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Crumbs regulates polarity and prevents light-induced degeneration of the simple eyes of *Drosophila*, the ocelli

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Abstract

The evolutionary conserved transmembrane protein Crumbs (Crb) regulates morphogenesis of photoreceptor cells in the compound eye of *Drosophila* and prevents light-dependent retinal degeneration. Here we examine the role of Crb in the ocelli, the simple eyes of *Drosophila*. We show that Crb is expressed in ocellar photoreceptor cells, where it defines a stalk membrane apical to the adherens junctions, similar as in photoreceptor cells of the compound eyes. Loss of function of *crb* disrupts polarity of ocellar photoreceptor cells, and results in mislocalisation of adherens junction proteins. This phenotype is more severe than that observed in mutant photoreceptor cells of the compound eye, and resembles more that of embryonic epithelia lacking *crb*. Similar as in compound eyes, *crb* protects ocellar photoreceptors from light induced degeneration, a function that depends on the extracellular portion of the Crb protein. Our data demonstrate that the function of *crb* in photoreceptor development and homeostasis is conserved in compound eyes and ocelli and underscores the evolutionarily relationship between these visual sense organs of *Drosophila*. The data will be discussed with respect to the difference in apico-basal organisation of these two cell types.

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Introduction

Invertebrates have developed a wide variety of visual systems, from very simple eyes to highly differentiated organs, which fulfil a broad range of functions. The visual system of adult *Drosophila melanogaster* consists of the two lateral compound eyes, a pair of extraretinal eyes, the eyelets (Helfrich-Forster *et al.*, 2002), and the ocelli (Stark *et al.*, 1989). The compound eye, the main light-sensing organelle, is composed of about 800 functional units called ommatidia, each of which is covered by a cornea and a crystalline cone, which together form the dioptric apparatus. Each ommatidium contains eight photoreceptor cells (PRCs), which are connected to each other and to cone and pigment cells by adherens junctions and are arranged in a highly stereotypic pattern. The outer PRCs, R1-R6, expand throughout the length of the retina, whereas the central PRCs, R7 and R8, only span the distal and proximal halves, respectively. The apical membrane of the PRCs is partitioned into the most apical rhabdomere, the light sensing organelle built from densely packed microvilli, which harbour the visual pigment rhodopsin, and the stalk membrane, localised between the rhabdomere and the *zonula adherens* (ZA). The rhabdomeres of all PRCs point towards the centre of the ommatidium and are separated from each other by the inter-rhabdomeral space (IRS), thus forming a so-called open rhabdom. The eyelet presents the adult remnant of the larval Bolwig's organ, which is considered as the most rudimentary visual sense structure in insects (Bolwig, 1946). Each Bolwig's organ consists of a bundle of 12 PRCs with loosely organised lamellae instead of densely packed microvilli present in the PRCs of the compound eye (Melzer & Paulus, 1989; Paulus, 1989).

The three ocelli of the adult fly are arranged in a triangle between the compound eyes on the vertex of the head. They serve to adjust the sensitivity of the compound eyes (Hu & Stark, 1980) and provide information about the fly's horizontal position (Krapp, 2009). Ocelli develop from specific progenitor cells of the eye-antennal imaginal disc of third instar larvae. The two lateral ocelli are derived from different discs, while the medial ocellus is formed by fusion of the anlagen present in each of the two discs (Royet & Finkelstein, 1995; 1996). Each ocellus is covered by a single dome-like corneal lens, with a diameter of ~40 μm, covering a thin corneagenous cell layer, which is followed by

about 80 PRCs. A layer of pigment cells surrounds the cluster of PRCs. Similar to compound eyes, the apical membrane of ocellar PRCs is highly expanded by tightly packed microvilli to form a rhabdomere. PRCs are connected to each other by adherens junctions (Stark *et al.*, 1989).

