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., 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 βH-spectrin (βH-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 AJ. 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 (Krahm et al., 2010a; Morais-de-Sa et al., 2010; Walther and Pichaud, 2010).

Results

Overexpression of CrbFLAGintra 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, CrbFLAGintra (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, elavGal4 and Rh1Gal4 (Kumar and Ready, 1995; Luo et al., 1994; Yao and White, 1994). In the developing eye, elavGal4 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 ommatidia. Each ommatidium contains eight PRCs that are arranged in a highly stereotypic pattern. The apical membrane of the photoreceptors is subdivided into the rhabdome 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 CrbFLAGintra in otherwise wild-type PRCs, using elavGal4 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 AJ at the basolateral side of individual PRCs (Fig. 1B,C,E,F, AJ 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 CrbFLAGintra using GMRGal4, 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 CrbFLAGintra 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 AJ 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 CrbFLAGintra under the control of elavGal4, 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),
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 CrbFLAGintra, 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 CrbFLAGintra, 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 CrbFLAGintra in late pupae and adult flies affects adherens junctions and localisation of polarity proteins**

To analyse the effects induced by overexpression of CrbFLAGintra in late pupae and adult, we used RhlGal4, 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 elavGal4, this later expression of CrbFLAGintra 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).
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 Rh1Gal4, 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 CrbFLAGintra 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. 2. Overexpression of CrbFLAGintra 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 CrbFLAGintra using elavGal4 (elav>CrbFLAGintra) (B,D,F,H). The sections were stained for Drosophila E-Cad (DE-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 CrbFLAGintra (J).
tightly regulated. Late expression of CrbFLAGintra 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, Rh1Gal4-induced CrbFLAGintra 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 overexpression of CrbFLAGintra under the control of Rh1Gal4 (Rh1>CrblFLAGintra) (CD). Flies were kept at 29°C to ensure high UAS-CrbFLAGintra expression. Overexpression of CrbFLAGintra 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 CrbFLAGintra using Rh1Gal4 (n=129). For each genotype, data were obtained from stalks of photoreceptors R1–R6 of two eyes from different flies. Overexpression of CrbFLAGintra 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 CrbFLAGintra using Rh1Gal4 that displays F-actin accumulation at the basolateral cortex. Scale bars: 1 μm (A–D); 2 μm (F).

**Early overexpression of CrbFLAGintra 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 CrbFLAGintra under the control of elavGal4 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). 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 CrbFLAGintra–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”), CrbFLAGintra 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 β₁-spect, 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). elavGal4-induced overexpression of CrbFLAGintra 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

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**Fig. 3. Rh1Gal4-driven overexpression of CrbFLAGintra 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 CrbFLAGintra under the control of Rh1Gal4 (Rh1>CrbFLAGintra) (CD). Flies were kept at 29°C to ensure high UAS-CrbFLAGintra expression. Overexpression of CrbFLAGintra 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 CrbFLAGintra using Rh1Gal4 (n=129). For each genotype, data were obtained from stalks of photoreceptors R1–R6 of two eyes from different flies. Overexpression of CrbFLAGintra 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 CrbFLAGintra using Rh1Gal4 that displays F-actin accumulation at the basolateral cortex. Scale bars: 1 μm (A–D); 2 μm (F).
K+-ATPase is excluded from the basolateral domain at sites with high levels of Sdt (B), E-Cad spots accumulate at the basolateral membrane (C), strong downregulation of endogenous Crb (A) at mid-pupal stages. Overexpression of CrbFLAGintra causes a delocalisation of polarity and junctional proteins in adult PRCs expressing CrbFLAGintra using Rh1Gal4. (A–D) Immunostainings on cross-sections of adult eyes overexpressing CrbFLAGintra under the control of Rh1Gal4. FLAG staining to visualise CrbFLAGintra is shown in green, Crb (A), Sdt (B), Drosophila E-Cad (DE-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). CrbFLAGintra 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 CrbFLAGintra 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 CrbFLAGintra (D’, D”, white arrows). Scale bars: 2 μm.

