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A role for the extracellular domain of Crumbs in morphogenesis of *Drosophila* photoreceptor cells

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Abstract

Morphogenesis of *Drosophila* photoreceptor cells includes the subdivision of the apical membrane into the photosensitive rhabdomere and the associated stalk membrane, as well as a considerable elongation of the cell. *Drosophila* Crumbs (Crb), an evolutionarily conserved transmembrane protein, organizes an apical protein scaffold, which is required for elongation of the photoreceptor cell and extension of the stalk membrane. To further elucidate the role played by different Crb domains during eye morphogenesis, we performed a structure-function analysis in the eye. The analysis showed that the three variants tested, namely full-length Crb, the membrane-bound intracellular domain and the extracellular domain were able to rescue the elongation defects of *crb* mutant rhabdomeres. However, only full-length Crb and the membrane-bound intracellular domain could partially restore the length of the stalk membrane, while the extracellular domain failed to do so. This failure was associated with the inability of the extracellular domain to recruit β_{Heavy}-spectrin to the stalk membrane. These results highlight the functional importance of the extracellular domain of Crb in the *Drosophila* eye. They are in line with previous observations, which showed that mutations in the extracellular domain of human CRB1 are associated with retinitis pigmentosa 12 and Leber congenital amaurosis, two severe forms of retinal dystrophy.

Keywords: *Drosophila*; Eye; Photoreceptor development; Polarity; Crumbs; EGF-like protein; Morphogenesis; Rhabdomere

Introduction

Animal cells adopt an amazingly wide range of different forms, and very often the shape of a cell is crucial for performing its specific functions. Cell shape is highly dynamic and can response to a variety of endogenous and environmental cues. This becomes obvious, for example, when cells become migratory, form protrusions, or undergo epithelial-mesenchymal transition. During these processes, cells have to adjust their shape or form cell-cell and cell-matrix junctions. Therefore, cell shape has to be tightly controlled during development and in the differentiated status.

The photoreceptor cell (PRC) of *Drosophila* is ideally suited to dissect the genetic and cellular requirements

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that orchestrate cell morphogenesis. During development, *Drosophila* PRCs are specified from epithelial cells of the eye imaginal discs of third instar larvae. In the pupae, PRCs undergo complex morphogenetic changes, which are initiated at ~37% pupal development, when the apical membrane undergoes a shift of 90°, so that it finally adopts a lateral position. During this process, the apical poles of all eight PRCs of an ommatidium are oriented towards the center, which later forms the interrhabdomeral space (IRS). Subsequently, the apical membrane is subdivided into the stalk, a supporting membrane immediately apical to the zonula adherens (ZA), and the rhabdomere, the central region of the apical membrane consisting of highly pleated microvilli that harbor proteins involved in phototransduction, among them the photopigment rhodopsin. The differentiation and expansion of the apical surface is accompanied by a remarkable extension of the cell along the proximo-distal axis (Longley and Ready, 1995). Several components have been identified, that participate in the regulation of this complex morphogenetic process (reviewed in (Knust, 2007; Tepass and Harris, 2006)). The scaffolding protein Bazooka, the *Drosophila* ortholog of Par-3, is involved in the specification of the apical membrane during the first half of pupal development, by recruiting PTEN, a PtdIns(3,4,5)P3-phosphatase, to the ZA, thereby subdividing the apical membrane into stalk and rhabdomere. PRCs without functional PTEN fail to differentiate the apical membrane. About 50% of the mutant PRCs lack the rhabdomere, while about 20% show split rhabdomeres, in which the interrupted membrane is positive for stalk-specific components, such as Crb or *D*PATJ (Pinal et al., 2006).

Following the separation of the apical membrane into rhabdomere and stalk, expansion of these two subdomains takes place. This process requires the function of the Crb protein complex, which is initially localized to the apical plasma membrane of developing PRCs, but becomes restricted to the stalk membrane during pupal development (Berger et al., 2007; Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002; Richard et al., 2006a). The core components of the Crb complex encompass Crb, the caroboxyterminus of which directly binds to Stardust (Sdt), a member of the membrane-associated guanylate kinase (MAGUK) family. Sdt, in turn, recruits the PDZ domain-containing proteins *D*Lin-7 and *D*PATJ into the complex (reviewed in (Assémat et al., 2008; Richard et al., 2006b; Bulgakova and Knust, 2009)). Crb, together with Sdt and *D*PATJ, controls the length of the stalk membrane, the extension of the rhabdomere and the maintenance of a continuous adhesion belt during elongation of the apical surface. The length of the stalk membrane is further controlled by *yurt*, which negatively regulates Crb activity (Laprise et al., 2006). Finally, the loss of any of the core components of the Crb complex results in light-dependent retinal degeneration in the adult eye (Bachmann et al., 2008; Berger et al., 2007; Johnson et al., 2002; Richard et al., 2006a).

Drosophila Crb is an evolutionarily conserved protein with a single membrane-spanning domain and a large extracellular domain, which is composed of 29 epidermal growth factor (EGF)-like and four laminin A globular domain-like repeats (Tepass et al., 1990). The small cytoplasmic domain of only 37 amino acids interacts with Sdt and the FERM (4.1/ezrin/radixin/moesin) protein Yurt via two highly conserved regions, the carboxyterminal PDZ-binding motif and the FERM-binding domain, respectively. Both Crb and Sdt are essential for the maintenance of epithelial cell polarity in the embryo (Bachmann et al., 2001; Grawe et al., 1996; Hong et al., 2001; Tepass and Knust, 1990, 1993; Tepass et al., 1990). Expression of the membrane-bound cytoplasmic domain of Crb can suppress the embryonic *crb* mutant phenotype to the same degree as expression of the full-length protein, and this function depends on the presence of the PDZ-binding motif ERLI and an intact FERM-binding domain (Klebes and Knust, 2000; Wodarz et al., 1995).

Little information is available concerning the functional domains of Crb required for retinal morphogenesis. Previous results indicated that the intracellular domain of Crb is able to rescue the defects in rhabdomere elongation of *crb* mutant PRCs (Johnson et al., 2002). Furthermore, overexpression of full-length Crb or the membrane-bound intracellular domain in otherwise wild-type eyes results in ectopic localization of ZA components and *D*PATJ during pupal development (Fan et al., 2003; Izaddoost et al., 2002). Finally, expression of either full-length Crb or its membrane-bound extracellular domain results in an expansion of the stalk membrane (Pellikka et al., 2002).

To further elucidate the role played by different domains of Crb during eye morphogenesis, we performed a structure-function analysis in wild-type and crb^{11A22} mutant eye clones. This analysis showed that the membrane-bound intracellular and the extracellular domain, when expressed individually, are able to rescue the elongation defects of PRCs. In contrast, only the membrane-bound intracellular domain could partially restore the length of the stalk membrane, while the Crb extracellular domain failed to do so.

Materials and methods

Fly strains

The following fly stocks were used: *w*, crb^{11A22}, *UAS-*Crb_{full}, *UAS-*Crb_{extra} (Klebes and Knust, 2000; Wodarz

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et al., 1995), *UAS-Crb*$_{FLAGintra}$ (this work; see below). Eyes mosaic for *crb* were generated by crossing *yw ey*FLP;; FRT82B w^+ *cl3R3*/*TM6B* females (Newsome et al., 2000) to *w*;;FRT82B*crb*^{11A22}/*TM6B* males.