Both compound eyes and ocelli possess a rhabdomeric type of photoreceptors, yet they differ from each other in various aspects. In compound eyes, the eight PRCs of each ommatidium develop in close association with accessory cells, e. g. pigment cells and cone cells, to which they are connected by adherens junctions. In contrast, all PRCs of an ocellus are surrounded by pigment cells and covered by a sheet of corneagenous cells. Rhabdomeres of the outer PRCs R1-R6 span the entire length of the cell (~85 μm), while ocellar rhabdomeres are about 7 μm in length and only span the distal portion of the cell. PRCs from compound eyes and ocelli also differ in the rhodopsin they express. Rhabdomeres of R1–R6 contain Rhodopsin1 (Rh1), which is encoded by *ninaE* (O'Tousa *et al.*, 1985; Zuker *et al.*, 1985), while the central R7 expresses the UV sensitive rhodopsins Rh3 or Rh4 (Zuker *et al.*, 1985; Feiler *et al.*, 1992; Montell, 1997), and R8 cells express Rh5 or Rh6, sensitive to blue and green light, respectively (Salcedo *et al.*, 1999). In contrast, ocelli express Rh2, which confers a spectral sensitivity between 350 nm (ultraviolet) and 445 nm (blue). Finally, rhabdomeres of the *Drosophila* compound eyes are clearly separated form each other by the interrhabdomeral space (IRS), while ocellar rhabdomeres directly face the neighbouring PRC (Stark *et al.*, 1989).

Besides the differences mentioned above, there are several molecular and developmental similarities between compound eyes and ocelli. Both develop from a common anlage, the visual field in the dorsal head neuroectoderm of the embryonic blastoderm (Green *et al.*, 1993). The retinal determination genes *twin of eyeless* (*toy*), *homothorax* (*hth*), *eyes absent* (*eya)* and *sine oculis* (*so)*, members of the Pax6, Meis, Eya and Six gene families, respectively, are required for the development of both compound eyes and ocelli (Pauli *et al.*, 2005; Blanco *et al.*, 2009; Brockmann *et al.*, 2011). Overexpression of *toy*, *ey* or *optix*, another member of the Six gene family, is sufficient to induce ectopic compound eyes (Halder *et al.*, 1995; Seimiya & Gehring, 2000; Onuma *et al.*, 2002), but insufficient for the induction of ectopic ocelli. However, co-expression of *toy* or *optix* together with *orthodenticle* (*otd*) is sufficient to induce ectopic ocelli, albeit

on the vertex of the head only.

Common to all PRCs is a highly polarised phenotype, characterised by an extremely expanded apical surface, highly specialised to accommodate the components of the light-induced signalling cascade. In addition, these cells have developed mechanisms to maintain cellular homeostasis, required to prevent damages induced by light stress. The evolutionarily conserved Crumbs (Crb) protein is required in rhabdomeric PRCs of *Drosophila* to ensure proper morphogenesis and prevents from light-dependent retinal degeneration (Johnson *et al.*, 2002; Pellikka *et al.*, 2002; Pocha *et al.*, 2011). Strikingly, mutations in human *CRB1* lead to progressive types of retinitis pigmentosa 12 (RP12) and Leber congenital amaurosis (LCA), two severe forms of retinal dystrophy (den Hollander *et al.*, 1999; den Hollander *et al.*, 2001; Lotery *et al.*, 2001a; Lotery *et al.*, 2001b). Crb/CRB1 is a type one transmembrane protein, with a highly conserved cytoplasmic tail of only 37 amino acids. The C-terminal PDZ-binding motif links Crb/CRB1 to a conserved protein scaffold, containing the core components Stardust (Sdt), *D*PATJ and *D*Lin-7 in flies, which correspond to MPP5/Pals1, PATJ and Veli/Lin-7 in vertebrates. Loss of any core component of the Crb complex results in light-dependent retinal degeneration in flies (Johnson *et al.*, 2002; Richard *et al.*, 2006; Berger *et al.*, 2007; Bachmann *et al.*, 2008; Bulgakova *et al.*, 2010). This similarity in function is striking, given the fact that *Drosophila* and vertebrates have two different types of PRCs, rhabdomeric and ciliary PRCs, respectively, which differ in the way they expand their highly elaborated apical surface. Apical membrane expansion in rhabdomeric PRCs is achieved by folding into tightly packed microvilli, while vertebrate PRCs achieve this expansion by folding the ciliary membrane (Arendt, 2003).

Here we show that the function of Crb is also required in the simple eyes of *Drosophila*, the ocelli. Expression studies show that, similar as in PRCs of the compound eye, Crb is localised between the adherens junctions and the rhabdomere and thus defines a stalk membrane also in these cells. Unlike PRCs of the compound eyes, ocellar PRCs lose apico-basal polarity upon loss of *crb* function, accompanied by mis-localisation of junctional proteins. Finally, Crb protects ocellar PRCs from light dependent degeneration. These results will be discussed in relation to the conserved role of Crb during eye evolution and the organisation of apico-basal polarity in these two visual

sense organs.

