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Increased levels of the cytoplasmic domain of Crumbs repolarise developing *Drosophila* photoreceptors

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Summary

Photoreceptor morphogenesis in *Drosophila* requires remodelling of apico-basal polarity and adherens junctions (AJs), and includes cell shape changes, as well as differentiation and expansion of the apical membrane. The evolutionarily conserved transmembrane protein Crumbs (Crb) organises an apical membrane-associated protein complex that controls photoreceptor morphogenesis. Expression of the small cytoplasmic domain of Crb in *crb* mutant photoreceptor cells (PRCs) rescues the *crb* mutant phenotype to the same extent as the full-length protein. Here, we show that overexpression of the membrane-tethered cytoplasmic domain of Crb in otherwise wild-type photoreceptor cells has major effects on polarity and morphogenesis. Whereas early expression causes severe abnormalities in apico-basal polarity and ommatidial integrity, expression at later stages affects the shape and positioning of AJs. This result supports the importance of Crb for junctional remodelling during morphogenetic changes. The most pronounced phenotype observed upon early expression is the formation of ectopic apical membrane domains, which often develop into a complete second apical pole, including ectopic AJs. Induction of this phenotype requires members of the Par protein network. These data point to a close integration of the Crb complex and Par proteins during photoreceptor morphogenesis and underscore the role of Crb as an apical determinant.

Key words: Morphogenesis, Polarity, Rhabdomere, Crumbs, Adherens junction

Introduction

Epithelia are sheets of adherent cells that separate different body compartments. They serve as diffusion barriers and are required for directed secretion and absorption. Epithelial cells are polarised along the apico-basal axis, with the apical surface facing the outside and the basal domain contacting the basal lamina. The lateral membrane is in contact with neighbouring cells and forms various junctions to seal the epithelium and ensure tissue integrity. In all epithelia studied so far, establishment and maintenance of a polarised phenotype depends on the integrated activity of several evolutionarily conserved proteins. Furthermore, epithelial polarity is closely linked to the formation of the zonula adherens (ZA), an adhesion belt encircling the apex of each cell. During development, epithelia undergo a variety of morphogenetic movements, which require assembly and remodelling of junctions. At the same time, epithelial cells have to maintain their polarised state, confronting them with a considerable challenge (Bryant and Mostov, 2008; Harris and Tepass, 2010; St Johnston and Ahringer, 2010).

Drosophila photoreceptor cells (PRCs) are ideally suited to study the genetic and cell biological basis that ensures maintenance of cell polarity and adhesion during morphogenetic processes. PRCs develop from a single-layered epithelium, the eye–antennal imaginal disc. They exhibit a pronounced apico-basal polarity, including a ZA, which they inherit from the epithelial cells of the disc. After specification and recruitment into ommatidial clusters, PRCs undergo a series of morphogenetic changes. Initially, their apical membranes undergo a shift of 90°, subsequently facing the ommatidial centre. In a second step, the apical membranes separate into the most apical rhabdomere and the adjacent stalk. The rhabdomere is formed by tightly packed microvilli, which harbour components of the phototransduction cascade. Concomitantly with the expansion and differentiation of the apical membrane, PRCs substantially elongate along the proximo-distal axis, forming the axon basally (Longley and Ready, 1995).

The transmembrane protein Crb is involved in orchestrating photoreceptor morphogenesis by controlling the remodelling of the ZA and by regulating the size of the stalk membrane. In addition, Crb ensures light-dependent survival of adult PRCs (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). Crb is also required in ectodermally derived epithelia in the *Drosophila* embryo, where it controls assembly of a continuous ZA and maintenance of apico-basal polarity (Grawe et al., 1996; Tepass, 1996; Tepass and Knust, 1990; Tepass and Knust, 1993; Tepass et al., 1990). Recent studies have shown that Crb is particularly important in tissues that undergo extensive morphogenetic movements, such as the embryonic epidermis during germband extension or the Malpighian tubules undergoing convergent extension, processes that require rapid remodelling of AJs to maintain tissue integrity (Campbell et al., 2009; Harris and Tepass, 2008).

crb encodes a type I transmembrane protein that consists of a large extracellular domain, containing an array of EGF-like repeats and four Laminin A G-domain-like domains, and a small intracellular part of 37 amino acids (Tepass et al., 1990). The cytoplasmic domain contains a FERM-binding site and a C-terminal PDZ-binding motif, which links Crb to the membrane-

associated guanylate kinase (MAGUK) Stardust (Sdt). Sdt, in turn, binds *Drosophila* Pals1-associated tight junction protein (PATJ) and *Drosophila* Lin-7 (also known as Veli) (Bachmann et al., 2008; Bachmann et al., 2001; Bachmann et al., 2004; Bulgakova and Knust, 2009; Hong et al., 2001; Klebes and Knust, 2000; Pielage et al., 2003). Several links between the cytoplasmic domain of Crb and members of the Par protein network have been described, including Par-6 and atypical protein kinase C (aPKC) (Goldstein and Macara, 2007; Hurd et al., 2003; Kempkens et al., 2006; Lemmers et al., 2004; Sotillos et al., 2004; Wang et al., 2004). With its FERM-binding motif, Crb binds to the FERM-domain-containing protein Yurt (Yrt), which negatively regulates Crb activity (Laprise et al., 2006). Recent reports suggest that there is a link between Crb and the Salvador–Warts–Hippo pathway via the FERM-domain-containing protein Expanded (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). Association of Crb with β_H-spectrin (β_H-spec), a major component of the apical spectrin-based membrane skeleton, points to an important connection of the Crb complex to the membrane-associated cell cortex (Medina et al., 2002; Pellikka et al., 2002; Richard et al., 2009).

Expression of the membrane-bound intracellular domain of Crb in *crb* mutant embryos and photoreceptors suppresses the *crb* mutant phenotype to the same extent as expression of full-length Crb (Richard et al., 2009; Wodarz et al., 1995). In addition, overexpression of this intracellular domain in wild-type embryonic epithelia or PRCs affects the stability of the ZA and the integrity of epithelia (Fan et al., 2003; Izaddoost et al., 2002; Klebes and Knust, 2000; Nam and Choi, 2003). To get a deeper understanding of the mechanisms by which Crb controls PRC morphogenesis, we performed a more detailed analysis of the dominant phenotype. We show that PRCs are particularly susceptible to increased levels of Crb at early stages, during which these cells undergo major remodelling of polarity and adhesion. PRCs lose integrity and often form ectopic apical membrane domains, including AJs. We further demonstrate that members of the Par protein network mediate the Crb-induced phenotype. Our results underscore the importance of the cooperation of the Crb complex and the Par protein network for normal photoreceptor morphogenesis, and will be discussed in the context of recently published data (Krahn et al., 2010a; Morais-de-Sa et al., 2010; Walther and Pichaud, 2010).

Results

Overexpression of $Crb_{FLAGintra}$ in developing photoreceptors affects ommatidial integrity and photoreceptor polarity

To understand the molecular function of the intracellular domain of Crb during photoreceptor morphogenesis, we expressed a FLAG-tagged version of the membrane-tethered intracellular domain, $Crb_{FLAGintra}$ (Richard et al., 2009), in otherwise wild-type photoreceptors. In order to activate expression at various developmental stages, we used two different Gal4 driver lines, *elav*Gal4 and *Rh1*Gal4 (Kumar and Ready, 1995; Luo et al., 1994; Yao and White, 1994). In the developing eye, *elav*Gal4 is expressed from late third-instar larvae onwards in all neuronal cells, with expression levels being higher in immature than in mature neurons (Robinow and White, 1988; Robinow and White, 1991). *Rhodopsin 1* (*Rh1*) expression starts at ~70% pupal development (pupal development) and is maintained throughout the life of the fly. The expression is restricted to the outer PRCs R1–R6, so that R7 and R8 can be used as internal controls (Kumar and Ready, 1995). Importantly, in the eye, both driver lines activate expression of transgene-encoded proteins exclusively in PRCs, which allowed us to specifically investigate the function of the intracellular domain of Crb in these cells.