For rescue experiments, stocks were established containing the different UAS-Crb transgenes as well as FRT82B*crb*^{11A22} (*w*; *UAS-Crb*/*UAS-Crb*; *FRT82Bcrb*^{11A22}/*TM6B*). These were crossed with the driver line *yw ey*FLP; *elav*Gal4/*elav*Gal4; FRT82B w^+ *cl3R3*/*TM6B*. At least two independent transgenic UAS-lines were tested for each construct. *Rh1*Gal4 was used to overexpress the various transgenes in R1-R6 PRCs (Tabuchi et al., 2000). *karst* mutants were *kst*^{1}/*kst*^{2} (a gift of Graham Thomas).

DNA constructs and establishment of transgenic lines

UAS-Crb$_{FLAGintra}$ was modified from the construct described previously (Klebes and Knust, 2000), in that the Myc epitope was replaced by a FLAG tag (Hopp et al., 1988). First, an EcoRI-XbaI fragment was obtained from the Myc-Intra$_{WT}$ construct and cloned into pBluescript KSII +/− (Stratagene, Amsterdam). To remove the Myc tag-encoding portion, the fragments were restricted with XhoII, resulting in the Myc tag-encoding region and two additional fragments. These two additional fragments were then religated in an EcoRI-XbaI-restricted pUAST vector (Brand and Perrimon, 1993). Orientation of the ligation was verified by sequencing. Because these constructs still contain a BglII restriction site between the regions encoding the signal peptide and the transmembrane domain, we amplified FLAG per PCR with primers flanked by BglII sites (5'-GATCCGATTACAAGGATGAT-GATGATAAGG-3' and 5'-GATCCCTTATCATCAT-CATCCTTGTAATCG-3') and cloned the FLAG-encoding region into the pUAST vectors containing the Crb$_{intra}$-encoding fragment.

P-element transformation of the constructs was done according to the procedure described by Spradling (1986). We used w^{1118} as recipient strains. At least 9 independent transgenic lines were established. Expression of transgenes encoding FLAG-tagged proteins was verified by staining with a anti-FLAG antibody (see below). Experiments were repeated with at least two independent UAS lines to eliminate position effects.

Antibodies and immunofluorescence analyses

Dissection of pupal eyes from staged pupae and staining of cryosections of adult retina were performed as previously described (Richard et al., 2006a). The following primary antibodies were used: rabbit anti-SdtMPDZ (1:500), rabbit anti-*D*PATJ (1:500) and rat anti-Crb (1:1000) (Richard et al., 2006a), rabbit anti-β_{Heavy}-spectrin (1:2000; a gift of G. Thomas), mouse monoclonal anti-ArmN2-7A1 (1:50; Developmental Studies Hybridoma Bank), mouse anti-FLAG M2 (1:500; Sigma). Fluorophore-labeled secondary antibodies (1:200) were purchased from Jackson Immuno-Research Laboratories (Cy2 or Cy3 conjugates) or Molecular Probes (Alexa-647). Rhabdomeres were visualized by labeling with Alexa-488-phalloidin at 1:40 (Molecular Probes). All images were processed and mounted using Adobe Photoshop 7.0 or Deneba CanvasX.

Semi-thin sections and transmission electron microscopy

Sections were prepared according to (Tepass and Hartenstein, 1994) with modifications. In brief, 0.1 M phosphate buffer (pH 7.4) was used to fix bisected heads in 25% glutaraldehyde, followed by simultaneous fixation in 1% osmium tetroxide/2% glutaraldehyde, followed by 2% OsO_4. After dehydration, eyes were embedded in Araldite, and semi-thin (2.5 μm) sections were cut on a Reichert OM U2 microtome and stained with toluidine. Ultrathin sections (0.1 μm thick) were contrasted and analyzed with a Zeiss EM9 S2. For stalk membrane measurements, EM negatives were scanned and digitized. Alternatively, the specimens were analyzed with a Tecnai 12 Biotwin (FEI Company (former Philips)) and photographed with a TemCam F214A digital camera at 4800× magnification. Stalk membranes were hand-traced with IMAGEJ software (http://rsb.info.nih.gov/ij/) at 200-300% magnification to reduce tracing errors. Only stalk membranes from R1-R6 with clearly identifiable ZAs were measured. Pixel measurements were transformed into microns using Adobe Photoshop 7.0. Statistical analyses were done using Microsoft Excel and the GraphPad freeware (www.graphpad.com).

Results

Expression of various transgene-encoded Crb proteins in wild-type PRCs

It has previously been shown that the membrane-bound cytoplasmic domain of Crb can suppress the *crb* mutant embryonic phenotype to a similar degree as full-length Crb, while a transgene that encodes just the extracellular domain failed to do so (Klebes and Knust, 2000; Wodarz et al., 1995). We were interested to know, which part of the Crb protein can suppress the *crb* mutant phenotype in the developing PRCs. Therefore, we overexpressed various transgenes in wild-type or *crb* mutant PRCs, using the

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GAL4/UAS system (Brand and Perrimon, 1993). The UAS-transgenes code either for full-length Crb (Crb_{full}), the extracellular domain lacking the transmembrane and cytoplasmic domain (Crb_{extra}) (Wodarz et al., 1995) or a FLAG-tagged intracellular domain of Crb, linked to the membrane ($Crb_{FLAGintra}$) (this work) (Fig. 1A). For expression of *crb* transgenes during PRC development, we used two different Gal4 lines: *elav*Gal4, which is active in PRCs throughout their development, but is turned down prior to eclosion (Lin and Goodman, 1994), and *Rh1*Gal4, which activates moderate levels of Gal4 in the outer PRCs, R1-R6, under the control of the *Rhodopsin1* promoter, starting in the last third of pupal development and lasting through adulthood (Kumar and Ready, 1995; Sheng et al., 1997).

The adult eye of *Drosophila melanogaster* is composed of about 800 individual units, the ommatidia. Each ommatidium contains eight PRCs, which exhibit a highly stereotypic arrangement. The actin-rich rhabdomeres, formed by the expanded photosensitive apical membrane, point towards the center of the ommatidium and are separated from each other by the IRS. The portion of the apical membrane between the rhabdomere and the ZA defines the stalk membrane, where the Crb complex is localized (Fig. 1B).

