption of either of these processes might interfere with the sorting machinery and ultimately with programmed cell death. Several genes, such as *shotgun*, which encodes the homophilic cell adhesion protein DE-cadherin (Tepass et al., 1996; Uemura et al., 1996), and *armadillo*, which codes for the *Drosophila* homologue of β-catenin (Peiffer and Wieschaus, 1990), are involved in the adhesion process itself. Other genes are known to control apicobasal polarity and adhesion in embryonic epithelia. One of these, *crumbs* (*crb*), encodes a large transmembrane protein whose extracellular domain is composed of 30 EGF-like repeats (Tepass and Knust, 1990; Tepass et al., 1990). The four C-terminal amino acids – ERLI – of its short cytoplasmic domain serve to recruit a multiprotein complex that forms apical to the zonula adherens (ZA). This complex contains the MAGUK (membrane-associated guanylate kinase) protein Stardust (Sdt) (Bachmann et al., 2001; Hong et al., 2001), the four-PDZ-domain protein DPATJ [previously known as Discs lost (Pielage et al., 2003)] (Klebes and Knust, 2000) and the single PDZ-domain protein Lin7 (Bachmann et al., 2004). Mutations in *crb* do not interfere with the formation or maintenance of the epithelial tissue structure of the eye imaginal disc, and the external morphology of the adult eye of such a mutant is essentially normal (Johnson et al., 2002). However, loss of *crb* during eye development prevents the elongation of the photoreceptor cells, which is manifested in the formation of shorter and thicker rhabdomeres, and results in shortening of the stalk membrane, i.e. the part of the apical membrane between the adherens junctions and the rhabdomere (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002).

We set out to analyse the role of cell adhesion at earlier stages of eye development, particularly at stages when cells are subjected to major rearrangements. We wished to determine to what extent perturbation of their adhesive properties might influence cell sorting. Here, we show that IrreC-rst colocalises with DE-cadherin in the zonula adherens at the border between primary pigment cells and IOCs in pupal eye discs. Elimination of the continuous belt of DE-cadherin in the apical regions of the cells, or its disruption, leads to ectopic localisation of IrreC-rst, which in turn prevents cell sorting and programmed cell death. Our data further suggest that the restriction of IrreC-rst to the membranes between primary pigment cells and IOCs is controlled by Notch signalling.

Materials and methods

Fly strains, overexpression analysis and conditions of culture

The Gal4 lines used for overexpression studies have been described in previous publications. *GMR-Gal4* [obtained from B. Hay (Hay et al., 1994)] is expressed in all developing cells posterior to the morphogenetic furrow; later on it becomes active throughout most of the pupal eye (Ellis et al., 1993; Freeman, 1996). *sev-Gal4* (obtained from E. Hafen) is expressed in a subset of photoreceptors and in the cone cells of the *Drosophila* eye (Tomlinson et al., 1987). *elav-Gal4* is expressed in the photoreceptors throughout larval and pupal eye development (Lin and Goodman, 1994; Luo et al., 1994). *Rh-Gal4* directs expression in photoreceptors R1-R6 from 75% p.d. onwards (Kumar and Ready, 1995). *UAS-CD2* (Dunin-Burkowski and Brown, 1995) was used as a reporter to confirm the expression patterns of the Gal4-driver lines used for these studies. To test the effects of overexpression of *crumbs* in the *Drosophila* eye we used *UAS-*crb_{intra}*38.1.2b*, the weaker of the two available *UAS-*crb_{intra} lines (Wodarz et al., 1995). This construct and *UAS-*$crb_{intra\Delta ERLI}$, which lacks the codons for the four C-terminal amino acids of the protein (Klebes and Knust, 2000), were expressed under the control of the Gal4 driver lines listed above. *GMR-Gal4>*crb_{intra}*38.1.2b* (in the text

referred to as *GMR-Gal4>crb$_{intra}$*) is a recombinant line carrying *GMR-Gal4* and *UAS-crb$_{intra}$38.1.2b*. Homozygous flies of this line die during late pupal development or shortly after hatching and exhibit very rough eyes. *UAS-CAD* (Sanson et al., 1996; Strutt et al., 1997) and *UAS-Rho^{N19}* (Strutt et al., 1997) were kindly provided by M. Mlodzik. The *irreC^{CT}* mutation (kindly provided by K. Fischbach) truncates the intracellular domain of the IrreC-rst protein (Ramos et al., 1993) and causes a severe rough-eye phenotype (Wolff and Ready, 1991). *N^{ts1}* (Shellenbarger and Mohler, 1975) pupae were aged as indicated in the figure legends, shifted to 32°C for 7 hours and dissected immediately afterwards (Gorski et al., 2000). Clones homozygous mutant for the null allele *shg^{IH}* (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998) were generated in the eye imaginal disc by mitotic recombination facilitated by the FRT/FLP system (Johnson et al., 2002), using flies kindly provided by F. Schweisguth. *eyFLP* expresses the FLP recombinase under the control of the *eyeless* promoter (Newsome et al., 2000).

All flies were raised on a standard cornmeal agar food at 25°C, if not indicated otherwise in the figure legends. The pupae were staged at either 25°C [100% pupal development (p.d.) corresponds to 103 hours)] or 20°C (100% p.d.=160 hours). Eye discs were dissected at the time points indicated in the text.

Scanning electron microscopy of adult compound eyes

Flies were decapitated using razor blades, and a longitudinal incision was made between the eyes. Specimens were collected in 30% ethanol and dehydrated by passage through a graded ethanol series (50%, 70%, 90%, 95%, 100%), followed by incubation in acetone (100%), tetramethyl-silane (TMS):acetone (1:1) and 100% TMS. Specimens were gold coated using a SEM UNIT 5100 and examined with a Leitz-AMR1000 scanning electron microscope.

Immunohistochemistry

Antibody staining of eye discs was performed as described earlier (Reiter et al., 1996). The pupae were allowed to develop for various times (usually between 16 and 42% p.d.) after puparium formation at 20 or 25°C. The retina-brain complex was dissected in PBT (PBS with 0.1% Triton X-100), fixed for 1 hour in 4% paraformaldehyde and blocked with normal goat serum. The following primary antibodies were used: mouse anti-IrreC-rst [mAb 24A5.1, 1:50; obtained from K. Fischbach (Schneider et al., 1995)]; rat anti-DE-cadherin or anti-α-Catenin [anti-DE-Cad1, anti-Cat2; 1:50 and 1:100, respectively; obtained from T. Uemura (Oda et al., 1994; Uemura et al., 1996)] and mouse anti-CD2 (1:500; Biozol). Anti-rabbit-Cy2 and anti-mouse-Cy3 (1:200, Transduction Laboratories) were used as secondary antibodies. Immunostained imaginal discs were embedded in glycerol/propylgallate and analysed with a Leica TCS NT confocal microscope. Images were processed and mounted using Adobe Photoshop 6.0 and Canvas 9.0.