The *Drosophila* eye is a compound eye composed of about 800 individual ommatidia. Each ommatidium contains eight PRCs that are arranged in a highly stereotypic pattern. The apical membrane of the photoreceptors is subdivided into the rhabdomere and the stalk membrane and points towards the interrhabdomeral space (IRS), a lumen that forms in the centre of each ommatidium (Fig. 1A). Early expression of $Crb_{FLAGintra}$, in otherwise wild-type PRCs, using *elav*Gal4 resulted in strong morphological defects, which was reflected by alterations in ommatidial integrity and photoreceptor apico-basal polarity (Fig. 1B–F). The most prominent phenotype was the formation of ectopic rhabdomeres and AJs at the basolateral side of individual PRCs (Fig. 1B,C,E,F, AJs highlighted with red arrows in Fig. 1F). In some cases, a complete reversion of apico-basal polarity was observed, with the basolateral membrane domain now facing the ommatidial centre (Fig. 1B, blue asterisk). Ommatidia with ectopic rhabdomeres often formed an additional lumen adjacent to ectopic rhabdomeres, which was reminiscent of the IRS and separated the PRCs from the neighbouring pigment cells (Fig. 1B,C,E, blue arrows). Unlike wild-type rhabdomeres of R1–R6, which span the entire depth of the retina (about 100 μm), ectopic rhabdomeres had a length of only 5–15 μm (Fig. 1J′, red arrows). Furthermore, PRCs often ruptured the basal lamina (Fig. 1H,H′,J–J″, indicated with black or white arrows). Besides formation of ectopic rhabdomeres, a variety of other phenotypes were observed, which comprise displacement of PRCs within the ommatidium (Fig. 1B,C), splitting of rhabdomeres (Fig. 1B, red arrowheads) and a decrease in the number of PRCs per ommatidium (Fig. 1D). Despite these strong morphological defects, we never observed a roughening of the eyes (data not shown), as seen upon overexpression of $Crb_{FLAGintra}$ using *GMR*Gal4, which drives expression in all ommatidial cells (Grzeschik and Knust, 2005; Hay et al., 1994).

Taken together, overexpression of the membrane-tethered intracellular domain of Crb during late larval and pupal development severely affects photoreceptor polarity and induces variable effects on their morphology and integrity.

Overexpression of $Crb_{FLAGintra}$ in developing photoreceptors results in delocalisation of polarity and junctional proteins

In adult PRCs, the core components of the Crb complex, Crb, Sdt, *Drosophila* PATJ and *Drosophila* Lin-7, colocalise at the stalk, the part of the apical membrane between AJs and rhabdomeres (Fig. 2A,C,E,G,I and data not shown) (Bachmann et al., 2008; Hong et al., 2003; Izaddoost et al., 2002; Johnson et al., 2002; Nam and Choi, 2003; Nam and Choi, 2006; Pellikka et al., 2002; Richard et al., 2006). In PRCs overexpressing $Crb_{FLAGintra}$ under the control of *elav*Gal4, localisation of Crb complex members was strongly affected. These proteins now also localised at the basolateral membrane (Fig. 2B′,B‴,D′,D‴, F′, F‴,H′,H‴). In cells with split rhabdomeres Sdt was present at the gap between the rhabdomeral parts (Fig. 2B′, arrowhead),

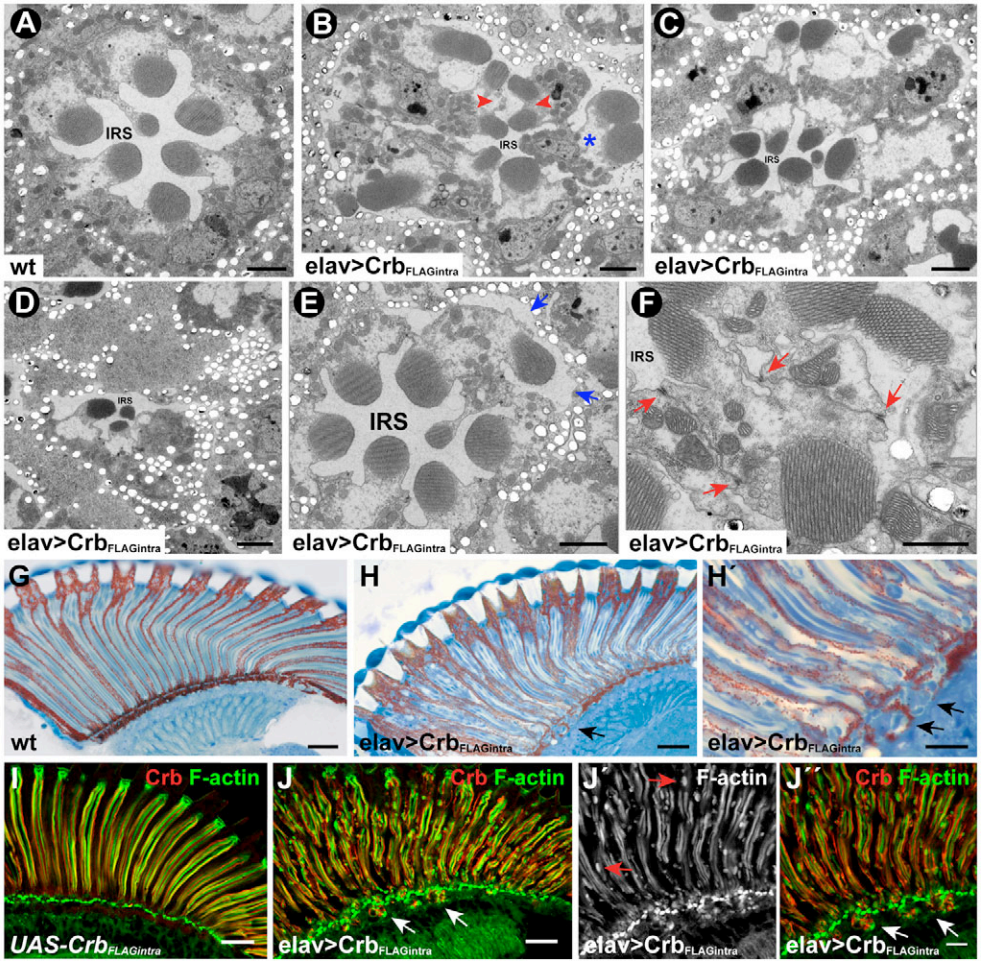

Fig. 1. Overexpression of Crb$_{FLAGintra}$ during late larval and pupal development induces morphological defects. (**A–F**) Electron micrographs of cross-sections of an adult wild-type ommatidium (A) and ommatidia overexpressing Crb$_{FLAGintra}$ using *elav*Gal4 (B–F). Overexpression of Crb$_{FLAGintra}$ during late larval and pupal development causes severe morphological defects, including: loss of photoreceptor adhesion (B,C); ectopic rhabdomeres at the basolateral membrane of the photoreceptors, which are often flanked by additional AJs (B,C,E,F, AJs are highlighted with red arrows in F); splitting of rhabdomeres (B, red arrowheads); reversion of apico-basal polarity (B, blue asterisk); a decrease in the number of PRCs per ommatidium (D); and ectopic lumen formation (E, blue arrows). (**G–J**) Longitudinal sections of adult control eyes (G,I) and eyes overexpressing Crb$_{FLAGintra}$ using *elav*Gal4 (H,H′,J–J″). Sections were stained with Toluidine Blue (G–H′) or phalloidin to label rhabdomeres and the basal lamina, and Crb to visualise the stalk membrane (I–J″). Black or white arrows (H,H′,J, J″) point to sites where PRCs have disrupted the basal lamina, the red arrows in J′ indicate ectopic rhabdomeres that typically span 5–15 µm of the retina. Scale bars: 2 µm (A–E); 1 µm (F); 20 µm (G,H,I,J); and 10 µm (H′,J″).

suggesting that an additional small stalk had formed. In many cases, ectopic rhabdomeres were flanked by short regions positive for Crb complex members (Fig. 2B′,D′,F′,H′) and the ZA component *Drosophila* E-Cadherin (*Drosophila* E-Cad) (Fig. 2B), which is consistent with the formation of ectopic stalks and AJs (see Fig. 1F), and indicates development of a complete ectopic apical pole.