Upon overexpression of Crb_{full} using *Rh1*Gal4 in otherwise wild-type PRCs, Crb was highly enriched at the stalk membrane (compare Fig. 1B and C). The enrichment becomes obvious when comparing the amount of Crb at the stalk membrane of R7, where *Rh1*GAL4 is not expressed (pointed out by arrowheads in Fig. 1B and C), with that at the stalk membranes of R1-R6. In addition, some Crb was detected at the rhabdomere base and in the cytoplasm. The strong enrichment of Crb at the stalk correlated with a huge expansion of the stalk membrane (not shown), which was also observed upon overexpression of a membrane-tethered extracellular domain (Pellikka et al., 2002). Otherwise, PRC morphogenesis was not affected. Sdt remained restricted to the stalk membrane (not shown). Upon overexpression of transgene-encoded Crb_{extra}, Crb was detected at similar amounts on the stalk membranes of R1-R6, which express the transgene, and R7, where it is not overexpressed. The amount was comparable to wild-type levels, suggesting that under this condition only endogenous Crb is found at the stalk membrane. In addition, Crb-positive vesicles were distributed throughout the cytoplasm (Fig. 1D), which probably contain transgene-encoded Crb_{extra}, since we never observed similar staining in wild-type PRCs. We could not detect any Crb extracellularly, i.e., in the IRS. This is in contrast to the accumulation of large amounts of extracellular Crb detected in the lumen of the embryonic salivary glands after Crb_{extra} overexpression (Wodarz et al., 1995). Crb_{extra} expression had no effect on stalk-membrane localization of Sdt (data not shown). Transgene-expressed $Crb_{FLAGintra}$ localized at the stalk membrane, but could also be detected at the baso-lateral membrane and the rhabdomere base (Fig. 1E). Its expression resulted in reduced levels of endogenous Crb at the stalk membrane and ectopic localization of Sdt at the baso-lateral membrane (not shown), a phenotype reminiscent to that observed in embryonic epithelia overexpressing Crb_{intra} (Klebes and Knust, 2000).

Crb_{full}, $Crb_{FLAGintra}$ and Crb_{extra} restore rhabdomere shape of *crb* mutant PRCs

In order to analyze whether the different transgenes can rescue the mutant phenotype, cross sections and longitudinal sections of adult eyes were analyzed. The regular trapezoid arrangement of the rhabdomeres in the wild-type retina is lost in *crb* mutant eyes (in all experiments the allele crb^{11A22} was used) (compare Fig. 2A and C). In mutant ommatidia, rhabdomeres are bulkier and often contact each other (Fig. 2C), a phenotype never observed in wild-type ommatidia. Wild-type PRCs span the entire retina from the lenses to the retinal floor (Fig. 2G). In contrast, rhabdomeres of *crb* mutant PRCs do not reach the basal lamina, but remain in the distal region of the retina (compare Fig. 2G and H) (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002).

When expressed in crb^{11A22} mutant cells using *elav*Gal4, Crb_{full} nearly completely restored the overall morphology of PRCs. The rhabdomeres appeared round and are clearly separated from each other as in wild-type ommatidia (compare Fig. 2A and D). They span the entire retina and reach the basal lamina, similar as wild-type PRCs (compare Fig. 2G and I). This result demonstrates that Crb_{full} is functional in this assay. The short membrane-tethered intracellular domain of Crb has been shown to suppress the *crb* mutant phenotype in the embryo to the same extend as full-length Crb (Klebes and Knust, 2000; Wodarz et al., 1995). When expressed in mutant PRCs using *elav*Gal4, $Crb_{FLAGintra}$ largely restored the shape of the rhabdomeres. Rhabdomeres were round and clearly separated from each other (Fig. 2E). Expression of $Crb_{FLAGintra}$ also rescued the elongation defect observed in the crb^{11A22} mutant PRCs (Fig. 2J). In the embryo, expression of the extracellular domain without any membrane anchor (Crb_{extra}) did not bring about any rescue (Wodarz et al., 1995). In contrast, when expressed in eyes using *elav*Gal4, the overall morphology of crb^{11A22} mutant PRCs and expansion of the rhabdomeres were almost completely re-established (Fig. 2F and K). Only few rhabdomeres were still slightly thicker than in wild-type.

Crb_{extra} does not restore the length of the stalk membrane and the localization of Sdt in *crb* mutant adult PRCs

In wild-type PRCs, the Crb protein complex is localized at the stalk membrane (see Fig. 1B), a portion

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of the apical plasma membrane of PRCs located between the rhabdomere and the ZA (highlighted in red in Fig. 2B). *crb* mutant PRCs not only fail to properly elongate their rhabdomeres, but they also exhibit a reduction in the length of the stalk membrane by about 50% in comparison to wild-type PRCs (Fig. 3). We addressed the question, which part of the Crb protein can restore the length of the stalk membrane, using the same transgenes as described above. Upon overexpression of either Crb_{full} or $Crb_{FLAGintra}$, the length of the stalk membrane was partially restored. In contrast, expression of Crb_{extra} had no effect on the length of the stalk of *crb* mutant PRCs (Fig. 3).

Loss of *crb* function in PRCs not only reduces the length of the stalk membrane, but also results in the loss of other Crb complex components, such as Sdt (compare Fig. 4A, B) or *D*PATJ (not shown) from the stalk membrane. We therefore tested the same transgenes for their capacity to recruit other Crb complex members to the stalk membrane of adult PRCs when expressed in *crb* mutant PRCs, using *elav*Gal4. Upon expression of Crb_{full}, transgene-encoded Crb was mostly found at the stalk membrane, where it co-localized with Sdt (Fig. 4C-C''). Expression of $Crb_{FLAGintra}$ brought some Sdt and *D*PATJ back to the stalk membrane (Fig. 4D, and data not shown). However, in *crb* mutant PRCs expressing Crb_{extra} no Crb protein could be detected and Sdt remained at the rhabdomere base as in *crb* mutant PRCs (compare Fig. 4B' and E').

Crb_{full}, $Crb_{FLAGintra}$ and Crb_{extra} recruit other Crb complex members to the stalk during pupal development

To get further insight into the function of the different transgenes during PRC development, we analyzed the localization of Crb complex members (Crb, Sdt, *D*PATJ), β_{Heavy}-spectrin and the ZA marker armadillo (Arm; β-catenin) during pupal eye development in different genetic backgrounds. In early wild-type pupal eyes before stalk membrane formation and rhabdomere elongation, i.e., at about 45% pupal development, F-actin staining highlights the forming rhabdomeres in the apical membrane of PRCs (Fig. 5A). Crb, Sdt, and