Detection of cell death

Third-instar larval imaginal discs and pupal eye discs 5-10 hours after the onset of interommatidial apoptosis, were stained with Acridine Orange according to Spreiji (Spreiji, 1971). Third-instar larval and pupal (31% p.d.) imaginal discs were rapidly dissected in cold PBS and stained in 0.5 μg/ml Acridine Orange for 15 minutes and 0.1 μg/ml Acridine Orange for 5 minutes, respectively. After a brief wash, the retinal preparations were mounted in PBS and immediately examined with a Zeiss Axiophot 2.

Results

Overexpression of Crumbs in the developing eye results in a rough-eye phenotype

Loss of *crumbs* (*crb*) function in the embryo results in the loss of apicobasal polarity and disrupts the integrity of several ectodermally derived epithelia (Tepass and Knust, 1990). Overexpression of the full-length protein or its membrane-bound cytoplasmic domain alone leads to a dominant phenotype, characterised by a redistribution of constituents of the adherens junctions, such as Armadillo, the *Drosophila* β-catenin and DE-cadherin, which ultimately also results in the formation of multilayered epithelia (Klebes and Knust, 2000; Wodarz et al., 1995). The *crb* function is not essential for the maintenance of epithelial cell polarity and adhesion in the imaginal discs, the single layered epithelia that give rise to most of the external structures of the adult fly. *crb* mosaic flies develop external structures (e.g. eyes, wings, legs) with nearly wild-type morphology. However, misexpression of *crb$_{intra}$*, a transgene that encodes the membrane-anchored 37-amino acid cytoplasmic domain of Crumbs, during larval/pupal stages of eye development leads to a dominant phenotype in the eye. Using either the *glass* multimer reporter *GMR-Gal4* or the *sev-GAL4* line to activate *UAS-crb$_{intra}$* results in a rough-eye phenotype that is characterised by perturbations of the regular pattern of the ommatidia and occasional bristle duplications (compare Fig. 1A,A′ with 1B,B′). The lenses are arranged in a square rather than in a hexagonal pattern in many cases. Use of other *UAS-crb* lines that express the construct at higher levels or encode the full-length Crumbs protein give rise to more severe phenotypes, in which the lenses are disrupted and may even become indistinguishable as individual units (not shown). The development of a rough-eye phenotype strongly depends on the presence of the four C-terminal amino acids of

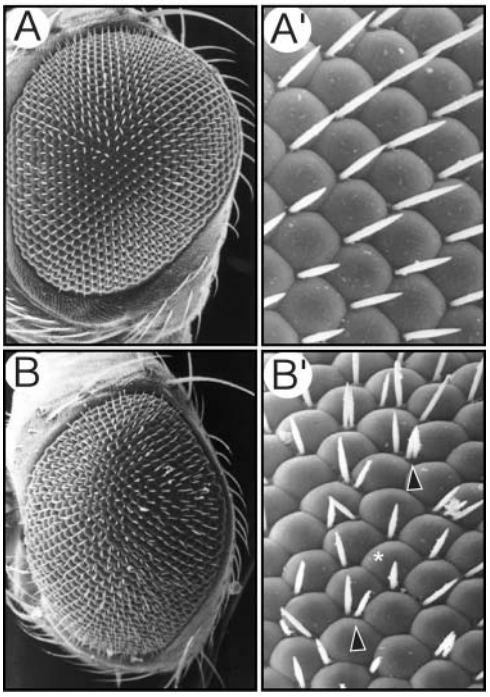

Fig. 1. Overexpression of Crb$_{intra}$ causes a rough eye phenotype. Scanning electron micrographs of adult eyes. (A,A′) Wild-type eyes show a regular, hexagonal arrangement of ommatidia. Bristles are found at alternating vertices of the hexagonal array. (B,B′) *sev-Gal4>UAS-crb$_{intra}$*. The roughening of the eye is induced by disorganisation of the array of facets; bristles are often doubled (arrowhead) and some lenses adopt a square shape (asterisk). Anterior is towards the left and dorsal towards the top.

the cytoplasmic domain, as the expression of a protein lacking these amino acids [*UAS-crb$_{intraΔERLI}$* (Klebes and Knust, 2000)] causes only a few bristle duplications and rare fusions of lenses (data not shown).

These findings raised the issue of where precisely Crb$_{intra}$ must be overexpressed in order to produce the rough-eye phenotype. It has previously been shown that overexpression of *crb$_{intra}$* in the developing eye leads to ectopic localisation of Armadillo and DPATJ, and affects the length of the adherens junctions in photoreceptor neurons (Fan et al., 2003; Izaddoost et al., 2002; Tanentzapf and Tepass, 2003). However, we never observed any roughening of the eyes upon overexpression of *crb$_{intra}$* driven by either *elav-Gal4* or *Rh-Gal4* (data not shown), which induce expression exclusively in photoreceptor cells. This suggests that the rough-eye phenotype results from persistent expression of *UAS-crb$_{intra}$* in the support cells, rather than in photoreceptor cells. To determine in which cells *GMR-Gal4* and *sev-Gal4* are expressed, we analysed their activity during pupal development, using the rat transmembrane protein CD2 (Dunin-Burkowski and Brown, 1995) as a reporter. *GMR-Gal4* was found to be expressed in all cell types (pigment, cone and photoreceptor cells) during the first half of pupal development, but later becomes restricted mainly to the photoreceptor cells. *sev-Gal4* is expressed in cone cells and in a subset of photoreceptor cells (data not shown). The only support cells that express Gal4 in both lines are the cone cells, suggesting that targeted expression of *UAS-crb$_{intra}$* in these cells is responsible for the mutant phenotype. The stronger roughening of the eye in *GMR-Gal4>UAS-crb$_{intra}$* flies in comparison with *sev-Gal4>UAS-crb$_{intra}$* could be due to the fact that expression in the pigment cells in *GMR-Gal4>UAS-crb$_{intra}$* may also contribute to the mutant phenotype.

Overexpression of *crb$_{intra}$* prevents sorting of interommatidial cells

Disruption of any one of several morphogenetic processes can lead to a rough-eye phenotype; among these are the prevention of cell sorting, inhibition of programmed cell death during pupal development (Hay et al., 1994; Reiter et al., 1996), and an increase or reduction in the number of photoreceptor neurons (Basler et al., 1991; Carthew and Rubin, 1990). Semi-thin sections of eyes overexpressing *crb$_{intra}$* reveal normal numbers of photoreceptor neurons (data not shown). Therefore we analysed pupal discs overexpressing *crb$_{intra}$* for defects in cell sorting. To do so, wild-type and *GMR-Gal4>UAS-crb$_{intra}$* pupal eye discs at different developmental stages were stained with an anti-DE-cadherin antibody to outline the cell membranes. In wild-type pupae at ~16% p.d., when the two primary pigment cells have been specified, two or more layers of interommatidial (lattice) cells (IOCs) separate individual ommatidia (Fig. 2A). Between 18 and 21% p.d., when the two primary (1°) pigment cells have completely enwrapped the cone cell quartet, the IOCs reorganise, so that each of them makes contact with at least two primary pigment cells in adjacent ommatidia. This results in the formation of a single row of lattice cells, aligned end-to-end, between individual ommatidia (Fig. 2B). The surplus cells are eliminated by programmed cell death, and the mature, hexagonal arrangement of the ommatidial cells becomes manifest (Fig. 2C). In pupal eyes that overexpress *crb$_{intra}$*, the number of IOCs is increased, and sorting of these cells fails to occur in many instances. As a consequence, even at ~42% p.d., there are too many IOCs [7.4 IOCs/ommatidium (total number of