A polarised phenotype strongly depends on functional membrane trafficking. This is reflected by the polarised accumulation of distinct proteins, including the light-receptor Rhodopsin 1 (Rh1), which predominantly localises in the rhabdomere, forming a crescent near the base (Fig. 2C) (Kumar and Ready, 1995), and the agrin- and perlecan-related matrix component Spacemaker (Spam, also known as Eyes shut, Eys) that is secreted into the IRS (Fig. 2E) (Husain et al., 2006; Zelhof et al., 2006). Upon overexpression of Crb$_{FLAGintra}$, Rh1 was also present in ectopic rhabdomeres (Fig. 2D–D‴, arrows) and Spam filled the additional lumen (Fig. 2F–F‴, arrows), formed between PRCs with ectopic rhabdomeres and pigment cells (see Fig. 1B–C,E). This indicates that the cytoplasmic domain of Crb reorganises the apical secretory machinery and that the ectopic lumen has characteristics of an IRS. We noticed, however, that there was not a complete reorganisation of the polarised trafficking machinery. In adult wild-type flies, the alpha-subunit of the Na^+/K^+-ATPase is present at the basolateral membrane of all PRCs and at the stalk of R7 and R8 (Fig. 2G) (Yasuhara et al., 2000). In cells expressing Crb$_{FLAGintra}$, the Na^+/K^+-ATPase was completely excluded from ectopic rhabdomeres, but was only diminished at sites with ectopic Crb (Fig. 2H–H‴). Strikingly, PRCs with an ectopic apical pole also showed Na^+/K^+-ATPase staining at the original apical stalk (Fig. 2H–H‴). This suggests that expression of the intracellular domain of Crb causes an intermingling of apical and basolateral proteins within the plasma membrane and thus loss of membrane identity.

To summarise, the membrane-tethered intracellular domain of Crb, when overexpressed in early PRCs, severely affects apico-basal polarity. Cells develop an additional apical pole, remodel their secretory transport machinery and exhibit a partial intermingling of apical and basolateral proteins.

Overexpression of Crb$_{FLAGintra}$ in late pupae and adult flies affects adherens junctions and localisation of polarity proteins

To analyse the effects induced by overexpression of Crb$_{FLAGintra}$ in late pupae and adult, we used *Rh1*Gal4, which starts expression after the stalk membrane and the rhabdomere have been separated from each other (Kumar and Ready, 1995). In contrast to results obtained using *elav*Gal4, this later expression of Crb$_{FLAGintra}$ produced no major defects in photoreceptor morphology and adhesion, and ectopic rhabdomeres developed in the distal part of the cells only occasionally (data not shown). However, the ZAs appeared split in many cells (Fig. 3C, arrows)

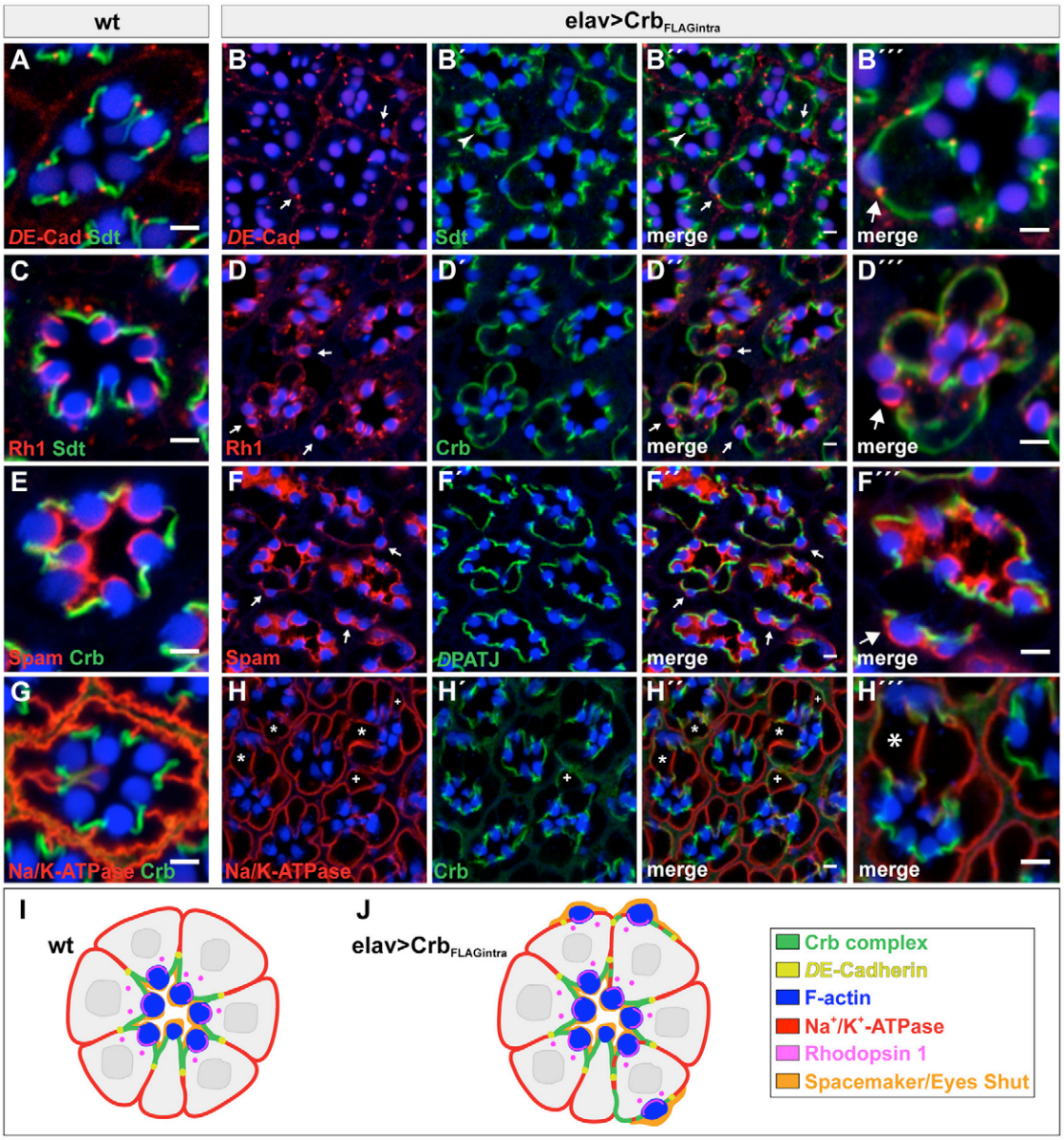

Fig. 2. Overexpression of $Crb_{FLAGintra}$ during late larval and pupal development affects photoreceptor apico-basal polarity. (**A–H**) Immunostainings on cross-sections of adult wild-type (wt) eyes (A,C,E,G) and eyes overexpressing $Crb_{FLAGintra}$ using *elav*Gal4 (elav>$Crb_{FLAGintra}$) (B,D,F,H). The sections were stained for *Drosophila* E-Cad (*D*E-Cad) (A,B), Rh1 (C,D), Spam (Eys) (E,F) or the alpha subunit of Na^+/K^+-ATPase (G,H) (in red), and Sdt (A–C), Crb (D,E,G,H) or *Drosophila* PATJ (F) (in green). B‴, D‴, F‴ and H‴ represent higher magnifications of one single ommatidium shown in B″, D″, F″ and G″, respectively. The F-actin stain (blue) shows the rhabdomeres. Sdt (B′), Crb (D′,H′) and *Drosophila* PATJ (F″) exhibit expanded expression domains and often localise adjacent to ectopic rhabdomeres. Additionally, Sdt localises between split rhabdomeres (B′,B″, white arrowhead). Note ectopic *Drosophila* E-Cad flanking ectopic rhabdomeres (B, B″,B‴, white arrows). Ectopic apical poles are positive for Rh1 (D,D″,D‴, arrows) and Spam (Eys) (F, F″, F‴, arrows). Na^+/K^+-ATPase is absent from ectopic rhabdomeres and diminished at membranes with high levels of ectopic Crb (H,H″,H‴, the decrease is indicated by a cross). In addition, Na^+/K^+-ATPase is present at the stalk of cells with ectopic rhabdomeres, which are not R7 (H,H″,H‴, some cells other than R7 are indicated by asterisks). Scale bars: 2 μm. (**I,J**) Schematic summary of the distribution of proteins (as shown in A–H) in wild-type ommatidia (I) and ommatidia overexpressing $Crb_{FLAGintra}$ (J).

and were often shifted to more basal positions (Fig. 3D, arrows). This was associated with a small but significant increase in stalk membrane length (Fig. 3E; supplementary material Table S1). In addition, F-actin, which predominantly localises in the rhabdomere and at low levels at the cortex in wild type, was enriched basolaterally (Fig. 3F).