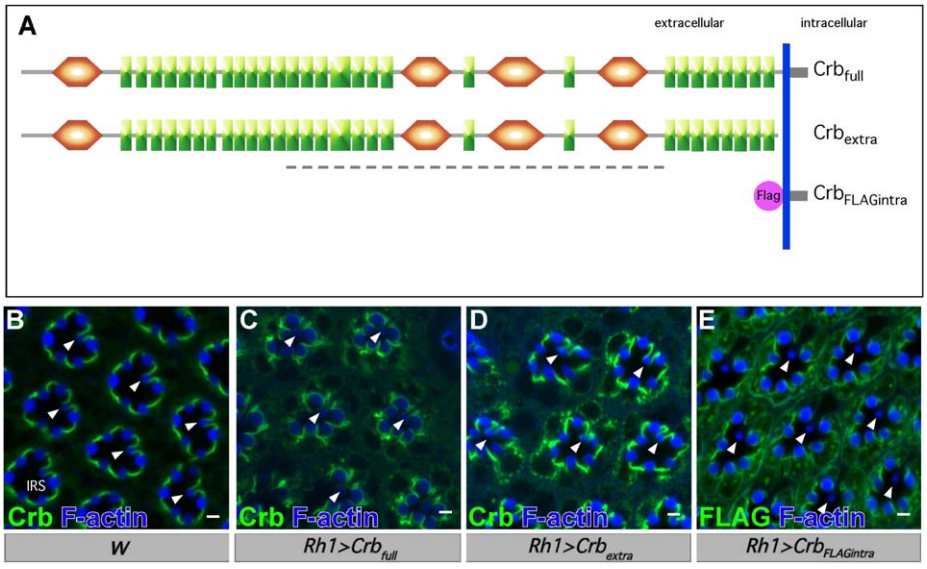

Fig. 1. Structure and expression of Crb transgenes. (A) Crb variants used here. Green rectangle: EGF-like repeats, brown hexagon: repeats with similarity to the globular domain of laminin A, blue bar: plasma membrane, grey bar: cytoplasmic domain. The constructs are based on the Crb-RA isoform (2146 amino acids). The dotted line indicates the region of the protein against which the anti-Crb antibody used in this work was raised. (B) The photoreceptors of wild-type ommatidia exhibit a highly stereotypic arrangement, as visualized by the patterning of the rhabdomeres, which are rich in F-actin (blue). Crb (green) is localized at the stalk membranes in wild-type (*w*) eyes. The rhabdomeres are separated from each other by the interrhabdomeral space (IRS). R7 cells are marked by arrowheads. (C-E) Expression of Crb_{full} (C), Crb_{extra} (D) and $Crb_{FLAGintra}$ (E) in wild-type (*w*) eyes using *Rh1*Gal4, which drives expression in the outer PRCs R1-R6. The Crb antibody detects an epitope in the extracellular domain (A, dotted line), and hence does not discriminate between endogenous and transgene-expressed protein in (C) and (D). Upon expression of Crb_{full} with *Rh1*Gal4 (B), transgene-encoded protein accumulated at the stalk membranes of R1-R6, while R7 cells (arrowheads), which do not express Rh1, only show endogenous Crb. Upon expression of Crb_{extra} with *Rh1*Gal4 (D), Crb staining at the stalk membrane of R1-R6 was about the same as that in R7 (arrowheads), where the transgene was not overexpressed. From this we conclude that the staining represents endogenous Crb, whereas the cytoplasmic staining in R1-R6 represents transgene-encoded Crb_{extra}. The different size of the IRS reflects the normal variation. Bars: 2 μm.

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*D*PATJ co-localize with F-actin at this stage. Arm is restricted to the ZAs, which are established between the PRCs, basal to the F-actin staining (Fig. 5A-A''' and data not shown). In crb^{11A22} mutant PRCs of comparable age, Crb complex members were no longer confined apically (Fig. 5C'' and data not shown), confirming that *crb* is essential for the stabilization of the complex already at early pupal stages (Izaddoost et al., 2002; Pellikka et al., 2002). In addition, Arm expanded into the baso-lateral membrane in the absence of *crb* function (Fig. 5B'').

crb^{11A22} mutant PRCs expressing either full-length Crb or the membrane-bound intracellular domain (Crb_{intra}) showed wild-type localization of Arm and Sdt at 45% pupal development (Fig. 5D'', 5E'' and data not shown). These results demonstrate that a wild-type cytoplasmic domain of Crb is sufficient for correct localization of Crb complex members and ZA markers

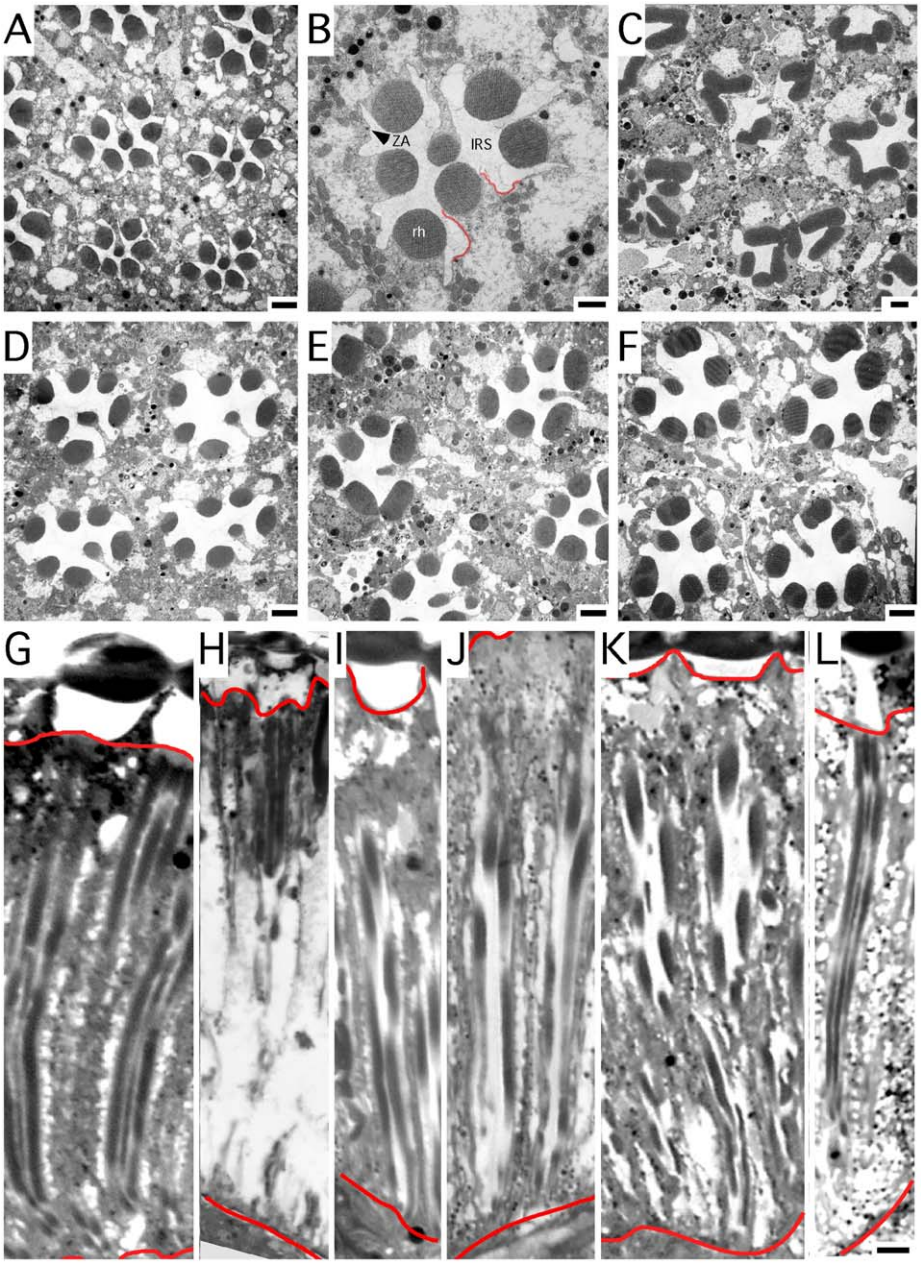

Fig. 2. Rescuing activity of different Crb variants. Cross sections of wild-type (A, B), crb^{11A22} (C), *elav*Gal > UAS-Crb_{full}; crb^{11A22} (D), *elav*Gal > UAS-$Crb_{FLAGintra}$; crb^{11A22} (E) and *elav*Gal > UAS-Crb_{extra}; crb^{11A22} (F) eyes. In (B), some stalk membranes in the wild-type ommatidium are outlined in red. Arrowhead: zonula adherens (ZA), IRS: interrhabdomeral space, rh: rhabdomere. Longitudinal sections of wild-type (G), crb^{11A22} (H), *elav*Gal > UAS-Crb_{full}; crb^{11A22} (I), *elav*Gal > UAS-$Crb_{FLAGintra}$; crb^{11A22} (J), *elav*Gal > UAS-Crb_{extra}; crb^{11A22} (K) and *karst* (L) eyes. In (G-L), the red lines indicate the distal (top) and proximal (bottom) end of the PRCs. Bars: 1 μm (B), 2 μm (A, C-F) and 5 μm (G-L).