Materials and Methods

Fly stocks and reagents

*white (*w*)* flies were used as wild-type control throughout the experiment. Eyes and ocelli mosaic for crb^{11A22}, a protein null allele, or crb^{8F105}, which encodes a truncated Crb protein (Wodarz *et al.*, 1993) were generated by crossing *yw ey*-FLP;;FRT82B *w*+ cl3R3/TM6B females (Newsome *et al.*, 2000) to w;;FRT82B *crb*/TM6B males. CRB:GFP-A flies, which express a GFP-tagged Crb protein that rescue lethality of *crb* mutant embryos (Huang *et al.*, 2009) were for used for immuno electron microscopy.

Antibodies and Immunofluorescence analysis

Staining of adult ocelli was done essentially as described for compound eyes (Richard *et al.*, 2006). Heads were cut with a razor blade and the proboscis was removed. Heads were fixed for 40 minutes in Stefanini's fixative at room temperature in shaking condition, washed three times with phosphate buffered saline (PBS) for 15 minutes each, and infiltrated at 4 °C with 10% sucrose in PBS for 2 hours and in 25% sucrose solution overnight. Heads were embedded in mounting media and frozen over dry ice. 12 µm sections were stained as described (Richard *et al.*, 2006). The following primary antibodies were used: rat anti-Crb2.8 (1:500; E. Theilenberg and E. Knust, unpublished), rabbit anti-*D*Lin-7 (1:300) (Bachmann *et al.*, 2004), rabbit anti-*D*PATJ (1:300) (Richard *et al.*, 2006), rabbit anti-SdtMPDZ (1:300) (Berger *et al.*, 2007), mouse anti-Na^+/K^+-ATPase (a5, alpha subunit), rat anti-*D*E-cadherin, mouse anti-Armadillo, mouse anti TRP (each 1:50; Developmental Studies Hybridoma Bank, DSHB), rabbit anti-aPKC (1:100; BD Biosciences), rabbit anti-Bazooka (1:500; A. Wodarz). Cy3-conjugated goat anti-HRP (1:100; Dianova) was used to label neuronal cells in the ocelli. Alexa 568- and Alexa 488-conjugated secondary antibodies (Molecular Probes, Inc.) were applied at 1:200 dilution. Alexa 647-conjugated phalloidin (1: 40; Invitrogen) was used to mark filamentous actin. Confocal images were taken on a LSM Meta confocal microscope

(Carl Zeiss). All images were processed with Fiji software and mounted using Adobe Illustrator CS4.

Immunoelectron microscopy

Crb::GFP-A flies (Huang *et al.*, 2009) were used to detect localisation of Crb in the ocelli by immunogold. Heads were cut and, after removal of the proboscis, placed into an Eppendorf tube containing 4% PFA diluted in PBS and kept at 4^{o}C for overnight. Heads were washed with PBS and dehydrated in 50%, 70%, 90%, 95% ethanol, for 10 minutes each. Heads were infiltrated overnight at -20 °C with a 95% EtOH/Lowicryl HM-20 mixture, followed by a second overnight infiltration with pure Lowicryl at -20 °C. Heads were put in gelatine capsules with Lowicryl and polymerised at -20 °C under UV inside an AFS (Automatic Freeze Substitution System, Leica) for two days. 70 nm sections were cut from appropriate areas and were collected on formvar coated nickel grids. Grids were washed with PBST (PBS +1% Tween-20) and blocked for one hour with 0.5% BSA and 10% gelatine at room temperature. Specimens were stained overnight at 4^{o}C with rabbit anti-GFP antibodies (1:200; Abcam), diluted with blocking solution. After washing with PBST, grids were incubated with proteinA/10-nm-gold (1:60; Cell Microscopy Centre) for one hour at room temperature, followed by several washings with PBS and water, and 2% uranyl acetate for 5 minutes for contrast enhancement. Digital images (Morada digital camera SiS) were taken using a FEI Tecnai 12 Bio Twin electron microscope operated at 80 kV, and mounted using Adobe Illustrator CS4.