When overexpressed using *Rh1*Gal4, the transgene-encoded membrane-tethered intracellular domain of Crb mostly localised at the stalk membrane, but, depending on the expression levels, could also be detected at the rhabdomere base and the basolateral membrane domain (Fig. 4A–D, no staining in R7, which was used as internal control, see Fig. 4A″–D″, white asterisks) (Richard et al., 2009). Overexpression of $Crb_{FLAGintra}$ led to a noticeable downregulation of endogenous Crb at the stalk membrane (Fig. 4A′, compare R1–R6 with R7) (Richard et al., 2009), indicating that the overall amount of Crb at the stalk is

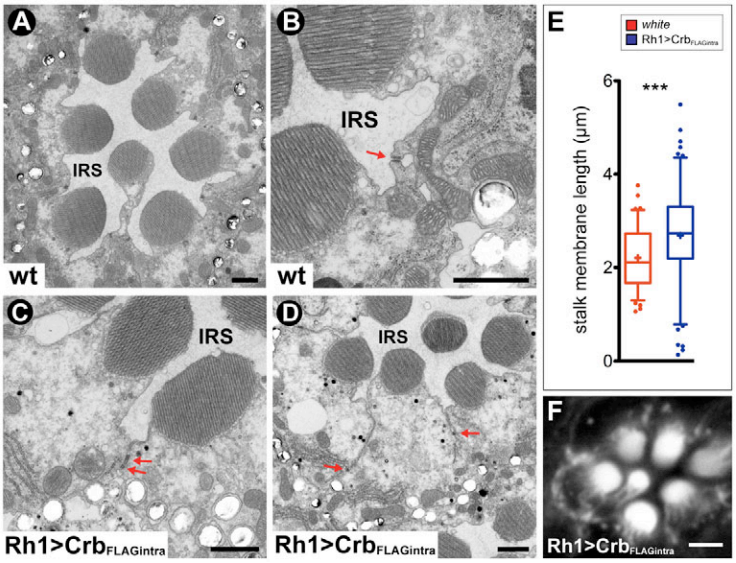

Fig. 3. *Rh1*Gal4-driven overexpression of $Crb_{FLAGintra}$ affects adherens junctions and stalk membrane length. (**A–D**) Electron micrographs of cross-sections of adult wild-type (wt) eyes (A,B) and eyes overexpressing $Crb_{FLAGintra}$ under the control of *Rh1*Gal4 (Rh1>$Crb_{FLAGintra}$) (C,D). Flies were kept at 29°C to ensure high *UAS-*$Crb_{FLAGintra}$ expression. Overexpression of $Crb_{FLAGintra}$ causes splitting of ZAs (C, red arrows). Additionally, ZAs are located more basally than in wild type (D, red arrows). (**E**) Box-and-whisker plots of the stalk membrane length of *white* flies (n=91) and flies overexpressing $Crb_{FLAGintra}$ using *Rh1*Gal4 (n=129). For each genotype, data were obtained from stalks of photoreceptors R1–R6 of two eyes from different flies. Overexpression of $Crb_{FLAGintra}$ causes a small but significant increase in stalk membrane length (***P<0.0001 as assessed by a two-sided Student's t-test for unequal variance). The central box covers the interquartile range with the median indicated by the line within the box and the mean by the cross. The whiskers extend to the 5th and 95th percentiles, the dots depict the most extreme values. (**F**) Cross-section of an adult ommatidium expressing $Crb_{FLAGintra}$ using *Rh1*Gal4 that displays F-actin accumulation at the basolateral cortex. Scale bars: 1 µm (A–D); 2 µm (F).

tightly regulated. Late expression of $Crb_{FLAGintra}$ also affected the localisation of the Crb complex members Sdt and *Drosophila* PATJ, which not only localised at the stalk but also at the basolateral membrane and occasionally at low levels at the rhabdomere base (Fig. 4B′, white arrows and data not shown). Similarly, *Drosophila* E-Cad accumulated ectopically at the basolateral membrane, with higher amounts of the Crb cytoplasmic domain resulting in more ectopic *Drosophila* E-Cad (Fig. 4C′–C″, white arrows). Furthermore, *Rh1*Gal4-induced $Crb_{FLAGintra}$ overexpression was sufficient to decrease the amount of the Na^+/K^+-ATPase at the basolateral membrane. However, it did not result in ectopic recruitment of Na^+/K^+-ATPase to the stalk of photoreceptors R1–R6, as observed upon *elav*Gal4-induced expression (compare Fig. 2H with Fig. 4D′,D″, white arrows).

These results show that the strength of the dominant phenotype caused by overexpression of $Crb_{FLAGintra}$ depends on the developmental stage at which expression is activated. Later $Crb_{FLAGintra}$ expression affects shape and positioning of AJs and perturbs localisation of polarity proteins without altering photoreceptor morphology. This suggests that more differentiated PRCs are less susceptible to increased $Crb_{FLAGintra}$ levels.

Early overexpression of $Crb_{FLAGintra}$ affects polarity already in developing PRCs

The data presented so far suggest that formation of ectopic apical poles and changes in photoreceptor morphology and ommatidial integrity are restricted to an early stage of pupal development. To analyse further the initial events, we studied the localisation of polarity and junctional proteins at approximately 45–55% of pupal development. At this time, the apical membrane becomes subdivided into the most apical rhabdomere and the stalk (Longley and Ready, 1995). PRCs overexpressing $Crb_{FLAGintra}$ under the control of *elav*Gal4 already exhibit severe morphological defects at mid-pupal development, discernable by the displacement of individual PRCs within the ommatidium (Fig. 5B; supplementary material Fig. S1B,F,H). F-actin, which is enriched at the apical membrane of wild-type cells and can be recognised as a single spot in the centre of each ommatidium, became mislocalised. We often observed at least two F-actin spots per ommatidium, which were formed by adjacent photoreceptors, with some cells contributing to more than one F-actin accumulation, indicating that they develop an additional apical pole (Fig. 5B and supplementary material Fig. S1B,F,H). We also noticed increased levels of F-actin at the basolateral cortex of the cells (Fig. 5B and supplementary material Fig. S1B,F,H). The ERM protein Moesin is required for photoreceptor morphogenesis and localises apically in early PRCs (supplementary material Fig. S1A,A′) (Karagiosis and Ready, 2004). Upon overexpression of $Crb_{FLAGintra}$, activated Moesin was present at sites with high F-actin concentrations (supplementary material Fig. S1B′–B‴). Whereas endogenous Crb was mostly localised apically (supplementary material Fig. S1D′–D‴), $Crb_{FLAGintra}$ itself showed a faint and spotty staining at all membranes (supplementary material Fig. S1F′–F‴). The Crb complex members Sdt and *Drosophila* PATJ as well as β_H-spec, a major component of the apical spectrin-based membrane skeleton, showed essentially the same distribution, but the staining was stronger at the apical membrane domain (Fig. 5B′–B‴; supplementary material Fig. S1H′–H‴; and data not shown). Par-6 and aPKC, two components of the Par protein network, which localise apically in wild-type photoreceptors during early pupal development, were similarly delocalised (Fig. 5D′,F′). In wild-type PRCs, the multi-PDZ-domain-containing protein Bazooka (Baz) colocalises with *Drosophila* E-Cad and Armadillo (Arm), the *Drosophila* β-Catenin, at the ZA at 40–55% of pupal development (Fig. 5C,E; supplementary material Fig. S1C′) (Hong et al., 2003). *elav*Gal4-induced overexpression of $Crb_{FLAGintra}$ caused an expansion or complete displacement of Baz, *Drosophila* E-Cad and Arm to the basolateral side of the cells (Fig. 5D,F; supplementary material Fig. S1D). In most cases Baz and AJ components were found adjacent to the Crb complex members Par-6 or aPKC, and only very rarely did we observe a partial overlap (Fig. 5D″,D‴,F″,F‴; supplementary material Fig. S1D″,D‴). In contrast to adult eyes, we did not observe an obvious decrease in the number of photoreceptor cells per ommatidium at this developmental stage.