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at early pupal stages. Strikingly, early pupal PRCs of crb^{11A22} mutant flies overexpressing the extracellular domain of Crb also accumulated Arm (Fig. 5F''), Sdt (Fig. 5G'') and *D*PATJ (not shown) apically, despite the fact that transgene-encoded Crb_{extra} protein was predominantly distributed throughout the cells.

Starting at about 50% pupal development, the apical membrane of wild-type PRCs becomes subdivided into the stalk, which abuts the ZA, and the rhabdomere. This separation is clearly manifested at 70% pupal development by the restriction of F-actin to the most apical part, the incipient rhabdomere, while Crb and the other members of the complex localize at the stalk, between the ZA and the rhabdomere (Fig. 6A-A'''). In *crb* mutant PRCs of this stage, *D*PATJ and Sdt remain delocalized in the absence of Crb and became progressively lost (Fig. 6B-B''' and data not shown). Similar as in early stages, expression of either Crb_{full} or $Crb_{FLAGintra}$ showed wild-type localization of *D*PATJ and Sdt (Fig. 6C'' and D''). In contrast, in PRCs expressing Crb_{extra} *D*PATJ was progressively lost from the stalk, while Sdt was removed from the stalk only during later pupal development (Fig. 6E'' and F'').

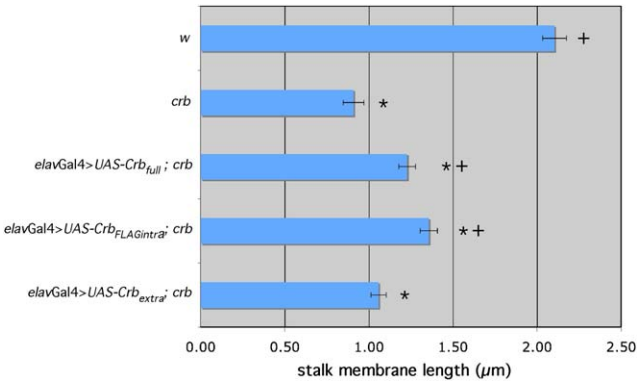

Fig. 3. Partial rescue of the stalk membrane length by different Crb-encoding transgenes. Plots of mean values ± S.E.M. of the stalk membrane length. From top to bottom: *w* (n = 109), crb^{11A22} (n = 121), *elav*Gal4 > UAS-Crb_{full}; crb^{11A22} (n = 180), *elav*Gal4 > UAS-$Crb_{FLAGintra}$; crb^{11A22} (n = 163) and *elav*Gal4 > UAS-Crb_{extra}; crb^{11A22} (n = 181). The stalk membranes were obtained from three eyes of different flies. Analysis includes the measurement of membranes from PRCs R1-R6, with a clearly identifiable ZA. Asterisk (*) or cross (+) indicate a significant difference compared to *w* or crb^{11A22}, respectively, with $p < 0.0001$ as assessed by Student's t-test (two-sided, unequal variance).

Crb_{full} and $Crb_{FLAGintra}$, but not Crb_{extra}, recruit β_{Heavy}-spectrin to the stalk during pupal development

The spectrin-based membrane cytoskeleton plays an important role in various processes, such as the generation of specialized membrane domains, endocytosis or vesicle transport (Bennett and Baines, 2001). β_{Heavy}-spectrin co-localizes with members of the Crb complex in the subapical region of the embryo (Medina et al., 2002; Wodarz et al., 1995) and at the stalk of PRCs (Pellikka et al., 2002). β_{Heavy}-spectrin becomes delocalized in *crb* mutant PRCs at 45% pupal development (compare Fig. 7A'-A'' with Fig. 7B'', B'''). Expression of full-length Crb protein or of $Crb_{FLAGintra}$ restored apical localization of β_{Heavy}-spectrin in *crb* mutant PRCs at early and late stages of pupal development (Fig. 7C'', C''' and data not shown). In contrast, expression of the extracellular domain of Crb completely failed to bring β_{Heavy}-spectrin back to its normal place (Fig. 7D'', D'''). The difference in the behavior of these transgenes with respect to β_{Heavy}-spectrin localization correlated with their different rescuing activity of stalk membrane length, in that only Crb_{full} and $Crb_{FLAGintra}$, but not Crb_{extra}, restored stalk membrane length (see Fig. 3). This suggests that β_{Heavy}-spectrin is a crucial factor for regulating stalk membrane length. Indeed, PRCs mutant for *karst* (*kst*), the gene encoding β_{Heavy}-spectrin, have shorter stalks (Pellikka et al., 2002). In contrast, elongation of rhabdomeres in PRCs of *kst* mutants is not affected (Fig. 2L).

Discussion

The apical plasma membrane of *Drosophila* PRCs is subdivided into the central rhabdomere and the adjacent stalk. *crb* mutant PRCs exhibit shortened stalk membranes and their rhabdomeres do not span the depth of the retina. Results presented here are the first showing a rescuing function for the extracellular domain of Crb in *Drosophila*. They further support the conclusion that elongation of the rhabdomeres and stalk membrane length are independently regulated. The latter was previously suggested based on the mutant phenotypes of various *sdt* alleles, some of which only affect the length of the stalk membrane without impairing elongation of the PRC (Berger et al., 2007). Our results now show that Crb_{full}, Crb_{intra} and Crb_{extra} can rescue defects in rhabdomere elongation, but only Crb_{full} and Crb_{intra} are able to partially restore stalk membrane length of *crb* mutant PRCs, while Crb_{extra} fails to do so.

The failure to fully elongate *crb* mutant rhabdomeres is associated with a fragmentation of the ZA during pupal development (Fan et al., 2003; Izaddoost et al., 2002; Pellikka et al., 2002). This defect is comparable to that observed in the embryonic Malpighian tubules, the excretory organs of insects. Epithelial integrity and polarity of Malpighian tubules of *crb* mutant embryos is