Transmission Electron Microscopy

Fixation of adult eyes for transmission electron microscopic analysis was performed as previously described (Richard *et al.*, 2006). Essentially, fly heads of appropriate genotypes were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight at 4°C. Heads were washed with PBS, followed by a secondary fixation with 2% osmium for one hour at room temperature in the dark. Heads were dehydrated in 50%, 70%, 90%, 2 x 100% ethanol and 2x propylene oxide. Heads were infiltrated with a mixture of resin and polypropylene oxide overnight at room temperature, and embedded in pure resin.

Samples were allowed to polymerise at 60 °C overnight. Semithin sections of 500 nm were cut (Leica Ultracut Microsytems) and stained with toluidine blue. Ultrathin sections of 70 nm were cut from the appropriate areas with a diamond knife and stained with 2% uranyl acetate and lead citrate for contrast enhancement. Digital images (Morada digital camera SiS) were taken using a FEI Tecnai 12 Bio Twin electron microscope, and mounted using Adobe Illustrator CS4.

Results

Members of the Crb complex define a stalk membrane in ocellar photoreceptors

Ocellar PRCs of wild-type *Drosophila* are elongated cells, situated below the corneagenous cells. They develop laterally located rhabdomeres, which span the distal third of the cell (Stark *et al.*, 1989; Yoon *et al.*, 1996) (Fig. 1A, B; Fig. 2A-A'', actin staining). PRCs are connected to their neighbours by adherens junctions (AJs) (Fig. 1B-D, arrows). Similar as in PRCs of the compound eye, AJs are separated from the rhabdomere by a smooth membrane, which we call, in analogy to PRCs of the compound eye, stalk membrane (Fig. 1D, white arrowhead). Supporting evidence for the identity of this membrane as stalk membrane came from staining sections with antibodies against the core components of the Crb complex, Crb, Sdt, *D*PATJ and *D*Lin-7, which define the stalk membrane in PRCs of the compound eye. All four proteins exhibited similar localisations and overlapped with each other at a site next to the F-actin staining, which marks the rhabdomere (Fig. 2A-A'', Fig. 3). To determine the localisation more precisely, we applied immuno-gold staining, using a GFP-tagged version of Crb, which has been shown to fully rescue the lethality loss of *crb* mutant animals (Huang et al., 2009). This staining revealed Crb (GFP) labelling on the stalk membrane, apical to the adherens junction and basal to the microvilli (Fig. 4).

To further define the localisation of Crumbs in the stalk membrane, we analysed the localisation of a basolateral marker, Na^+/K^+-ATPase, a heterodimeric integral membrane protein highly conserved during evolution (Lebovitz *et al.*, 1989; Takeyasu *et*

al., 1991). Na^+/K^+-ATPase resides in the baso-lateral surface of most epithelial cells (Fambrough & Bayne, 1983; Kashgarian *et al.*, 1985). In only a few epithelia Na^+/K^+-ATPase is restricted to the apical domain, e. g. in the retinal pigment epithelium of rats (Gundersen, 1991) or in the salivary glands of cockroaches (Just & Walz, 1994). In arthropod photoreceptors, Na^+/K^+-ATPase is concentrated in the non-receptive compartment, i. e. on the baso-lateral membrane (Yasuhara *et al.*, 2000; Friedrich, 2006), where it functions to maintain ionic gradients, electric potential and osmotic balance. In ocellar PRCs, Na^+/K^+-ATPase was confined to membranes basal to F-actin and Crb staining (Fig. 5A-A''), further strengthening the Crb positive membrane as apical stalk membrane.

Crb controls rhabdomere organisation, adhesion and cell polarity in ocellar photoreceptor cells

In compound eyes, loss of function of *crb* leads to bulkier rhabdomeres, which often stick together and fail to expand throughout the length of the PRC. In addition, stalk membranes are reduced in length (Johnson *et al.*, 2002; Pellikka *et al.*, 2002; Richard *et al.*, 2009). Given that the Crb complex is localised to the stalk membrane of ocellar PRCs, we were interested to study the effects of loss of *crb* in mosaic eyes carrying large clones mutant for *crb*. While in wild-type (*w*) ocelli rhabdomeres are localised below the lens and nicely aligned, rhabdomeres of crb^{11A22} mutant ocelli were heavily disorganised and scattered throughout the depth of the ocelli (compare Fig. 1A, B with Fig. 1E and F; Fig. 2A-A'' with Fig. 2 C-C''). Rhabdomeres had variable shapes, microvilli were not properly aligned and often splayed at the margins of the rhabdomere (Fig. 1G, arrowheads). Abnormally formed, electron dense structures, which could be remnants of AJs, were irregularly scattered along the membranes (Fig. 1G, arrows). In most cases, stalk membranes could not be distinguished in crb^{11A22} mutant ocellar PRCs (compare Fig. 1D and H) and cell shape was strongly affected. Crb staining was completely abolished (Fig. 2C-C'').