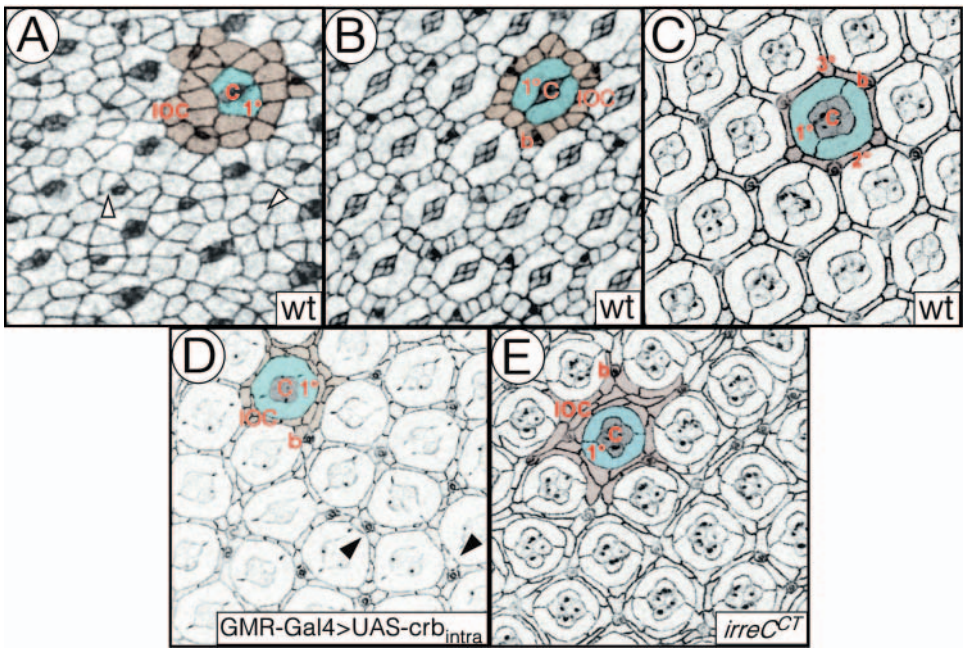

Fig. 2. Interommatidial cells in pupal eye imaginal discs overexpressing *crb$_{intra}$* or mutant for *IrreC-rst* are not properly sorted and surplus cells fail to be eliminated. (A-C) Wild-type; (D) *GMR-Gal4>UAS-crb$_{intra}$*; (E) *irreC^{CT}*. Anti-DE-cadherin antibody was used to visualise the plasma membranes. (A) At the onset of cell sorting (16% p.d.), formation of the primary pigment cells (1°; blue) around the cone cell quartet (C) is complete. Sorting of the interommatidial cells (IOC; brown) is in progress. Most ommatidial clusters are still separated by more than one row of IOCs (arrowheads). (B) At 21% p.d., cell sorting has been completed. The IOCs (brown) now form single rings of lattice cells, aligned head-to-tail, separating the ommatidial clusters from each other. b, bristle. (C) At 42% p.d. all surplus cells have been eliminated by apoptosis. The remaining cells are now arranged in a regular hexagonal pattern, in which secondary (2°) and tertiary (3°) pigment cells, as well as bristles (b), can be distinguished. (D) Discs overexpressing Crb$_{intra}$, examined at 42% p.d., after the time at which cell death occurs in the wild type. Cells are not properly rearranged and not all surplus cells have been eliminated; occasionally two rows of cells are left between ommatidial clusters (see also Fig. 5G,H). In several instances an 'astral' arrangement of the 2° and 3° pigment cells can be observed, in which four cells, instead of the usual three, surround a bristle (arrowheads, compare with C). (E) An *irreC-rst^{CT}* retina at 40% p.d. Here, none of the surplus cells has been eliminated, leaving the ommatidial clusters surrounded by two or three rows of cells. The arrangement is similar of that seen in discs overexpressing Crb$_{intra}$, although the phenotype is stronger, because the cell sorting is now disrupted in all instances (compare with D).

ommatidia counted: 495), in comparison with 4.9 IOCs/ommatidium in wild-type eyes (total number counted: 50 ommatidia)]. Many of the surplus IOCs are aligned side-by-side instead of end-to-end, so that two cell rows are found between many ommatidial cell clusters (Fig. 2D). The secondary and tertiary pigment cells are often arranged 'astrally': four cells, instead of the usual three, surround a bristle cell (compare Fig. 2C with 2D, arrowheads). As a result, the ommatidia adopt a square rather than a hexagonal shape, as also observed in SEM images of adult eyes (see Fig. 1B,B′). Ommatidia with one additional cone cell (11/495 ommatidia) or primary pigment cell (66/495) per ommatidium are also seen, albeit less frequently.

Overexpression of *crb$_{intra}$* perturbs the distribution of IrreC-rst and DE-cadherin

Mutations in *irregular chiasmC-roughest* (*irreC-rst*) give rise to a phenotype that is very similar to that described here (Reiter et al., 1996; Tanenbaum et al., 2000; Wolff and Ready, 1991). IOCs do not sort correctly, and each developing ommatidium is separated by two or three cell rows from its neighbours (Fig. 2E). *irreC-rst* encodes a transmembrane protein of the immunoglobulin superfamily that becomes localised at the interface between primary pigment cells and IOCs between 16 and 21% p.d. in wild-type discs, i.e. during the period when cell sorting takes place, and can also be found at this site at later stages of development (Reiter et al., 1996) (Fig. 3A,J). In