These data demonstrate that expression of the membrane-tethered intracellular domain of Crb under the control of

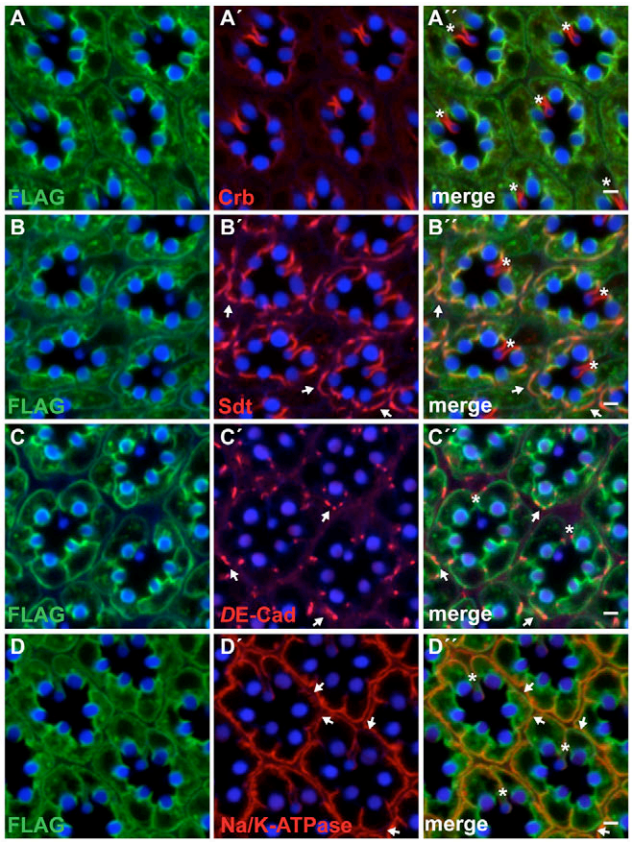

Fig. 4. Delocalisation of polarity and junctional proteins in adult PRCs expressing $Crb_{FLAGintra}$ using *Rh1*Gal4. (A–D) Immunostainings on cross-sections of adult eyes overexpressing $Crb_{FLAGintra}$ under the control of *Rh1*Gal4. FLAG staining to visualise $Crb_{FLAGintra}$ is shown in green, Crb (A), Sdt (B), *Drosophila* E-Cad (*D*E-Cad) (C) and alpha subunit of Na^+/K^+-ATPase (D) in red. The F-actin stain (blue) shows the rhabdomeres. *Rh1* is only expressed in R1–R6, so that R7 served as internal control (R7 is highlighted by white asterisks in A″–D″). $Crb_{FLAGintra}$ predominantly localises at the stalk membrane but can also be detected at the rhabdomere base, intracellularly and at the basolateral membrane (A–D). Overexpression of $Crb_{FLAGintra}$ causes a strong downregulation of endogenous Crb (A′). Sdt is not only present at the stalk but also localises basolaterally (B′, white arrows). Similarly, *Drosophila* E-Cad spots accumulate at the basolateral membrane (C′, white arrows). Na^+/K^+-ATPase is excluded from the basolateral domain at sites with high levels of $Crb_{FLAGintra}$ (D′,D″, white arrows). Scale bars: 2 μm.

*elav*Gal4 already affects photoreceptor morphology and polarity at mid-pupal stages.

The Crb-induced dominant phenotype depends on the Par protein network

In order to identify downstream components that are required to mediate the Crb overexpression phenotype, we performed genetic interaction studies. For these, we reduced the gene dose of candidate genes in PRCs overexpressing $Crb_{FLAGintra}$ using *elav*Gal4 and looked for a modification of the overexpression phenotype. As expected, flies overexpressing $Crb_{FLAGintra}$ and heterozygous for the null allele sdt^{K85} showed a strong suppression of the dominant phenotype (Fig. 6; supplementary material Table S2). This is consistent with the delocalisation of Sdt in pupal and adult PRCs overexpressing $Crb_{FLAGintra}$ and indicates that Sdt is involved in this process. By contrast, we did not observe any significant suppression using the sdt^{EH681} allele (Fig. 6; supplementary material Table S2). This is in agreement with the finding that the molecular lesion in sdt^{EH681} does not affect Sdt-D and Sdt-H, the two Sdt isoforms predominantly expressed in the retina (Bulgakova et al., 2010; Hong et al., 2001). No obvious suppression was observed when using *Df(3L)My10* (data not shown), which removes *Drosophila* PATJ, suggesting that this protein is not required to mediate the function of $Crb_{FLAGintra}$. β_H-spec colocalises with the Crb complex in pupal PRCs, is mislocalised in *crb* mutant clones (Pellikka et al., 2002; Richard et al., 2009) and is recruited to ectopic sites upon expression of $Crb_{FLAGintra}$ (this work). Therefore, we analysed the $Crb_{FLAGintra}$-induced dominant phenotype after removing one copy of *karst* (*kst*), which encodes β_H-spec. $kst^{14.1}$, which carries a small deletion that results in a truncated protein of approximately half the size of wild-type β_H-spec (Medina et al., 2002), significantly suppressed the overexpression phenotype (supplementary material Fig. S2 and Table S2). Similar to the results observed with *sdt* and *kst*, removal of one functional copy of *par-6*, using the null allele $par\text{-}6^{\Delta 226}$ or *aPKC* also gave rise to a strong suppression of the dominant phenotype (Fig. 6; supplementary material Table S2). Four different alleles of *aPKC* were analysed, including the null allele $aPKC^{k06403}$ and three hypomorphic alleles, $aPKC^{psu69}$, $aPKC^{psu141}$ and $aPKC^{psu265}$. Whereas $aPKC^{psu69}$ cannot bind Par-6, the proteins encoded by $aPKC^{psu141}$ and $aPKC^{psu265}$ exhibit a strongly reduced kinase activity (Kim et al., 2009). All four *aPKC* alleles showed a significant suppression of the $Crb_{FLAGintra}$-induced dominant phenotype (Fig. 6; supplementary material Fig. S2 and Table S2), demonstrating that both binding to Par-6 and a functional kinase domain are required to mediate the dominant phenotype. The null allele baz^{Xi106} did not show any significant modification of the $Crb_{FLAGintra}$ gain-of-function phenotype (Fig. 6; supplementary material Table S2), though the localisation of Baz was severely affected in mid-pupal photoreceptors overexpressing $Crb_{FLAGintra}$. Similarly, removing one copy of *shg*, which encodes *Drosophila* E-Cad, did not result in any obvious suppression of the dominant phenotype (data not shown). Recently, it was demonstrated that phosphorylation of Baz by aPKC is required to exclude Baz from the apical membrane of the photoreceptors of early pupae, which restricts AJs to more basal positions (Walther and Pichaud, 2010). Simultaneous overexpression of $Crb_{FLAGintra}$ and a non-phosphorylatable version of Baz, GFP–Baz-S980A (Krahn et al., 2010a; Morais-de-Sa et al., 2010), caused a significant suppression of the Crb overexpression phenotype (Fig. 6, supplementary material Table S2).

Lethal giant larvae (Lgl), a member of the Scribble (Scrib) network, is required for basolateral membrane identity in *Drosophila* embryonic epithelia (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Because the $Crb_{FLAGintra}$ overexpression phenotype of PRCs mimics the *lgl* loss-of-function phenotype (Grzeschik et al., 2007), we tested whether lowering the *lgl* gene dose, using the null allele lgl^{27S3}, is sufficient to enhance further the dominant phenotype. However, we did not observe a significant enhancement of the $Crb_{FLAGintra}$ overexpression phenotype (data not shown). By contrast, removing one functional copy of *yurt*, a negative regulator of Crb activity in PRCs and embryonic epithelia (Laprise et al., 2006), led to a significant enhancement of the $Crb_{FLAGintra}$ overexpression phenotype (supplementary material Fig. S2 and Table S2).