elavGal4 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 used the gene dose of candidate genes in PRCs overexpressing CrbFLAGintra using elavGal4 and looked for a modification of the overexpression phenotype. As expected, flies overexpressing CrbFLAGintra and heterozygous for the null allele sdt^{E681} 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 CrbFLAGintra 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 CrbFLAGintra. βH-spec colocalises with the Crb complex in pupal PRCs, is mislocalised in crb mutant clones (Pellicka et al., 2002; Richard et al., 2009) and is recruited to ectopic sites upon expression of CrbFLAGintra (this work).

Therefore, we analysed the CrbFLAGintra-induced dominant phenotype after removing one copy of karst (kst), which encodes βH-spec. kst^{447}, which carries a small deletion that results in a truncated protein of approximately half the size of wild-type βH-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-6^{A226} 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^{E604G} and three hypomorphic alleles, aPKC^{p549}, aPKC^{p7141} and aPKC^{p5265}. Whereas aPKC^{p549} cannot bind Par-6, the proteins encoded by aPKC^{p7141} and aPKC^{p5265} exhibit a strongly reduced kinase activity (Kim et al., 2009). All four aPKC alleles showed a significant suppression of the CrbFLAGintra-induced dominant phenotype (Fig. 6; supplementary material Fig. S2 and Table S2), though the localisation of Baz was severely affected in mid-pupal photoreceptors overexpressing CrbFLAGintra. 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 CrbFLAGintra 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 CrbFLAGintra 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^{2752}, is sufficient to enhance further the dominant phenotype. However, we did not observe a significant enhancement of the CrbFLAGintra 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 CrbFLAGintra overexpression phenotype (supplementary material Fig. S2 and Table S2).
These results are in agreement with observations from our protein localisation studies in the pupae and suggest that Sdt, βTrH spec and members of the Par protein network mediate the CrbFLAGintra-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 GMRGal4 or hsGal4 (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 elavGal4 and RhIGal4, which exclusively activate transgene expression in PRCs, thus avoiding any phenotypic effects induced by CrbFLAGintra expression in non-neuronal cells. In addition, these two lines allowed us to distinguish effects induced upon early and late transgene expression, respectively.

Strikingly, CrbFLAGintra 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,
we show that early expression of CrbFLAGintra 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 CrbFLAGintra 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 CrbFLAGintra 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 AJs. In contrast to results presented here, 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, CrbFLAGintra encoded by a different transgene, accumulated in the rhabdomere. The phenotype that we observed for CrbFLAGintra also differs from the one described for RhlGal4-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 CrbFLAGintra 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 β1-spec, which might stabilise the CrbFLAGintra 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 CrbFLAGintra-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 β1-spec, Par-6 and aPKC are also recruited into the CrbFLAGintra complex and both are required to mediate the CrbFLAGintra-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