The function of adherens junction is to assist, assemble and maintain epithelial cell

sheets by mediating cell adhesion, communication and cytoskeletal integration. Proteins required for adherens junction development and maintenance include the homophilic adhesion protein E-cadherin and associated cytosolic proteins, such as α- and β-catenin, organised into a multi-protein complex, which forms an adhesion belt in the apex of the cells. The *Drosophila* orthologues of E-cadherin and β-catenin, Shotgun and Armadillo, play an important role in AJ formation and stability in epithelia and in the retina (Harris & Peifer, 2006; Meng & Takeichi, 2009; Harris & Tepass, 2010). Results obtained from electron microscopic studies described above revealed severe structural disorganisation of the junctions in *crb* mutant ocellar PRCs. To confirm this result, we analysed the localisation of adherens junction proteins in wild-type and *crb* mutant ocelli, using antibodies to the known components *D*E-cadherin and Armadillo. Unlike *D*E-cadherin in PRCs of the compound eye, which spans the entire retina, *D*E-cadherin staining in wild-type ocelli was restricted to sites at the distal and proximal ends of the rhabdomeres (Fig. 5C-C''). While the proximal staining adjacent to the F-actin staining highlights the junctions between the PRCs, the distal *D*E-cadherin staining marks junctions between the corneagenous cells. In *crb* mutant clones, *D*E-cadherin was no longer detectable at these sites, but was reduced to small speckles of variable intensities scattered throughout the ocelli (Fig. 5D-D''). Occasionally, *D*E-cadherin showed co-localisation with rhabdomeral actin (Fig. 5D'', arrowhead). Similar as *D*E-cadherin, Armadillo was also mis-localised in *crb* mutant PRCs (not shown). Loss of both junctional proteins and data from electron microscopic studies support the conclusion that in ocelli, *crb* is required to maintain proper adherens junctions between the PRCs.

Given the defective localisation of junctional markers in *crb* mutant PRCs, we were interested to study the localisation of additional proteins in the ocelli. Therefore we stained for other polarity markers, including members of the Crb complex. *D*PATJ and *D*Lin-7, which span the length of the rhabdomere in wild-type ocellar PRCs (Fig. 3 and Fig. 5 A' and C') were no longer visible in *crb* mutant ocelli (Fig. 5B' and D' and data not shown). The *Drosophila* Par-3 orthologue Bazooka and the atypical protein kinase C (aPKC), members of the Par-protein network, are apically localised in *Drosophila* embryonic epithelia. While aPKC was enriched in the apical cytoplasm of ocellar PRCs (Fig. 5E-E'), Bazooka showed nuclear localisation (data not shown). Both stainings were

no longer detected in *crb* mutant ocelli (Fig. 5F-F' and data not shown). The baso-lateral marker Na^+/K^+-ATPase showed an aberrant distribution and was often detectable immediately below the cone (Fig. 5B-B''), suggesting a loss of cell polarity in *crb* mutant PRCs.

TRP (**T**ransient-**r**eceptor **p**otential) is a multifunctional molecule conserved from *Drosophila* to human, which serves as anchoring protein as well as cation channel. Some TRP family members are functioning in the sensory system. In *Drosophila* compound eyes, TRP is part of the signalplex, which includes rhodopsin and other components of the light-dependent signalling cascade, and is involved in the light-dependent translocation of TRPL [(Li & Montell, 2000; Tsunoda *et al.*, 2001; Bähner *et al.*, 2002); reviewed in: (Venkatachalam & Montell, 2007)]. In PRCs of wild-type ocelli, TRP co-localised with F-actin in the rhabdomeres, below the corneal lens, marking the apical compartment of the cell (Fig. 5G-G''). In *crb* mutant ocelli, TRP still localised, together with actin, in the delocalised rhabdomeres (Fig. 5H-H'').