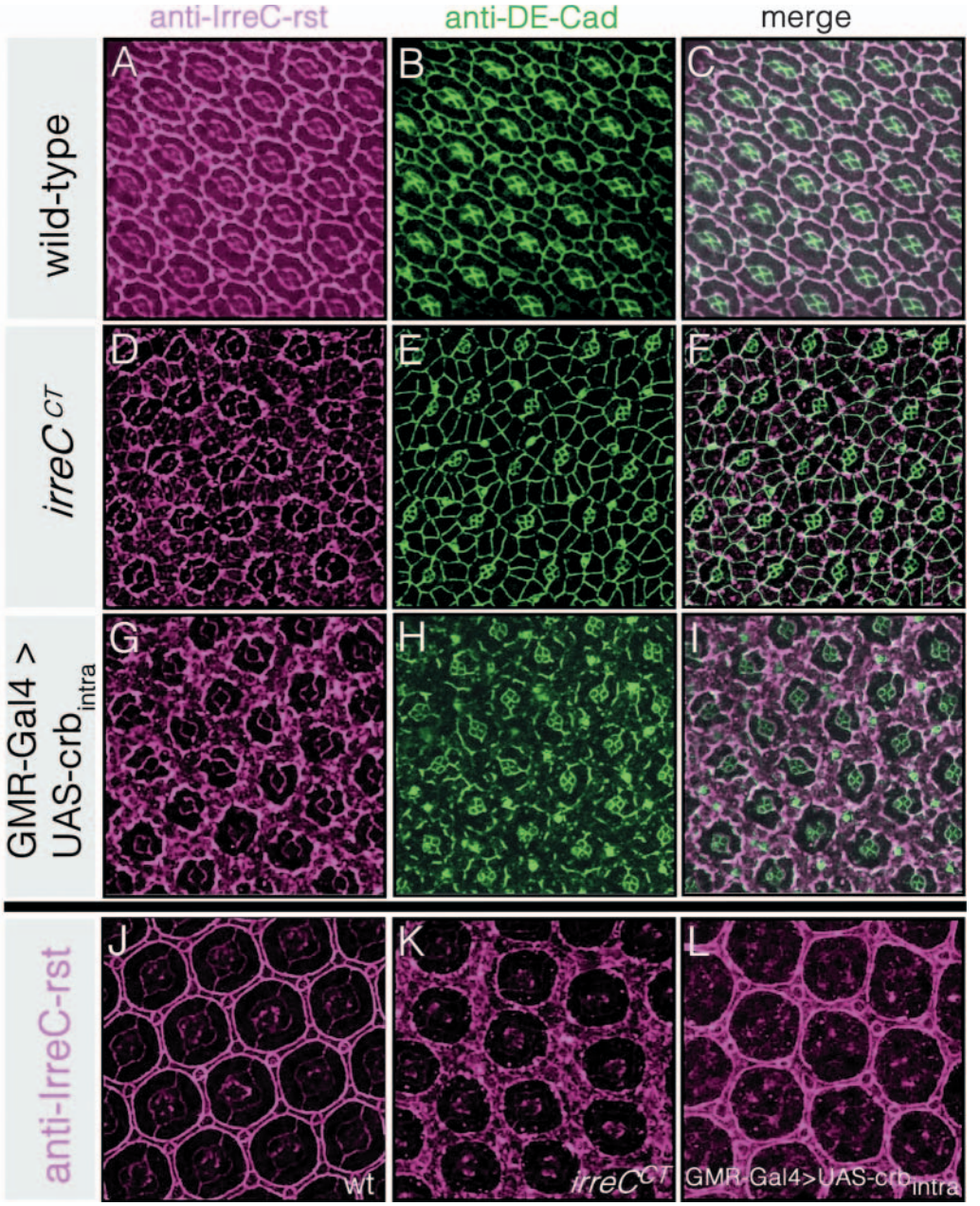

Fig. 3. Defects in IrreC-rst distribution in pupal eyes discs overexpressing *crb$_{intra}$* or mutant for *irreC-rst*. (A-C,J) Wild-type; (D-F,K) *irreC^{CT}*; (G-I,L) *GMR-Gal4>UAS-crb$_{intra}$*. (A-C) At 21% p.d., after completion of cell sorting, the IOCs form a single layer of lattice cells between the ommatidia. DE-cadherin (green) marks the apical regions of all cells. By contrast, IrreC-rst (violet) accumulates almost exclusively at the borders between primary pigment cells and IOCs (1°/IOC), where it colocalises with DE-cadherin (C). (D-F) In an *irreC-rst^{CT}* retina at 18% p.d., staining of IrreC-rst protein at the apical membranes of the 1°/IOC cell border is discontinuous. The protein localises in small patches along the membrane of the IOCs. The ommatidial clusters are surrounded by two or three rows of IOCs, outlined by staining for DE-cadherin. The distribution of DE-cadherin is unaffected by the mislocalisation of IrreC-rst (violet) and the protein still appears at the apex of all cells (green). (G-I) In a retina overexpressing Crb$_{intra}$ at 22% p.d., DE-cadherin (green) and IrreC-rst (purple) are discontinuously scattered in the apical cell membranes where they mostly colocalise. In addition, IrreC-rst also is also found diffusely distributed and in vesicles in the cytoplasm. (J,K) Crb$_{intra}$-overexpressing pupal retina at 42% p.d. (L), after the phase of cell death. The distribution of IrreC-rst now resembles the wild-type pattern (compare L with J). IrreC-rst accumulates, as in wild-type (42% p.d.), at the 1°/IOC border and around the bristles. In an *irreC-rst^{CT}* retina at 40% p.d. (K) there is no continuous zone of IrreC-rst protein at the apical membranes that form the 1°/IOC border. Instead, the protein is localised in vesicles in the cytoplasm.

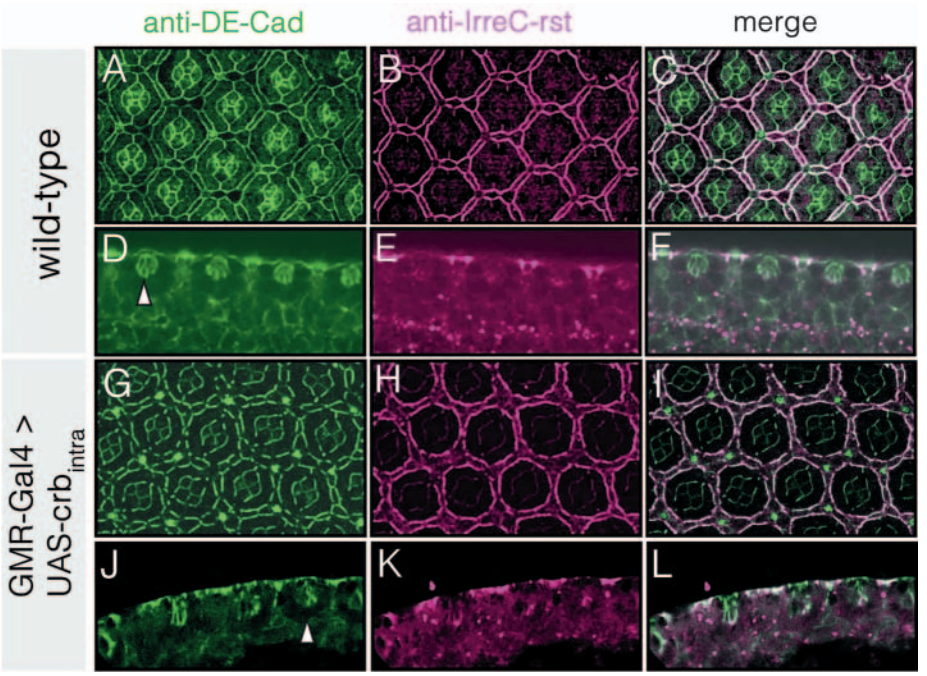

Fig. 4. IrreC-rst colocalises with DE-cadherin in the zonula adherens. (A-C) In the wild-type retina at 30% p.d., DE-cadherin (green) marks the apical region of all accessory cells, cone cells, primary pigment cells, IOCs and bristles (see also Fig. 3B). At this stage and later (Fig. 3J), IrreC-rst (violet) accumulates almost exclusively at the borders between primary pigment cells and IOCs (1°/IOC). DE-cadherin and IrreC-rst colocalise at the border between IOCs and primary pigment cells. (D-F) Longitudinal sections through a wild-type retina at 35% p.d. confirm the colocalisation in the apical region of the pigment cells, at the site of the adherens junctions. DE-cadherin is also found at the adherens junctions of the photoreceptor cells (arrowhead), which have not yet begun to elongate. (G-I) Retina overexpressing Crb_{intra} at 30% p.d. DE-cadherin (green) and IrreC-rst (violet) colocalise at the border between IOCs and primary pigment cells. The staining pattern is nearly the same as in wild type, showing only a few interruptions. (J-L) Longitudinal sections through a Crb_{intra}-overexpressing retina at 38% p.d. confirm the colocalisation of IrreC-rst and DE-cadherin in the apical region of the pigment cells, at the site of the adherens junctions. The structure of the adherens junction of photoreceptor cells (arrowhead) is less regular than in wild type.