![Fig. 6. Genetic interactions between the Crb complex and the Par protein network are required to induce the CrbFLAGintra overexpression phenotype.](image)
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 CrbFLAGintra Lgl is removed from CrbFLAGintra 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 CrbFLAGintra would be consistent with the finding that the lgl loss-of-function phenotype of PRCs mimics the CrbFLAGintra 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., 2010a; Morais-de-Sa et al., 2010; Wallther and Pichaud, 2010). We suggest that the following scenario occurs upon CrbFLAGintra overexpression. First, removal of Lgl from the basolateral membrane enables Baz to spread basolaterally. However, under these conditions, Baz becomes immediately excluded from CrbFLAGintra-positive 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 CrbFLAGintra and a non-phosphorylatable version of Baz (GFP–Baz–S980A) strongly suppressed the CrbFLAGintra 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-Sa 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 CrbFLAGintra. Alterations in PRC adhesion might also explain 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)P3 is enriched at the apical membrane, whereas PtdIns(4,5)P2 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 CrbFLAGintra 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 CrbFLAGintra. Given these data, it is tempting to speculate that ectopic CrbFLAGintra 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 Rh1Gal4-driven overexpression of UAS-CrbFLAGintra, 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 w1118 as served as wild-type controls, UAS-CrbFLAGintra (Richard et al., 2009), UAS-GFP-BazS980A (kindly provided by Andreas Wodarz, University of Göttingen, Göttingen, Germany), UAS-BazS980A:GFP (kindly provided by Daniel St Johnston, The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK). elavGal4 (Bloomington Drosophila Stock Center) (Luo et al., 1994), Rh1Gal4 (Bloomington Drosophila Stock Center), GMRGal4 (Bloomington Drosophila Stock Center), sd(1)Lsd(2) (Berger et al., 2007; Eberl and Hilfiker, 1998; Hong et al., 2001), Df(3)LMy10/Blat (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-6(226) (Petronczki and Knoblich, 2001), aPKC96403aPKC96403 aPKC96403 aPKC96403, aPKC96403 (kindly provided by Andreas Wodarz) (Kim et al., 2009; Luschig et al., 2004; Wodarz et al., 2000), baz(2155) (Wieschaus et al., 1984), shibire (Nüsslein-Volhard et al., 1984), lgl(226) (kindly provided by Helena Richardson, Peter MacCallum Cancer Centre, Melbourne, Australia) (Brumby et al., 2002; Grzeschik et al., 2007), yrt(12) (kindly provided by Ulrich Tepass, University of Toronto, Toronto, Canada) (Laprise et al., 2006).

To identify downstream components that mediate the UAS-CrbFLAGintra-induced dominant phenotype, a UAS-CrbFLAGintra/Cyo; elavGal4/TM3,Db 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 CrbFLAGintra 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 expressing the intracellular domain of Crb alone. The strength of the CrbFLAGintra overexpression phenotype varies with genetic background; we used the siblings from the cross with the mutant allele that carried UAS-CrbFLAGintra and elavGal4, 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 elavGal4) caused any phenotype. All images for analysis were taken from cryosections of adult eyes of the different genotypes that were stamed 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 graphs was drawn using Prism (GraphPad Software).
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), mouse anti-Sdt-PDZ antibody (B1-8, 1:200) (Bulgakova et al., 2010), mouse anti-Drosophila-PATJ antibody (1:1000) (Richard et al., 2006), rabbit anti-DcDC2 antibody (1:50) (Berger et al., 2007), mouse 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 (1:200, kindly provided by Andreas Wodarz) (Kim et al., 2009), mouse anti-Arm antibody (N2 7A1, 1:50, DSHB), rabbit anti-Baz antibody (1:400, kindly provided by 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-phallolidin at 1:400 (Invitrogen). Images were taken on a Zeiss LSM 510 and processed using ImageJ/Fiji, Adobe Photoshop CS3 and Adobe Illustrator CS3 for image assembly.

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References


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Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.091223/-/DC1
Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion.


Table S1. Stalk membrane length upon overexpression of Crb\textsubscript{FLAGintra} under the control of \textit{Rh1Gal4}.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strength \textit{Crb\textsubscript{FLAGintra}} overexpression phenotype mean ± s.e.m</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{white} (\textit{w}^{1118})</td>
<td>2.21±0.07</td>
<td>91</td>
</tr>
<tr>
<td>\textit{Rh1}⇒\textit{Crb\textsubscript{FLAGintra}}</td>
<td>2.69±0.09</td>
<td>129</td>
</tr>
</tbody>
</table>