PRCs of ocelli and compound eyes show different apico-basal organisation

We noticed, that *D*E-cadherin in ocellar PRCs is restricted to the proximal ends of the rhabdomeres, while staining of Crb complex members (e. g. *D*Lin-7) runs parallel to the rhabdomere (Fig. 5C'). This is in contrast to PRCs of the compound eye, where staining of Crb complex members and *D*E-cadherin run parallel to the rhabdomeres (marked by actin), spanning the entire retina (Fig. 6B' and data not shown; see (Johnson *et al.*, 2002). This led us to speculate that the apico-basal organisation of the two types of PRCs might be different.

To address this question, we compared the localisation of Na^+/K^+-ATPase in ocellar and compound eye PRCs. In the *Drosophila* compound eye, the alpha subunit of the Na^+/K^+-ATPase is localised in the baso-lateral surface of all PRCs, opposite to the apical membrane, marked by the rhabdomere (Yasuhara *et al.*, 2000) (Fig. 6A-A''). This becomes particularly obvious in longitudinal sections, in which Na^+/K^+-ATPase staining is detected parallel to the F-actin and Crb staining (Fig. 6B-B''). In ocellar PRCs,

however, Na^+/K^+-ATPase was not adjacent to the rhabdomeres, but rather confined to membranes basal to the F-actin staining (Fig.6 C-C''), suggesting a different apico-basal organisation of the PRCs in these two visual sense organs.

Crb protects ocelli from light-dependent degeneration

A known function of Crb is to protect compound eyes from light-dependent retinal degeneration (Johnson *et al.*, 2002). This function is conserved from *Drosophila* to human, since mutations in the human orthologue *CRB1* lead to RP12 and LCA, two severe forms of retinal dystrophy (den Hollander *et al.*, 1999; den Hollander *et al.*, 2001). As shown here, ocelli exhibit strong morphogenetic defects, which were more severe than in *crb* mutant PRCs of the compound eyes. Therefore, we asked whether ocelli of *crb* mutant PRCs are also responsive to continuous light stress. Newly eclosed flies (both *w* and those carrying *crb* clones) were exposed to seven days of constant light and analysed for any degeneration in the ocelli. At least six ocelli from different individuals and 10 photoreceptors in each section were analysed.

In wild-type ocelli exposed to light, the photoreceptor layer remained unaffected in comparison to those kept under day/night conditions (Fig. 7A) or in complete darkness (not shown). Some light-induced holes formed (Fig. 7B), which were also observed in *w* PRCs of compound eyes (Bachmann *et al.*, 2008). In contrast, PRCs of *crb* mutant ocelli showed severe characteristics of degeneration after light exposure (Fig. 7C). Remnants of dying PRCs contained darkly stained cytoplasm, and microvilli were often extended and showed signs of dissolution (Fig. 7C). Lysosomes appear as dark organelles and multivesicular bodies were abundant in the cytoplasm (Fig 7D). We conclude, that *crb* is not only required in PRCs of compound eyes, but also in those of the ocelli, to protect from light-dependent retinal degeneration.

Drosophila Crb is contains a large extracellular domain composed of an array of epidermal growth factor (EGF)-like repeats (Tepass et al., 1990). The small cytoplasmic domain of 37 amino acids is highly conserved and interacts via its C-terminal PDZ-binding motif and its FERM-binding motif with Sdt and Yurt, respectively (Klebes & Knust, 2000; den Hollander *et al.*, 2001; Laprise *et al.*, 2006; Richard *et al.*, 2006).

Expression of the cytoplasmic domain of Crb is able to rescue the defects in rhabdomere elongation and to partially rescue the length of the stalk membrane in PRCs of the compound eye (Johnson *et al.*, 2002; Richard *et al.*, 2009), while overexpression of either full-length Crb or its membrane bound extracellular domain in an otherwise wild-type background results in an expansion of the stalk membrane (Izaddoost *et al.*, 2002; Pellikka *et al.*, 2002; Richard *et al.*, 2009). To find out which part of Crb is responsible for the ocellar phenotype observed, we analysed the weaker allele crb^{8F105}. This allele encodes a truncated protein, in which the last 23 amino acids of the intracellular domain are lacking (Wodarz *et al.*, 1993). In agreement with previous findings from compound eyes, we could still detect some Crb protein in crb^{8F105} mutant ocelli (data not shown). The phenotype of crb^{8F105} mutant ocelli was weaker than that of crb^{11A22} cells. Most of the rhabdomeres were still confined to the distal part of the retina and exhibited normal shapes, but they were not as regularly aligned as in wild-type. AJs did not properly form in crb^{8F105} mutant PRCs. Unlike in wild-type ocelli and similar as in crb^{11A22} mutant ocelli, we frequently detected triangular structures scattered between the PRCs (Fig. 7F). In agreement with the anti-*D*E-cadherin antibody staining (Fig. 5D'), we suggest that these structures correspond to displaced, and probably abnormal, adherens junctions. Strikingly, when exposed to constant light, crb^{8F105} mutant PRCs did not show major signs of degeneration (Fig. 7E). This result is in agreement with that obtained in crb^{8F105} mutant PRCs of the compound eye, which also did not degenerate upon light exposure (Johnson *et al.*, 2002).