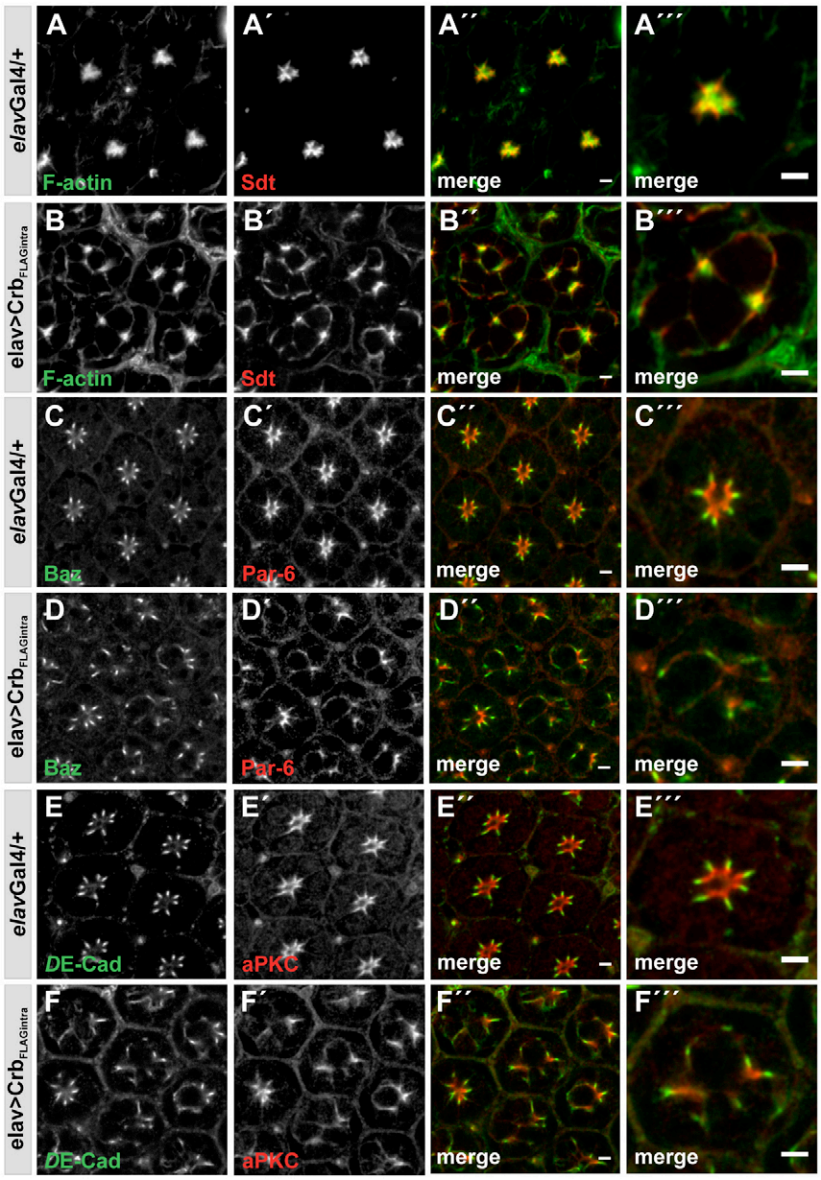

Fig. 5. Overexpression of $Crb_{FLAGintra}$ causes morphological defects and delocalisation of apical and junctional proteins in mid-pupae. Optical cross-sections of pupal control eyes (*elav*Gal4/+) (**A**,**C**,**E**) and eyes overexpressing $Crb_{FLAGintra}$ using *elav*Gal4 (elav>$Crb_{FLAGintra}$) (**B**,**C**,**F**) at 45–55% of pupal development. F-actin (A,B), Baz (C,D) and *Drosophila* E-Cad (*D*E-Cad) (E,F) staining is shown in green, Sdt (A,B), Par-6 (C,D) and aPKC (E,F) staining is in red. A‴–F‴ represent higher magnifications of one single ommatidium shown in A″–F′, respectively. Note that F-actin is already delocalised at this early developmental stage and often forms more than one spot per ommatidium (compare A with B). Sdt colocalises with F-actin spots and can also be found basolaterally (B′–B″). Baz (D) and *Drosophila* E-Cad (F), which normally localise at the ZA (C,E), spread towards the basolateral membrane and are often found adjacent to Par-6 (D′–D‴) and aPKC (F′–F‴), which exhibit a similar delocalisation as Sdt. Scale bars: 2 μm.

These results are in agreement with observations from our protein localisation studies in the pupae and suggest that Sdt, β_H-spec and members of the Par protein network mediate the $Crb_{FLAGintra}$-induced dominant phenotype and act downstream of the cytoplasmic domain of Crb.

Discussion

Drosophila Crb is required to orchestrate photoreceptor morphogenesis by controlling remodelling of the ZA, but the underlying mechanisms are not entirely understood. The data presented here demonstrate that increased levels of the membrane-bound intracellular domain of Crb cause severe defects in ommatidial integrity, suggesting impairments in cell adhesion, and alterations in apico-basal polarity, as seen by the formation of additional apical membrane domains at the basolateral side of the cells. To our knowledge, Crb is the only transmembrane protein described so far that can change the identity of the plasma membrane of epithelial cells. Previous studies analysing UAS-*crb*-induced changes in PRC morphology have used *GMR*Gal4 or *hs*Gal4 (Fan et al., 2003; Grzeschik and Knust, 2005; Izaddoost et al., 2002; Nam and Choi, 2003), both of which are expressed in all retinal cells. Here, we used *elav*Gal4 and *Rh1*Gal4, which exclusively activate transgene expression in PRCs, thus avoiding any phenotypic effects induced by $Crb_{FLAGintra}$ expression in non-neuronal cells. In addition, these two lines allowed us to distinguish effects induced upon early and late transgene expression, respectively.

Strikingly, $Crb_{FLAGintra}$ can only affect PRC shape and adhesion when expressed during late larval and early pupal development, which is in agreement with previous data (Fan et al., 2003). During this period, PRCs undergo substantial morphogenetic changes to adopt their final shape. It is noteworthy that the epithelial cells of the imaginal disc are already well polarised, with an elaborated ZA encircling the apices of the cells (Longley and Ready, 1995). Therefore, the transition from a larval epithelial cell into the highly modified PRC does not require establishment of polarity, but rather mechanisms that control remodelling of polarity and AJs. Here,

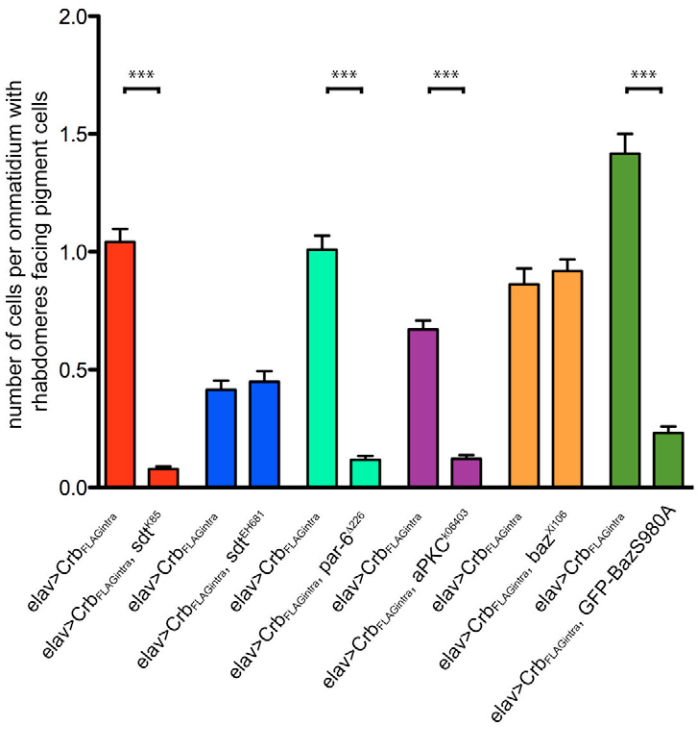

Fig. 6. Genetic interactions between the Crb complex and the Par protein network are required to induce the $Crb_{FLAGintra}$ overexpression phenotype. Means ± s.e.m. of the strength of the $Crb_{FLAGintra}$ overexpression phenotype and its modification upon removal of single wild-type copies of candidate genes or simultaneous expression of additional transgenes. $Crb_{FLAGintra}$ was expressed under the control of *elav*Gal4. For quantification of the phenotype, the number of cells per ommatidium with rhabdomeres facing the pigment cells was counted. Owing to variations within different genetic backgrounds, siblings from the same cross were used as internal controls (indicated by the same colour, labelled with elav>$Crb_{FLAGintra}$). Introducing one copy of the loss-of-function alleles sdt^{K85}, $par\text{-}6^{\Delta 226}$ or $aPKC^{k06403}$, as well as simultaneous expression of a non-phosphorylatable version of Baz (GFP–Baz-S980A), results in a significant suppression of the $Crb_{FLAGintra}$ overexpression phenotype (***$P<0.0001$ in all cases, as assessed by two-sided Student's *t*-tests for unequal variance).