Mean values ± standard error of the mean (±s.e.m.) of the stalk membrane length of \textit{white} flies and flies overexpressing \textit{Crb\textsubscript{FLAGintra}} under the control of \textit{Rh1Gal4}. Flies were kept at 29°C. \textit{n}, number of stalk membranes measured.
Table S2. Strength of the Crb<sub>FLAGintra</sub> overexpression phenotype and its modification upon introduction of mutant alleles or additional transgenes of candidate genes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strength Crb&lt;sub&gt;FLAGintra&lt;/sub&gt; overexpression phenotype</th>
<th>n</th>
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<tbody>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control sdt&lt;sup&gt;E85&lt;/sup&gt;)</td>
<td>1.04 +/- 0.05</td>
<td>421</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; sdt&lt;sup&gt;E85&lt;/sup&gt;</td>
<td>0.08 +/- 0.01</td>
<td>691</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control sdt&lt;sup&gt;El681&lt;/sup&gt;)</td>
<td>0.42 +/- 0.04</td>
<td>253</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; sdt&lt;sup&gt;El681&lt;/sup&gt;</td>
<td>0.45 +/- 0.04</td>
<td>227</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control kst&lt;sup&gt;14.1&lt;/sup&gt;)</td>
<td>1.10 +/- 0.05</td>
<td>384</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; kst&lt;sup&gt;14.1&lt;/sup&gt;</td>
<td>0.32 +/- 0.03</td>
<td>466</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control par-&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;226&lt;/sup&gt;)</td>
<td>1.01 +/- 0.06</td>
<td>320</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; par-&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;226&lt;/sup&gt;</td>
<td>0.12 +/- 0.02</td>
<td>478</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control aPKC&lt;sup&gt;306403&lt;/sup&gt;)</td>
<td>0.67 +/- 0.04</td>
<td>431</td>
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<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; aPKC&lt;sup&gt;306403&lt;/sup&gt;</td>
<td>0.12 +/- 0.02</td>
<td>581</td>
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<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control aPKC&lt;sup&gt;sta69&lt;/sup&gt;)</td>
<td>0.69 +/- 0.05</td>
<td>262</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; aPKC&lt;sup&gt;sta69&lt;/sup&gt;</td>
<td>0.11 +/- 0.02</td>
<td>251</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control aPKC&lt;sup&gt;sta141&lt;/sup&gt;)</td>
<td>0.56 +/- 0.06</td>
<td>215</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; aPKC&lt;sup&gt;sta141&lt;/sup&gt;</td>
<td>0.03 +/- 0.01</td>
<td>264</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control aPKC&lt;sup&gt;sta265&lt;/sup&gt;)</td>
<td>0.58 +/- 0.06</td>
<td>187</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; aPKC&lt;sup&gt;sta265&lt;/sup&gt;</td>
<td>0.03 +/- 0.01</td>
<td>348</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control baz&lt;sup&gt;Xi106&lt;/sup&gt;)</td>
<td>0.86 +/- 0.07</td>
<td>204</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; baz&lt;sup&gt;Xi106&lt;/sup&gt;</td>
<td>0.91 +/- 0.05</td>
<td>382</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control GFP-BazS980A)</td>
<td>1.42 +/- 0.08</td>
<td>175</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; GFP-BazS980A</td>
<td>0.23 +/- 0.03</td>
<td>234</td>
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<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; (internal control yrt&lt;sup&gt;75&lt;/sup&gt;)</td>
<td>1.20 +/- 0.05</td>
<td>463</td>
</tr>
<tr>
<td>elav&gt;Cr&lt;sub&gt;FLAGintra&lt;/sub&gt; yrt&lt;sup&gt;75&lt;/sup&gt;</td>
<td>2.10 +/- 0.07</td>
<td>349</td>
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</table>

Mean values +/- standard error of the mean (s.e.m.) of the number of cells per ommatidium with rhabdomeres facing pigment cells that served as read-out for the strength of the Crb<sub>FLAGintra</sub>-mediated overexpression phenotype. n = number of counted cells. At least 5 eyes were analysed for each genotype.