Discussion

The evolution of compound eyes and ocelli from a common primordial visual organ dates back to more than 500 million years (Bitsch & Bitsch, 2005; Friedrich, 2006). Similar as in epithelial cells and PRCs of the compound eye, localisation of the Crb complex immediately apical to the adherens junction defines a stalk membrane in ocellar PRCs, which is comparable to that of PRCs of the compound eye and to the subapical region in embryonic epithelia. Similar as in the *Drosophila* compound eye, Crb is required in the

ocelli for correct morphogenesis of the PRCs and protects them from light-dependent degeneration. However, ocelli lacking *crb* function show a much stronger phenotype compared to that of *crb* mutant PRCs of the compound eye. While the latter are still hold together in ommatidial clusters by apical AJs and point their apical surface, the rhabdomere, towards the centre of the ommatidium (Izaddoost *et al.*, 2002; Johnson *et al.*, 2002; Pellikka *et al.*, 2002), *crb* mutant ocellar PRCs have scattered AJs with abnormal morphology. In addition, unlike PRCs of the compound eyes, ocellar PRCs lose their polarity and regular alignment in the absence of *crb*. The phenotype in *crb* mutant ocelli is more similar to that of embryonic epithelia lacking *crb.* Here, loss of *crb* results in a reduction/mislocalisation of junctional proteins, followed by tissue breakdown in many epithelia (Tepass *et al.*, 1990; Grawe *et al.*, 1996; Tepass, 1996). A similar phenotype is observed in the retinal epithelium of zebrafish embryos mutant for *oko meduzy* (*ome*), one of the zebrafish *crb* orthologues (Wei & Malicki, 2002). In these mutants, the junctional complex, which is normally confined to an apical region close to the ventricular surface, is localised ectopically and exhibits an aberrant morphology, and epithelial integrity is compromised (Malicki & Driever, 1999).

Why are PRCs of the compound eye less susceptible to the lack of *crb*? During the first half of pupal development, the apical surface of the compound eye PRCs adopts a lateral position, with the apical surfaces of all eight PRCs of an ommatidium facing each other. This is followed by a tremendous expansion of the apical surface, including the zonula adherens (ZA) and the stalk membrane, which finally span the entire retina (Longley & Ready, 1995) (Fig. 8, top). As a consequence, staining of Crb, *D*E-cadherin and Na^+-K^+-ATPase parallels the rhabdomere and expands throughout the depth of the retina (see Fig. 6 B-B''). Our data suggest that the topology of ocellar PRCs is distinct from that of PRCs of the compound eye (compare Fig. 6B-B'' with Fig. 6C-C''). In this model, the apical membrane is expanded, probably asymmetrically, without affecting the ZA, which stays parallel to the surface of the lens, rather than perpendicular as in compound eyes (Fig. 8, bottom). As a consequence, staining of Crb, but not Na^+-K^+-ATPase or *D*E-cadherin, parallels the rhabdomere. Na^+-K^+-ATPase staining is restricted to membranes proximal to the rhabdomere (see Fig. 6C), while *D*E-cadherin is only detectable proximal to the rhabdomere (data not shown). This interpretation is compatible

with immuno-EM localisation of Crb along the membrane next to the rhabdomere (see Fig. 4). Further investigations on the development of ocellar PRCs are needed to support this model.