mutants homozygous for *irreC-rst*^{CT}, an amorphic allele which encodes a non-functional protein with a truncated cytoplasmic domain, IrreC-rst protein is no longer contiguously expressed along the 1°/IOC boundary, but exhibits a patchy distribution at the membrane and in the cytoplasm; this fragmentation becomes more pronounced at later stages (Reiter et al., 1996) (Fig. 3D,K).

To address the issue of whether defective sorting of IOCs in discs that persistently express *crb*$_{intra}$ is associated with misdistribution of IrreC-rst, the expression of this protein was analysed in *GMR-Gal4>UAS-crb*$_{intra}$ pupal eye discs. At the time when cell sorting occurs (between 16 and 21% p.d.), IrreC-rst is discontinuously expressed along the interface between 1° pigment cell and IOCs, but is also found at the other surfaces of the IOCs and is diffusely distributed in the cytoplasm, a pattern that is reminiscent to that of IrreC-rst expression in *irreC*^{CT} mutant discs (compare Fig. 3G with 3D). However, from 25% p.d. onwards, the distribution gradually comes to resemble the wild-type pattern, so that at 42% p.d. the protein is nearly exclusively concentrated at the borders between primary and secondary pigment cells (compare Fig. 3J with 3L).

Embryos that overexpress *crb*$_{intra}$ develop multilayered epithelia, in which components of the zonula adherens (ZA), such as the homophilic cell-adhesion molecule DE-cadherin, are dispersed along the lateral membranes instead of being concentrated in the ZA (Klebes and Knust, 2000). To find out whether the failure to properly localise IrreC-rst and sort IOCs in eye discs overexpressing *crb*$_{intra}$ is the result of a disruption of epithelial structure and/or misdistribution of DE-cadherin, we analysed the structure of the epithelium and the distribution of DE-cadherin. Persistent expression of *crb*$_{intra}$ indeed disrupts the continuity of the DE-cadherin layer at the boundary between primary pigment cell and IOC, and between the IOCs themselves (compare Fig. 3B with 3H). By contrast, DE-cadherin localisation is not affected in *irreC-rst* mutant eye discs (Fig. 3E), suggesting that DE-cadherin acts upstream of IrreC-rst localisation. As both DE-cadherin and IrreC-rst are misdistributed in *GMR-Gal4>UAS-crb*$_{intra}$ pupal eye discs at the time when cell sorting occurs, we wondered whether the two proteins colocalise to the ZA. Indeed, we found that the two proteins do colocalise apically (Fig. 4A-F). In eye discs that overexpress *crb*$_{intra}$, however, partial colocalisation is observed at the time when cell sorting occurs (Fig. 3G-I), but at all stages both proteins are restricted to apical regions (Fig. 4G-L). However, at 42% of p.d., both proteins exhibit a nearly wild-type pattern of expression, being concentrated along the apical perimeter of the cells (Fig. 2D, Fig. 3L). These data thus suggest that *crb*$_{intra}$ overexpression disturbs the continuous apical distribution of DE-cadherin precisely at the time when cell rearrangements occur, but, unlike the case in the embryo, it does not markedly affect either apicobasal polarity or the overall structure of the epithelial tissue.

Overexpression of *crb*$_{intra}$ prevents programmed cell death

A second feature of *irreC-rst* mutant discs is the absence of programmed cell death during larval and pupal stages of development. To analyse whether inhibition of programmed cell death contributes to the mutant phenotype seen in discs that overexpress *crb*$_{intra}$, larval and pupal eye discs were stained with Acridine Orange. In the eye-antennal imaginal discs of wild-type third-instar larvae, zones of apoptosis can be detected in two regions: in a row of cells just ahead of the morphogenetic furrow, and a broader stripe posterior to the morphogenetic furrow (Fig. 5A) (Wolff and Ready, 1991). A

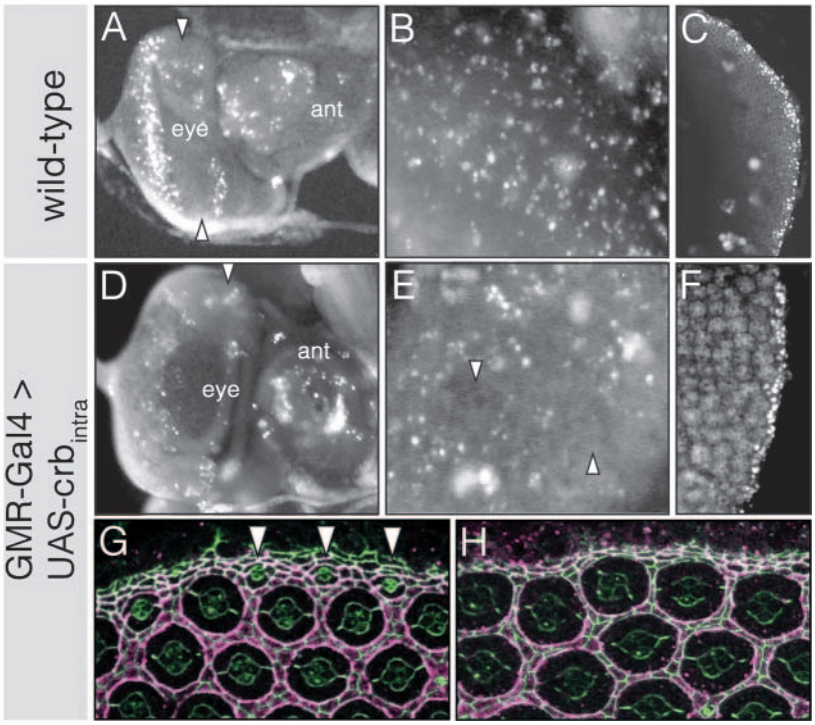

Fig. 5. Crb_{intra} expression prevents programmed cell death. (A-C) Wild-type, (D-H) *GMR-Gal4>UAS-crb$_{intra}$*. (A-F) Apoptotic cells visualised by Acridine Orange staining. (A-C) In the wild-type third-instar eye disc (A; eye, posterior to the left), dying cells appear anterior and posterior to the morphogenetic furrow (arrowheads). Cell death is also observed in the antennal region of the disc (ant). In pupae, fluorescent fragments of dying cells are visible throughout the disc at 31% p.d. (B) and in the perimeter clusters at 40% p.d. (C). (D-F) Cell death in *GMR-Gal4>UAS-crb$_{intra}$* larval discs (D) is strongly reduced posterior to the morphogenetic furrow (arrowhead), but still present anterior to the furrow and in the antennal disc (where *GMR-Gal4* is not expressed). In pupal discs at 31% p.d. (E) cell death is locally suppressed (arrowheads), while at 40% p.d. cell death in perimeter clusters is not affected (F). (G,H) Elimination of perimeter clusters in pupal imaginal discs overexpressing Crb_{intra}, visualised by immunostaining for IrreC-rst (violet) and DE-cadherin (green). At 38% p.d. (G), the perimeter clusters can still be identified (arrowheads). At 42% p.d. (H), the perimeter clusters have been eliminated.