we show that early expression of $Crb_{FLAGintra}$ interferes with this process. Similar conclusions were drawn from studies in the Malpighian tubules, where proper Crb levels are essential for maintenance of polarity and epithelial integrity only during the process of tube elongation, which depends on major cell rearrangements (Campbell et al., 2009). Once most of the morphogenetic changes and remodelling of the ZA have been completed, PRCs are less susceptible to elevated $Crb_{FLAGintra}$ levels. This is reflected by the observation that cells in which the intracellular domain of Crb is expressed during late pupal development and in the adult, exhibit a normal polarised shape, although junctional and polarity proteins are severely mislocalised in these cells. Two explanations might account for this difference. First, the apical and basolateral membrane domain, as well as the ZA, might be more stable at later stages, so that ectopic apical and junctional components recruited by $Crb_{FLAGintra}$ are unable to affect apico-basal polarity and AJs. Second, some of the downstream factors required for ectopic apical pole formation might no longer be available at later stages. In fact, Baz is removed from the ZA at ~60% of pupal development and becomes enriched in the rhabdomere, similar to aPKC (Hong et al., 2003). Furthermore, Par-6 can be found at the basolateral membrane in adult PRCs (Bulgakova and Knust, 2009). Although the polarised shape is unaffected, PRCs overexpressing $Crb_{FLAGintra}$ during later stages display defects in ZA positioning and show an increase in stalk membrane length, the development of which is regulated by *crb* (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). In contrast to results presented here, Pellikka et al. (Pellikka et al., 2002) did not find any alterations in the length of the stalk membrane upon expression of the cytoplasmic domain of Crb when using the same Gal4 driver. The difference might be explained by the fact that in their experiments, Crb_{intra}, encoded by a different transgene, accumulated in the rhabdomere. The phenotype that we observed for $Crb_{FLAGintra}$ also differs from the one described for *Rh1*Gal4-driven overexpression of full-length Crb and a version in which the cytoplasmic part was replaced by GFP, both of which caused a massive stalk membrane expansion without altering the position of the ZA (Pellikka et al., 2002; Richard et al., 2009).

Loss-of-function studies show that *crb* is not required for the development of an apical pole (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002), yet, as shown here, overexpression of its cytoplasmic tail is sufficient to induce formation of ectopic apical membranes. This raises the question of how ectopic apical poles develop under these conditions. Our results, from localisation studies and genetic interactions, indicate that, once initiated, development of an ectopic apical membrane domain relies on the same events and requires identical components to those required for formation of the original apical domain. We suggest that $Crb_{FLAGintra}$ assembles a new Crb-dependent membrane-associated protein platform at the basolateral membrane domain, enabling the recruitment of effector proteins essential to develop apical features. One of these is β_H-spec, which might stabilise the $Crb_{FLAGintra}$ complex by linking it to the underlying spectrin-based membrane skeleton. In fact, removal of one copy of *kst* strongly suppresses the overexpression phenotype and F-actin accumulates at $Crb_{FLAGintra}$-positive membranes. In addition, the actin-based cytoskeleton is likely to be directly involved in the formation of ectopic rhabdomeres, as rhabdomeres are composed of microvilli and the terminal web, both of which are actin-rich structures.

In addition to β_H-spec, Par-6 and aPKC are also recruited into the $Crb_{FLAGintra}$ complex and both are required to mediate the $Crb_{FLAGintra}$-induced overexpression phenotype, as demonstrated by genetic interactions. Furthermore, by using different hypomorphic alleles of aPKC, we could prove that the function of aPKC in this process depends on its ability to bind Par-6 and the

presence of an intact kinase domain. In the embryonic epidermis, aPKC ensures apical identity by phosphorylation of the tumour suppressor Lgl, thereby excluding it from the apical domain and restricting its activity to the basolateral side of the cells (Hutterer et al., 2004). Lgl, on the other hand, prevents Baz from promoting apical membrane characteristics basolaterally (Bilder et al., 2003; Tanentzapf and Tepass, 2003). We propose that, upon overexpression of $Crb_{FLAGintra}$, Lgl is removed from $Crb_{FLAGintra}$-positive sites through phosphorylation by aPKC, which weakens basolateral membrane identity. Unfortunately, the anti-Lgl antibody did not work in our hands, but the observation that other basolateral markers are absent from ectopic rhabdomeres and diminished at membranes surrounding ectopic rhabdomeres supports this assumption. Furthermore, removal of Lgl from the basolateral membrane upon overexpression of $Crb_{FLAGintra}$ would be consistent with the finding that the *lgl* loss-of-function phenotype of PRCs mimics the $Crb_{FLAGintra}$ overexpression phenotype (Grzeschik et al., 2007). This is similar to the situation in *Drosophila* embryonic epithelia (Bilder et al., 2003; Tanentzapf and Tepass, 2003), and suggests that there is a conserved mechanism for both cell types. Moreover, it might explain why lowering the dose of *lgl* does not cause an enhancement of the overexpression phenotype. By contrast, we found an enhancement with *yrt*, which negatively regulates Crb activity (Laprise et al., 2006), demonstrating that our experimental approach is suitable for the identification of enhancers. Besides Lgl, aPKC also phosphorylates Baz, as shown in the *Drosophila* follicle epithelium, the embryonic epidermis and PRCs. Phosphorylation of Baz is required to exclude it from the apical membrane, thereby restricting AJs to more basal positions. Apical exclusion of Baz also requires Crb, which prevents binding of Baz to Par-6 (Krahn et al., 2010b; Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). We suggest that the following scenario occurs upon $Crb_{FLAGintra}$ overexpression. First, removal of Lgl from the basolateral membrane enables Baz to spread basolaterally. However, under these conditions, Baz becomes immediately excluded from $Crb_{FLAGintra}$-positives sites by the same mechanisms occurring at the original apical domain. Delocalisation of Baz, in turn, affects AJs and alters the adhesive properties of the cells, as Baz localisation defines the position of the ZA (Harris and Peifer, 2005; Morais-de-Sa et al., 2010). The model is consistent with observations from genetic interactions, which have shown that simultaneous expression of $Crb_{FLAGintra}$ and a non-phosphorylatable version of Baz (GFP–Baz-S980A) strongly suppressed the $Crb_{FLAGintra}$ overexpression phenotype (Krahn et al., 2010a; Morais-de-Sa et al., 2010). This suppression could be the result of Baz S980A either binding to aPKC–Par-6, as suggested by Morais-de-Sá et al. (Morais-de-Sa et al., 2010), or to Sdt, as recently demonstrated by Krahn et al. (Krahn et al., 2010a), therefore preventing aPKC–Par-6 or Sdt from binding to $Crb_{FLAGintra}$. Alterations in PRC adhesion might also explain the disruption of the basal lamina and the elimination of PRCs. As no obvious decrease in cell number was noticed at 45–55% of pupal development, elimination is likely to occur during late pupal development.

Formation of distinct membrane domains also requires polarised protein trafficking. The ectopic localisation of Rh1 and Spam (Eys) upon overexpression of $Crb_{FLAGintra}$ during late larval and pupal development suggests that the apical secretory machinery becomes reorganised under these conditions. In *Drosophila* PRCs, delivery of various apical proteins, including Rh1, depends on the small GTPase Rab11 and the exocyst component Sec6 (Beronja et al., 2005; Satoh et al., 2005). A redistribution of these proteins upon overexpression of $Crb_{FLAGintra}$ in developing PRCs might account for the delivery of apical transport vesicles to $Crb_{FLAGintra}$-positive membranes, which facilitates the formation of a second apical pole. In case of cells with reversed apico-basal polarity the majority of apical vesicles might be targeted to the ectopic apical pole so that the original apical membrane domain receives only minor amounts of apical proteins, with it eventually adopting basolateral membrane identity.

Another crucial component in polarised vesicle delivery and targeting are phosphoinositides (Vicinanza et al., 2008). In developing *Drosophila* PRCs, PtdIns(3,4,5)P_3 is enriched at the apical membrane, whereas PtdIns(4,5)P_2 predominantly localises at the ZA (Pinal et al., 2006). Studies in MDCK (Madin–Darby canine kidney) cells have shown that ectopic localisation of either of the above two phosphoinositides is sufficient to cause a switch from one membrane identity to the other (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). Strikingly, Baz recruits the lipid phosphatase PTEN (phosphatase and tensin homolog) to the AJs of PRCs and embryonic epidermal cells (Pinal et al., 2006; von Stein et al., 2005), and Baz is delocalised upon $Crb_{FLAGintra}$ expression in pupal PRCs. Mutations in, or overexpression of, *PTEN* cause severe morphogenetic defects, including loss of PRCs and absence or splitting of rhabdomeres (Pinal et al., 2006), phenotypes that we also observed upon overexpression of $Crb_{FLAGintra}$. Given these data, it is tempting to speculate that ectopic $Crb_{FLAGintra}$ and its associated proteins cause a modification in the lipid composition of the basolateral membrane domain, thereby remodelling the polarity of PRCs.