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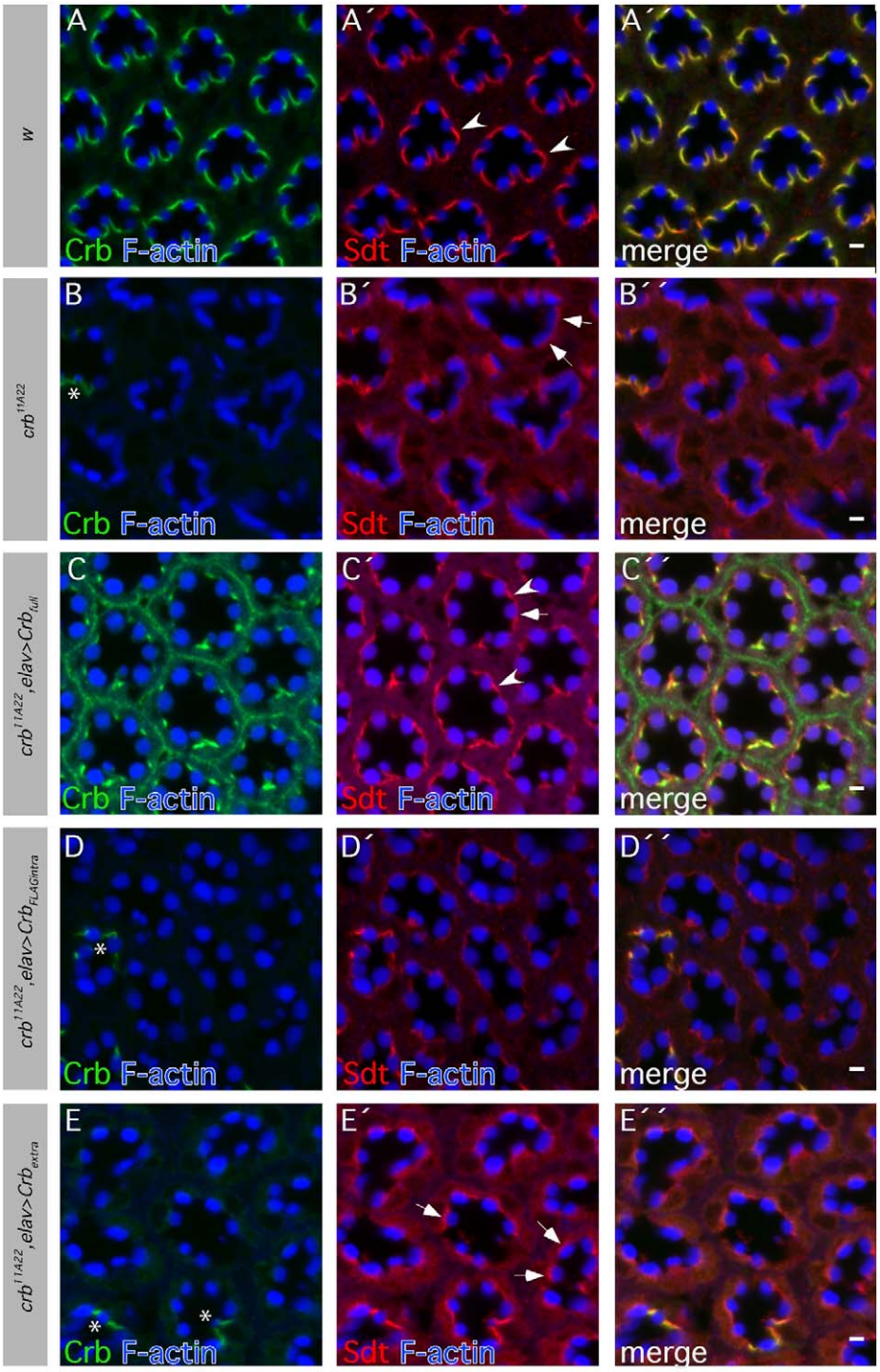

Fig. 4. Localization of Crb and Sdt in adult crb^{11A22} mutant PRCs upon expression of different Crb transgenes. Cross-sections of adult wild-type eyes, (*w*) (A), crb^{11A22} mutant eyes (B) and crb^{11A22} mutant eyes expressing different transgenes with *elav*Gal4 (C-E) were stained with anti-Crb (green), anti-Sdt (red) and Alexa-660-phalloidin to stain F-actin (blue). Note localization of Sdt at the stalk in wild-type PRCs (arrowhead in A') and at the rhabdomere base in *crb* mutant PRCs (arrows in B'). Sdt is localized mostly at the stalk upon Crb_{full} and Crb_{intra} expression (arrowheads in C'), but mostly remains at the rhabdomere base upon Crb_{extra} expression (E', arrows). Crb staining in the pigment cells in (C) is unspecific staining. Asterisks in (B, D E) mark ommatidia with wild-type PRCs, which express endogenous Crb. Bars: 2 μm.

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completely abolished during convergent extension movements, which takes place in the second half of embryogenesis. During this process, new cell contacts are formed, the apical surface expands, and the epithelium transforms from a cuboidal to a squamous epithelium (Campbell et al., 2009). Both processes, i.e., convergent extension of Malpighian tubules and elongation of the PRC, require building and remodeling of junctions. Similar as in the developing wing undergoing morphogenesis (Classen et al., 2005), remodeling of junctions during PRC elongation may also depend on recycling of E-cadherin. Strikingly, although Crb_{extra} lacks the transmembrane and cytoplasmic domain, it can recruit apical Sdt and *D*PATJ in *crb* mutant PRCs, which, in turn, are important to stabilize the ZA during elongation. So far, we can only speculate on the mechanism responsible for this function, since no ligand of the extracellular domain is known. It is possible that the extracellular domain recruits a still unknown protein to the apical membrane, which could act redundantly to organize an Sdt-based membrane-associated protein scaffold.

Beside defects in rhabdomere elongation, *crb* mutant PRCs have shorter stalks, and overexpression of full-length Crb, but not a secreted Crb protein elongates the stalk ((Pellikka et al., 2002) and own observation). One way to explain these results is to assume that the Crb complex is involved in the formation of an apical, membrane-associated platform, which participates in recycling and/or delivery of apical proteins. The observations that β_{Heavy}-spectrin co-localizes with the Crb complex at the stalk, and loss of Crb results in loss of β_{Heavy}-spectrin may imply a role of β_{Heavy}-spectrin in stabilizing this platform. In fact, previous results suggested that β_{Heavy}-spectrin plays a role in stabilizing membrane domains (Bennett and Gilligan, 1993; Williams et al., 2004). Overexpression of the C-terminal domain of β_{Heavy}-spectrin, which lacks the membrane-binding domain, results in an expansion of the plasma membrane of some embryonic epithelia, possibly by sequestering components of the endocytic machinery, such as dynamin (Williams et al., 2004). In line with this, reduction of β_{Heavy}-spectrin leads to shorter stalk membrane length (Pellikka et al., 2002), but does not affect rhabdomere elongation (this work). Thus, the opposite effects generated by *crb* loss-of-function mutations and Crb overexpression on stalk membrane length may be mediated by the loss or stabilization of β_{Heavy}-spectrin, and hence enhanced or decreased endocytosis, respectively (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). β_{Heavy}-spectrin could only be restored at the stalk membrane upon expression of Crb variants with an intact cytoplasmic domain, suggesting that this domain provides a link to the cytoskeleton. This link could be achieved either by building a plasma membrane-associated protein scaffold mediated by Sdt, *D*PATJ and *D*Lin-7, which then, directly or indirectly, recruits β_{Heavy}-spectrin. In the embryo, the ERM protein moesin could be co-immunoprecipated with Crb and β_{Heavy}-spectrin, indicating that moesin may link the Crb complex with the spectrin-based membrane skeleton (Medina et al., 2002). In pupal PRCs moesin and Crb co-localize apically during the first half of pupal development, but upon forming the stalk, moesin becomes associated with the rhabdomere base (Karagiosis and Ready, 2004). This excludes moesin as a partner of Crb for the stabilization of the stalk membrane. Although Yurt binds to Crb via the FERM-binding site in vitro, β_{Heavy}-spectrin is still correctly localized at the stalk membrane in *yurt* mutant PRCs, making Yurt unlikely to act as a linker between Crb and the cytoskeleton (Laprise et al., 2006). The observation that the cytoplasmic domain of Crb with a mutated FERM-binding domain ($Crb_{intraY10A}$; Klebes and Knust, 2000) was unable to rescue stalk membrane shortening of *crb* mutant PRCs (data not shown) supports the importance of this domain, and suggests that another, yet unknown interactor may provide a link between Crb and the spectrin-based cytoskeleton.