second phase of apoptosis occurs between 23 and 31% p.d., when all that cells that have not been recruited into ommatidial clusters are eliminated (Fig. 5B). During the third phase, between 38% and 42% p.d., the ommatidia at the perimeter are removed (Fig. 5C) (Cagan and Ready, 1989; Hay et al., 1994; Wolff and Ready, 1991). Larval eye imaginal discs from the *GMR-Gal4>UAS-crb$_{intra}$* line exhibit a strong reduction in the number of Acridine Orange-positive cells posterior to the morphogenetic furrow (Fig. 5D). In the pupal eye discs, however, suppression of apoptosis is less pronounced and is only visible in small patches (Fig. 5E). The partial suppression observed may be attributable to the somewhat irregular expression of GMR-Gal4, which is also reflected by the fact that defects in cell sorting are not observed throughout the retina (see Fig. 2D). Nevertheless, the phenotype suggests that pupal eye discs of *GMR-Gal4>UAS-crb$_{intra}$* animals do contain more cells because of a marked decrease in the incidence of apoptosis during larval stages. Furthermore, these supernumerary cells are not eliminated at the pupal stage, as there is no compensatory enhancement of apoptosis in the pupal discs. However, the phenotype differs from that of *irreC-rst* mutant discs, in which cell death is completely blocked at both larval and pupal stages (Reiter et al., 1996; Wolff and Ready, 1991). This difference accounts for the difference in the severity of the cell-sorting defect (compare Fig. 2D with 2E). Interestingly, persistent expression of *crb$_{intra}$* does not prevent programmed cell death in the perimeter clusters at the perimeter of the disc (Fig. 5F).

Taken together, the data suggest that overexpression of *crb$_{intra}$* prevents cell death in the larva and interferes with the formation of a continuous belt of DE-cadherin in the epithelial cells of early pupal eye discs; the latter phenotype is also seen in embryonic epithelia upon overexpression of the membrane-anchored intracellular domain of Crb. As a consequence, IrreC-rst, which normally colocalises with DE-cadherin, exhibits a patchy expression pattern and accumulates at ectopic positions. This, in turn, apparently prevents the correct sorting of IOCs and ultimately results in a rough-eye phenotype.

Assembly of the apical belt of DE-cadherin is a prerequisite for the correct distribution of IrreC-rst

To test the idea that a continuous belt of DE-cadherin in the apical regions of the cells is required for the correct localisation of IrreC-rst, we analysed IrreC-rst expression in clones mutant for a putative null allele of its coding gene *shotgun* (*shg^{IH}*) (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998), which produce no protein detectable with DCAD1 (Oda et al., 1998) and in pupal discs that overexpressed DE-cadherin (*GMR-Gal4>UAS-DE-Cad*). Loss of *shg* prevents the accumulation of IrreC-rst at all membranes (Fig. 6A-C), while persistent expression of DE-cadherin leads to a patchy distribution of α-catenin, another component of the adherens junction (Fig. 6D-F). IrreC-rst becomes mislocalised during the stage when cell sorting occurs, but colocalises with α-catenin in many of the patches at the membrane between primary pigment cells and IOCs, and can also be detected at the borders between IOCs (Fig. 6D-F). The defects in cell sorting are milder than in discs overexpressing Crb_{intra} (not shown).

The assembly of cadherin-based adherens junctions is also regulated by Rho1, a member of the Rho family of small GTPases. In the *Drosophila* embryo, *Rho1* is essential for proper adherens junction formation and/or maintenance (Bloor and Kiehart, 2002; Magie et al., 2002). This led us to examine pupal eye discs that persistently expressed a dominant-negative version of Rho1, Rho1^{N19} (Strutt et al., 1997). At the stage of cell sorting (18% p.d.), the IOCs in these discs show an aberrant distribution of DE-cadherin. Only small amounts of protein are found dispersed in the cell membranes, while the majority of the protein is found in the cytoplasm (Fig. 6G).