Materials and Methods

Fly stocks and genetics

Flies were kept at 25°C except for *Rh1*Gal4-driven overexpression of *UAS-*$Crb_{FLAGintra}$, where flies were kept at 29°C in order to ensure high $Crb_{FLAGintra}$ expression. The following stocks and mutant alleles were used: OregonR and w^{1118} served as wild-type controls, *UAS-*$Crb_{FLAGintra}$ (Richard et al., 2009), *UAS-GFP-BazS980A* (kindly provided by Andreas Wodarz, University of Göttingen, Göttingen, Germany), *UAS-*Baz^{S980A}*:GFP* (kindly provided by Daniel St Johnston, The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK), *elav*Gal4 (Bloomington *Drosophila* Stock Center) (Luo et al., 1994), *Rh1*Gal4 (Bloomington *Drosophila* Stock Center), *GMR*Gal4 (Bloomington *Drosophila* Stock Center), sdt^{K85}, sdt^{EH681} (Berger et al., 2007; Eberl and Hilliker, 1988; Hong et al., 2001), *Df(3L)My10* (Bhat et al., 1999), *kst14.1* (kindly provided by Graham Thomas, The Pennsylvania State University, University Park, PA) (Medina et al., 2002; Thomas et al., 1998), $par\text{-}6^{\Delta 226}$ (Petronczki and Knoblich, 2001), $aPKC^{k06403}$, $aPKC^{psu69}$, $aPKC^{psu141}$, $aPKC^{psu265}$ (kindly provided by Andreas Wodarz) (Kim et al., 2009; Luschnig et al., 2004; Wodarz et al., 2000), baz^{Xi106} (Wieschaus et al., 1984), shg^{IG29} (Nüsslein-Volhard et al., 1984), lgl^{27S3} (kindly provided by Helena Richardson, Peter MacCallum Cancer Centre, Melbourne, Australia) (Brumby et al., 2004; Grzeschik et al., 2007), yrt^{75} (kindly provided by Ulrich Tepass, University of Toronto, Toronto, Canada) (Laprise et al., 2006).

To identify downstream components that mediate the *UAS-*$Crb_{FLAGintra}$-induced dominant phenotype, a *UAS-*$Crb_{FLAGintra}$/CyO; *elav*Gal4/TM6B stock was established and crossed to mutant alleles (deficiencies or UAS-driven transgenes) of candidate genes. To quantify any modification in the strength of the $Crb_{FLAGintra}$ overexpression phenotype, we counted the number of cells per ommatidium with rhabdomeres facing the pigment cells and compared this with the data obtained for eyes overexpressing the intracellular domain of Crb alone. As the strength of the $Crb_{FLAGintra}$ overexpression phenotype varies with genetic background, we used the siblings from the cross with the mutant allele that carried *UAS-*$Crb_{FLAGintra}$ and *elav*Gal4, but not the mutant allele, as internal controls. At least five eyes from different flies were analysed for each genotype. In addition, we tested whether removal of one functional copy of the candidate gene alone (or overexpression of the UAS-transgene using *elav*Gal4) caused any phenotype. All images for analysis were taken from cryosections of adult eyes of the different genotypes that were stained for endogenous Crb and F-actin. Statistical significance was assessed by a two-sided Student's *t*-test for unequal variance in Microsoft Excel. The graph was drawn using Prism (GraphPad Software).

Immunohistochemistry on pupal and adult eyes

For stainings of pupal eyes, staged pupae (45–55% of pupal development) were collected. The retina–brain complex was dissected in PBS, fixed for 50 minutes in 4% paraformaldehyde on ice, washed in PBT (PBS with 0.1% Triton X-100) and incubated with primary antibodies overnight at 4℃. After washing in PBT, the retina–brain complexes were incubated with secondary antibodies and Alexa-Fluor-488–phalloidin for 2 hours at room temperature. Stained eyes were washed with PBT and mounted in glycerol and propylgallate.

Cryosections were performed to stain adult eyes. Heads were cut off and bisected. Eyes were fixed in Stefanini's fixative (8% formaldehyde, 75 mM Pipes and 15% picric acid) for 40 minutes at room temperature and washed in PBS. Cryopreservation was performed in 10% sucrose for 30 minutes, followed by an incubation in 25% sucrose overnight at 4℃. Eyes were embedded in tissue-freezing medium (GSV-1, Slee Technik or NEG50, Thermo Scientific), deep frozen on dry ice and stored at −80℃ until used. Cryoscetions (10–12 µm) were made with a Microm Cryo-Star HM560M cryostat and collected on coated glass slides (Marienfeld). Cryosections were surrounded with a layer of hydrophobic compound (ImmedgePEN, Vector). Sections were permeabilised in PBT (PBS with 0.1% Triton X-100) for 40 minutes before incubation with primary antibodies overnight at 4℃. Sections were washed in PBT before incubation with secondary antibodies and Alexa-Fluor-488–phalloidin for 2 hours at room temperature. After washing in PBT, sections were mounted in DABCO-containing (Sigma) Mowiol (Calbiochem).

The following primary antibodies were used: rat anti-Crb2.8 antibody (1:1000) (Richard et al., 2006), rabbit anti-Sdt-PDZ antibody (1:500) (Berger et al., 2007), mouse anti-Sdt-PDZ antibody (B1-8, 1:200) (Bulgakova et al., 2010), rabbit anti-*Drosophila*-PATJ antibody (1:500) (Richard et al., 2006), rat anti-*D*E-Cad antibody (DCAD2, 1:50, DSHB), mouse anti-Na^+/K^+-ATPase antibody (a5, alpha subunit, 1:500 concentrate, DSHB), rabbit anti-Rh1 antibody (1:1000) (Satoh et al., 2005), mouse anti-Rh1 (4C5, 1:100, DSHB), mouse anti-Spam antibody (21A6, 1:100, DSHB) (Zelhof et al., 2006), mouse anti-FLAG M2 antibody (1:1000, Sigma), rabbit anti-FLAG antibody (1:200, Sigma, F7425), rabbit anti-Baz antibody (1:400, kindly provided by Andreas Wodarz) (Wodarz et al., 1999), guinea pig anti-Par-6 antibody (1:1000, kindly provided by Andreas Wodarz) (Kim et al., 2009), mouse anti-Arm antibody (N2 7A1, 1:50, DSHB), rabbit anti-PKC ζ C20 (1:1000, Santa Cruz Biotechnology), rabbit anti-phosphorylated-Ezrin/Radixin/Moesin (1:100, Cell Signalling Technology), and rabbit anti-β_H-spec (1:500, kindly provided by Graham Thomas) (Thomas and Kiehart, 1994). Secondary antibodies conjugated to Cy2, Cy3 (Jackson ImmunoResearch Laboratories) or Alexa Fluor 647 (Invitrogen) were used at 1:200. Rhabdomeres were visualised by labelling F-actin with Alexa-Fluor-488–phalloidin at 1:40 (Invitrogen). Images were taken on a Zeiss LSM 510 and processed using ImageJ/Fiji, Adobe Photoshop CS3 and Adobe Illustrator CS3 for image assembly.

Transmission electron microscopy and quantification of stalk membrane length

Fixation of adult eyes for transmission electron microscopic analysis was performed as previously described (Richard et al., 2006). Semi- (2.5 µm) and ultra-thin (0.2 µm) sections were cut using a Leica Ultracut UCT. Semi-thin sections were stained with Toluidine Blue and analysed on a Zeiss AxioImager.Z1. Ultra-thin sections were contrasted and analysed using a FEI Tecnai 12 Bio Twin. For quantitative analysis of the stalk membrane length, tiled images were taken at a magnification of 4800× using a TemCam F2114A digital camera. The stalk membranes of 10–12 ommatidia, obtained from two eyes of different flies, were measured for each genotype using ImageJ. Only stalks from R1–R6 were considered for the quantitative analysis. Statistical significance was assessed by a two-sided Student's *t*-test for unequal variance in Microsoft Excel. The graph was drawn using Prism (GraphPad Software).

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