While some of the interaction partners of the cytoplasmic domain of Crb have been described, much less is known about the partners and function of the extracellular domain. The fact that the overall organization, i.e., the modular organization of EGF-like and laminin A-like repeats, is conserved from flies to mammals (Richard et al., 2006b) points to a functional conservation. In human, mutations in *CRB1* result in retinitis pigmentosa (RP)12- and Leber congenital amaurosis (LCA)-associated blindness (den Hollander et al., 2004), reminiscent of the light-induced retinal degeneration observed in *crb* mutant fly eyes (Johnson et al., 2002). In total, over 70 different sequence variants have been identified in more than 184 *CRB1* alleles of patients with retinal dystrophies, and nearly all of them have been mapped to the extracellular domain (den Hollander et al., 2004). So far, we do not have evidence that the extracellular domain encoded by Crb_{extra} is secreted when expressed in PRCs. While we could detect extracellular Crb upon Crb_{extra} expression in embryonic salivary glands (Wodarz et al., 1995), no Crb protein could be detected outside the PRCs, e.g. in the IRS, upon expression of Crb_{extra} in wild-type or *crb* mutant PRCs. This could be either due to lack of secretion in PRCs, to instability of the protein after secretion or to experimental conditions, during which the extracellular protein is washed out. Currently, we cannot distinguish between these possibilities.

In the mouse, a *Crb1* mRNA is expressed in the developing epidermis that encodes a secreted Crb1 isoform, This form contains only the N-terminal part of the extracellular domain, and lacks the transmembrane and cytoplasmic domain (Watanabe et al., 2004).

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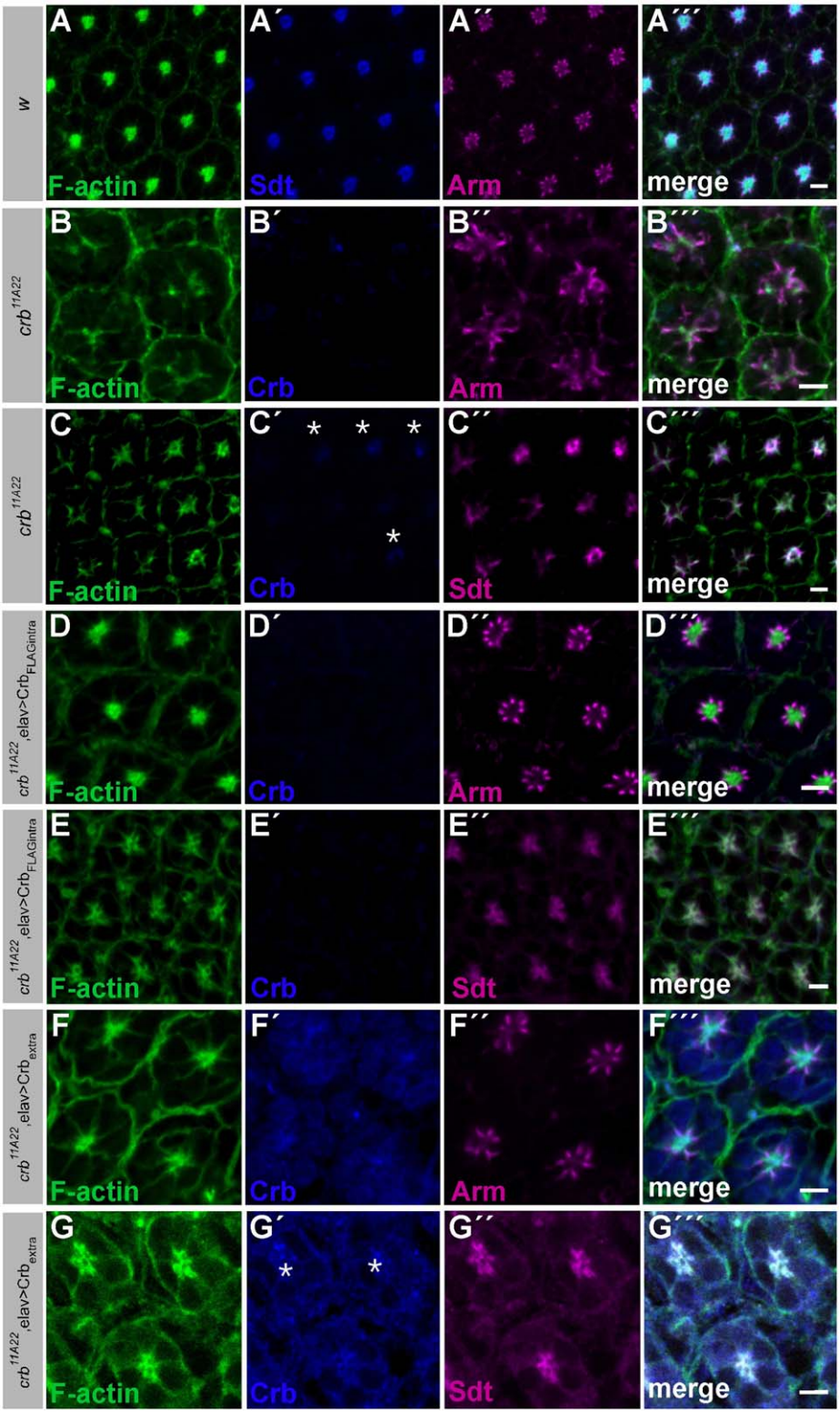

Fig. 5. Localization of Crb, Sdt and Arm in pupal PRCs at 45% pupal development in crb^{11A22} mutant PRCs upon expression of different Crb transgenes. Optical cross sections of pupal eyes. Wild-type (*w*) eyes, (A), crb^{11A22} mutant eyes (B), and eyes mutant for crb^{11A22} and expressing different transgenes with *elav*Gal4 (C-G) were stained with Alexa-488-phalloidin (green), anti-Crb (B'-G'; blue) or anti-Sdt (A'; blue), anti-Arm (A'', B'', D'', F''; magenta) and anti-Sdt (C'', E'', G''; magenta). Asterisks in (C') and (G') mark ommatidia with wild-type PRCs, which express endogenous Crb. Bars: 2 μm.