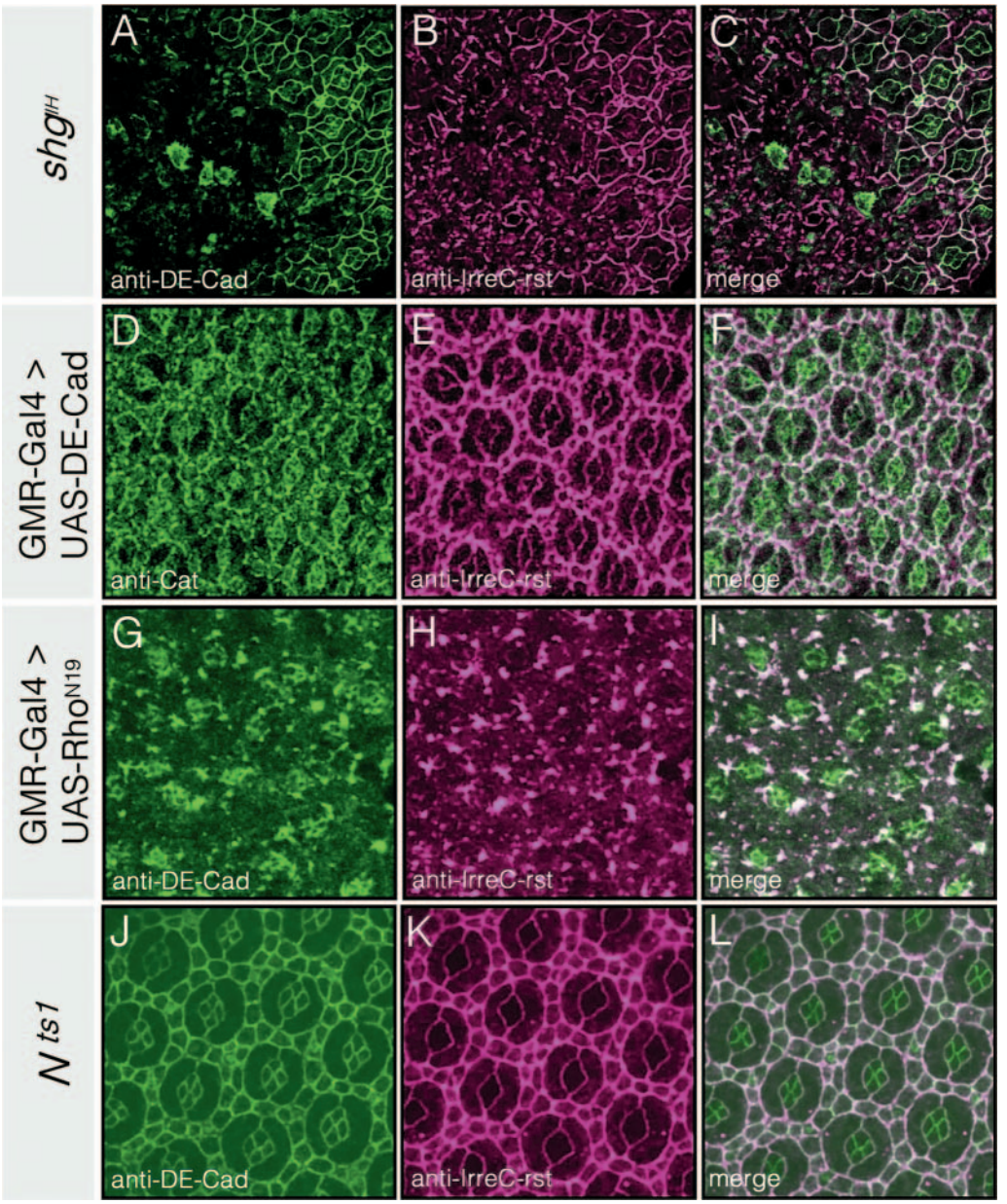

Fig. 6. Disruption of the adherens belt perturbs IrreC-rst localisation. (A-C) Clones homozygous mutant for *shg*^{IH} in a retina at 26% p.d., identified by the absence of DE-cadherin staining (green). IrreC-rst (violet) is not localised and appears as diffuse staining in the cytoplasm, while in the region heterozygous for *shg*^{IH} it colocalises with DE-cadherin at the 1°/IOC border. (D-F) *GMR-Gal4<UAS-CAD* retina at 22% p.d. exhibits a patchy distribution of α-catenin (green) in the apical regions of the cells and diffuse staining in the cytoplasm. IrreC-rst (violet) is colocalised with α-catenin at the 1°/IOC borders, but is also found at IOC/IOC borders. (G-I) In a *GMR-Gal4<UAS-Rho*^{N19} retina at 18% p.d. the continuous adherens junctions, as revealed by staining for DE-cadherin (green), are eliminated. Single cells or ommatidia are difficult to discern and only minor speckles of immunoreactivity can be detected in the plasma membranes of IOCs and primary pigment cells. The majority of the protein appears as diffuse staining in the cytoplasm. IrreC-rst (violet) adopts an identical pattern. [Flies raised at 18 (shown here) or 25°C die as pharate adults with severe head defects and very rough eyes that are only one-third the size of wild-type eyes.] (J-L) *Notch*^{ts1} discs heat-shocked at 19% p.d. In the absence of *Notch* function during cell sorting, IrreC-rst (violet) becomes ubiquitously distributed on all plasma membranes. Only partial cell sorting has taken place, leaving two rows of IOCs between ommatidia in many cases. DE-cadherin (green) is localised in a continuous apical belt but now also colocalises with IrreC-rst on all plasma membranes.

This suggests that, as in the embryo, *Rho1* is required during pupal eye development for the recruitment of the adherens junction component DE-cadherin into a continuous belt in the apical region of the cell. In agreement with the model proposed above, localisation of IrreC-rst is also severely affected in *GMR-Gal4>UAS-Rho*^{N19} discs; only speckles of immunoreactivity can be detected at the cell boundary, and these are not restricted to the 1°/IOC border but appear throughout the plasma membranes of the IOCs. However, most of these patches still colocalise with DE-cadherin (Fig. 6G-I).

To summarise, IrreC-rst is colocalised with DE-cadherin in epithelial cells of pupal eye discs, and misdistribution of adherens junction components induces the mislocalisation of IrreC-rst, which then affects sorting of IOCs. However, although DE-cadherin forms a continuous belt in the apical regions of all cells (including all IOCs) in wild-type discs, IrreC-rst colocalises with DE-cadherin only at the border between 1° pigment cells and IOCs (Fig. 3A-C). What factor(s) might be responsible for the spatial restriction of IrreC-rst to this border? It has recently been shown that the removal of *Notch* or *Delta* function during cell-sorting results in the ubiquitous distribution of IrreC-rst to all plasma membranes and the prevention of programmed cell death (Gorski et al., 2000). We analysed whether this might be the result of defective DE-cadherin localisation. Antibody staining reveals no influence of *Notch* on the continuous apical localisation of DE-cadherin, but shows that IrreC-rst now colocalises with the latter on all plasma membranes of the IOCs (Fig. 6J-L). This

suggests that Notch acts downstream of DE-cadherin in the control of IrreC-rst localisation. It is therefore tempting to speculate that it is the Notch pathway, which provides local signalling between the lattice cells to direct cell death (Miller and Cagan, 1998), that prevents the accumulation of IrreC-rst at the borders between IOCs and thus restricts its localisation to the 1°/IOC cell boundary.

Discussion

Pattern formation in the *Drosophila* eye disc depends on a well-balanced system of signals that promote either the survival or the death of cells, mediated by the EGF and Notch receptor pathways, respectively. In addition, the morphogenetic events, which take place in a single-layered epithelium, crucially depend on factors that regulate the maintenance of cell polarity and cell shape, and modulate cell adhesion. Sorting of interommatidial cells (IOCs) during pupal development, which results in the conversion of several parallel rows of cells into a single ring, requires the weakening of pre-existing adhesive cell contacts and the establishment of new ones without interrupting the epithelial integrity of the tissue. During tissue morphogenesis, epithelial cells use different strategies to modify their adhesive contacts (reviewed by Tepass et al., 2002). One of these consists of regulating the amount and/or distribution of the homophilic cell-adhesion molecule E-cadherin, one of the central components of the adherens junctions. The first in vivo evidence for this kind of regulation came from the analysis of the *Drosophila* egg chamber. Here, the localisation of the oocyte at the posterior pole depends on a higher level of expression of DE-cadherin in the oocyte and the posterior follicle cells, when compared with the nurse cells and other follicle cells (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). Differential adhesion can also be regulated by alterations in the composition or activity of intracellular binding partners, or by the integration of various other molecules into the adhesive complexes. No change in the distribution of the adherens junction components DE-cadherin and α-catenin could be detected in wild-type discs undergoing rearrangements of the IOCs. This behaviour contrasts with epithelial rearrangements during morphogenesis of the *Drosophila* tracheal system, which are associated with alterations in the amount of DE-cadherin, controlled by *Drosophila* Rac, another member of the Rho GTPase family (Chihara et al., 2003). This in turn suggests that other cytoplasmic or transmembrane proteins are involved in the modulation of adhesion in IOCs. The adhesion protein IrreC-rst is involved in the control of the cell sorting process. Its predominant localisation at the border between primary pigment cells and IOCs (at the 1°/IOC border) has been suggested to provide an attractive interface that controls sorting. According to this proposal, IOCs tend to maximise their contacts with primary pigment cells (Reiter et al., 1996). Failure to restrict IrreC-rst to this border results in the inability to sort the IOCs properly. Although IrreC-rst behaves as a homophilic adhesion molecule when expressed in cell culture, data from expression analysis argue for the presence of a different, as yet unknown, partner in the primary pigment cell (Reiter et al., 1996).

We were particularly interested in the relationship between the localisation of DE-cadherin, a component of the zonula adherens (ZA) and IrreC-rst. We show that in wild-type discs IrreC-rst colocalises with DE-cadherin at the 1°/IOC border in the apical ZA of the cell and that removal of DE-cadherin completely abolishes IrreC-rst accumulation. Nothing is yet known about how IrreC-rst may integrate into the ZA at this border. In vertebrates, the Ca^{++}-independent cell adhesion molecule nectin, a transmembrane protein of the immunoglobulin superfamily, has been implicated in the organisation of cadherin-based adherens junctions, tight junctions and synapses (reviewed by Takai and Nakanishi, 2003). It is recruited into cadherin-based adherens junctions through interactions with the PDZ domain of l-afadin, an F-actin-binding protein (Takahashi et al., 1999). Intriguingly, the C-terminal sequence of IrreC-rst (T-A-V) matches the consensus binding site for class I PDZ domains (S/T-X-V) (Harris and Lim, 2001). Interestingly, the protein encoded by the mutant allele *irreC-rst*^{CT}, which lacks the C-terminal 175 amino acids of the wild-type form (Reiter et al., 1996), is no longer recruited into the ZA (Fig. 4G-I). It is, however, unlikely that IrreC-rst acts as a general adhesion molecule in IOCs of pupal eye discs, because the epithelial tissue structure is stable in the absence of *irreC-rst* function, as deduced from the formation of the continuous apical belt of DE-cadherin in i*rreC-rst* mutants (see Fig. 4G).

The continuous belt of DE-cadherin can be disrupted by a number of different genetic conditions, such as overexpression of the membrane-bound intracellular domain of Crumbs, of DE-cadherin itself, or of a dominant-negative version of the monomeric GTPase Rho1. Overexpression of the membrane-bound intracellular domain of Crumbs in embryonic epithelia has previously been shown to lead to a redistribution of DE-cadherin throughout the plasma membrane and the formation of multilayered tissues (Grawe et al., 1996; Klebes and Knust, 2000). By contrast, IOCs overexpressing Crb_{intra} exhibit a fragmented DE-cadherin belt, which remains localized in the apical zone of the cells, and apicobasal organisation and tissue integrity are not affected. This suggests that IOCs may contain additional adhesion components which are independent of, or less affected by, Crb. Support for this view comes from the phenotype of discs lacking *crb* function, in which the apical belt of DE-cadherin expression is fragmented, yet there is no major effect on polarity or adhesion of the epithelium, the cells undergo nearly normal sorting and IrreC-rst is still restricted to the membrane at the 1°/IOC border (N.G. and E.K., unpublished). Overexpression of $Crb_{intra\Delta ERLI}$ does not interfere with sorting, suggesting that a protein complex similar to the one that controls apicobasal polarity in embryonic epithelia (which includes Stardust, DPATJ and D-Lin7) contributes to the development of the dominant phenotype.

Overexpression of DE-cadherin similarly results in the fragmentation of the adhesion belt and defects in cell sorting. In various tissues, overexpression of full-length DE-cadherin can also reduce Wingless signalling by sequestering Armadillo from the cytoplasmic pool, thus making it unavailable to transduce the Wingless signal (Sanson et al., 1996). However, we can exclude the possibility that the defects in sorting are the result of a suppression of Wingless signalling. Inactivation of components of the Wingless pathway in eye imaginal discs induces the initiation of ectopic morphogenetic furrows (Ma and Moses, 1995; Treisman and Rubin, 1995), and this phenotype was not observed upon overexpression of DE-

cadherin. Overexpression of DE-cadherin in eye discs therefore seems to interfere with adhesion, rather than Wingless signalling.

Rho GTPases play central roles in the organisation of the actin cytoskeleton and in cell adhesion (reviewed by Hall, 1998; van Aelst and Symons, 2002). In mammals, inhibition of Rho activity results in the removal of cadherins from epithelial cell junctions (Braga et al., 1999; Braga et al., 1997; Takaishi et al., 1997), while increased Rho activity induces an invasive and metastatic phenotype (Schmitz et al., 2000). Members of the Rho GTPase family are recruited into the adherens junctions by direct interactions with junctional components. Thus, in *Drosophila*, Rho1 localises to the adherens junctions and interacts directly with α-catenin and $p120^{ctn}$, a homologue of β-catenin. As in pupal epithelia expressing a dominant-negative form of Rho1, *Rho1* mutant embryos exhibit a diffuse distribution of components of the ZA, such as DE-cadherin and α- and β-catenin (Magie et al., 2002). Rho1 may either act directly on the accumulation of cadherins at the junctions, or indirectly by recruiting accessory proteins, which then modulate the amounts or activity of junctional and/or cytoskeletal proteins. Rho1 plays a different role in tracheal epithelia insofar as its inactivation does not disrupt DE-cadherin localisation, but rather interferes with the formation of the apical surface and the tracheal lumen (Lee and Kolodziej, 2002).

Although it is evident that DE-cadherin plays a crucial role in the accumulation of IrreC-rst at the adherens junctions, other mechanisms are required to explain the asymmetric localisation and restriction of the latter to the 1°/IOC boundary. Reiter et al. (Reiter et al., 1996) have speculated that an as yet unknown ligand expressed in the primary pigment cell may account for this restricted accumulation. As an alternative – but not mutually exclusive – model, we suggest that signalling between the IOCs, mediated by Notch, which is expressed in IOCs during pupal development (Kooh et al., 1993), prevents the accumulation of IrreC-rst at their borders. Interplay between adhesion and signalling molecules also directs other processes in which cellular polarisation is involved in tissue remodelling. The growth of the wing imaginal disc along the proximodistal axis, for example, is the result of cell shape changes and cell rearrangements during pupal development, which are controlled by the atypical cadherins Fat and Dachsous, as well as Four-Jointed, which is assumed to be a secreted molecule. This process, in turn, is responsible for the asymmetric localisation of components that control planar polarity, such as Frizzled, Dishevelled or Strabismus, which serves to ensure that bristles and hairs adopt a common orientation (for reviews, see Adler, 2002; Eaton, 2003). During germ band elongation in the *Drosophila* embryo, adherens junction remodelling in intercalating ectodermal cells is facilitated by the polarised expression of non-muscle myosin II at the anteroposterior and of Bazooka at the dorsoventral cell boundaries (Bertet et al., 2004; Zallen and Wieschaus, 2004). Future experiments will demonstrate whether cell sorting in pupal eye discs makes use of any of the components known to be involved in these processes.

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