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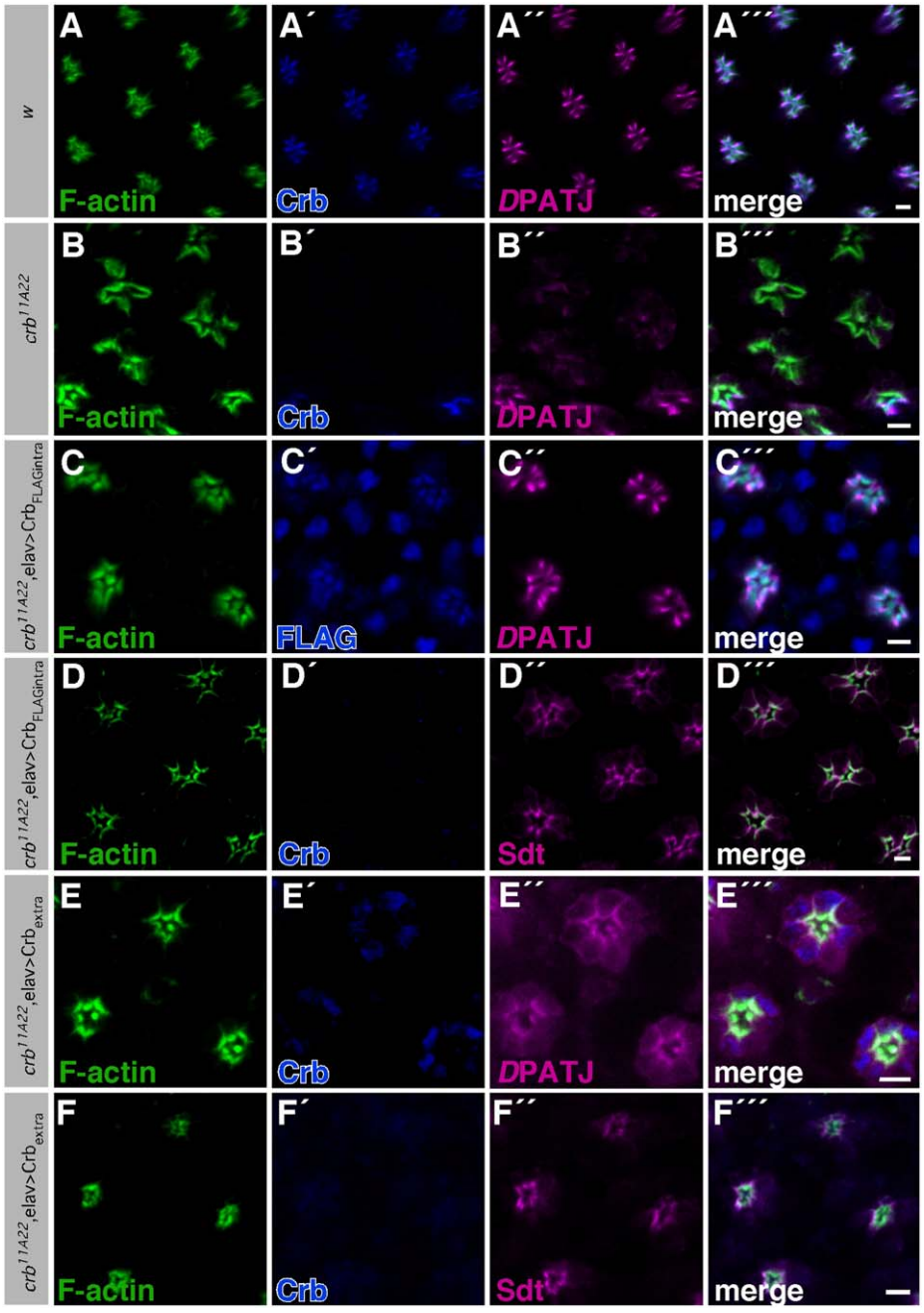

Fig. 6. Localization of Crb, Sdt or *D*PATJ in pupal PRCs at 70% pupal development in crb^{11A22} mutant PRCs upon expression of different Crb transgenes. Optical cross-sections of pupal eyes. Wild-type (*w*) (A) and crb^{11A22} mutant (B) eyes and those mutant for crb^{11A22} and expressing different Crb-encoding transgenes with *elav*Gal4 (C-F) were stained with Alexa-488-phalloidin (green), anti-Crb or anti-FLAG (blue), and anti-Sdt or anti-*D*PATJ (magenta). Bars: 2 μm.

Similarly, cDNA analysis suggests that the human retina expresses a secreted form of *CRB* (den Hollander et al., 1999). Future studies will reveal the role and possible interaction partners of the extracellular domain of *Drosophila* Crb and thereby provide a deeper understanding of its function in PRCs, which certainly will shed light on the role of the mammalian CRB proteins.

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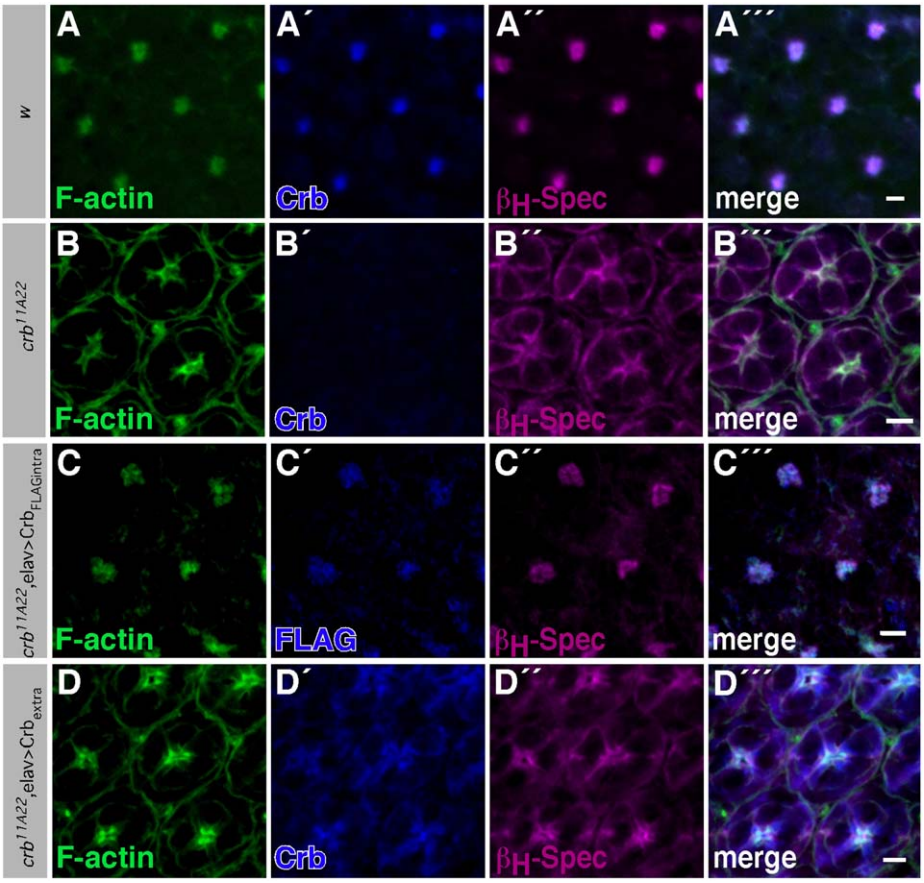

Fig. 7. Localization of Crb and β_{Heavy}-spectrin in pupal PRCs at 45% pupal development in crb^{11A22} mutant PRCs upon expression of different Crb transgenes. Optical cross sections of pupal eyes. Wild-type (*w*) (A) and crb^{11A22} mutant (B) eyes and eyes mutant for crb^{11A22} and expressing different Crb-encoding transgenes with *elav*Gal4 (C-D) were stained with Alexa-488-phalloidin (green), anti-Crb or anti-FLAG (blue), and anti-β_{Heavy}-spectrin (magenta). Bars: 2 μm.

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