In PRCs of the compound eyes, stable AJs are required for proper elongation of the apical surface during pupal development, which includes an enormous elongation of the rhabdomere and the ZA. In *crb* mutant PRCs, ZAs are interrupted during this elongation process, but are largely restored in adult PRCs, at least in their distal regions (Izaddoost *et al.*, 2002; Johnson *et al.*, 2002; Pellikka *et al.*, 2002). This suggests that the retina of the compound eyes have developed additional mechanisms that act during cellular reorganisation to ensure proper adhesion between PRCs of an ommatidium. Alternatively, the extracellular matrix that forms the interrhabdomeral space, which is missing in ocelli, could provide a mechanical support to prevent complete disruption of ommatidial structures. Finally, junctional connections between PRCs and cone and pigment cells, as well as integrin-mediated adhesion to the retinal floor (Longley & Ready, 1995) may contribute to ommatidial stability. In ocelli, however, pigment cells are situated at the periphery. PRCs are linked to each other by AJs, but not to the corneagenous cells. Therefore, lack of a *crb*-dependent control of E-cadherin trafficking, localisation and/or stability has more severe consequences on cell polarity and tissue integrity in ocellar PRCs.

In both compound eye and ocellar PRCs Crb protects from light-dependent degeneration. *crb* mutant ocelli exposed to constant light show common signs of degeneration, such as abnormal lysosomes and abundant multi-vesicular bodies, features that were also observed in degenerating ocelli of *rdgB* mutants (Yoon *et al.*, 1996). Occasionally observed extension and dissolution of microvilli were also reported from other insect species exposed towards UV light (Meyer-Rochow & Mishra, 2007; Mishra & Meyer-Rochow, 2008). The protective function of *crb* is not restricted to rhabdomeric PRCs, since RP12 or LCA patients with mutations in *CRB1* gradually lose their vision and become blind at an early age (den Hollander *et al.*, 1999; den Hollander *et al.*, 2001; Lotery *et al.*, 2001a; Lotery *et al.*, 2001b). Similarly, mice with a conditional knock-down of Pals1, the vertebrate orthologue of *Drosophila sdt*, exhibit severe defects in retinal layering and in the structure of the retinal pigment epithelium and showed

progressive programmed cell death (Park *et al.*, 2011). The common role of the Crb complex in both rhabdomeric and ciliary PRCs suggests a conserved function required for the maintenance of cell homeostasis during morphogenetic and light stress.

Strikingly, in PRCs of compound eyes and ocelli the extracellular domain of Crb is sufficient to protect from light-dependent degeneration. This is in agreement with mutations mapped in RP12 and LCA patients, many of which have been localised to the extracellular domain (den Hollander *et al.*, 2004). Some of these mutations affect amino acids that are conserved between flies and human, such as a conserved amino acid in a Ca^{++}-binding EGF-like repeat (Siemiatkowska, 2011). Elucidation of the molecular function of the extracellular domain will certainly shed light on the mechanisms by which Crb controls PRC survival under light stress.

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Figure legends

Fig. 1

***crb* is required for maintenance of the ocellar organisation**

(**A-D**) Transmission electron micrographs of wild-type (*w*) ocelli. Longitudinal sections (A-C) and a transverse section (D) reveal the cornea (c), the corneagenous cells (cc) and the array of photoreceptors, visible by nicely aligned rhabdomeres (R). PRCs are connected to neighbouring PRCs by adherens junctions (arrows). Note the stalk membrane between the AJs and the rhabdomere (white arrowhead in D)**.** (**E-H**) Transmission electron micrographs of *crb* mutant ocelli. The cornea (c) and the PRCs are disorganised. The rhabdomeres (R) are scattered throughout the retina rather than being aligned below the cone and are often splayed at their margins (arrowheads in F and G). No junctions can be detected between the corneagenous cells and the PRCs (F, G, longitudinal section), or between neighbouring PRCs (H, compare with D). Instead, electron dense triangular structures are frequently visible between adjacent plasma membranes (arrows in F and G). Stalk membranes are not discernable. Scale bars: A, E: 2 μm; B, F: 1.4 μm; C, D, G: 900 nm; H: 700 nm.

Fig. 2:

Localisation of Crb protein in wild type and *crb* mutant ocelli

Immunostainings of longitudinal sections of wild-type (A-A'') and *crb* mutant (C-C''') ocelli, labelling Crb (green), neuronal membranes (anti-HRP; red) and F-actin (blue). B shows a cartoon (modified from (Yoon *et al.*, 1996)) to demonstrate the localisation of F-actin and Crb, using the same colours. Crb staining is visible along the entire length of the rhabdomeres in wild-type (A-A''), and is completely missing in *crb* mutant cells (C-C''). PRCs are not properly aligned as revealed by anti-HRP staining (C') and rhabdomeres are disorganised and scattered throughout the depth of the retina (C''). *D*PATJ, a member of the Crb complex that co-localises with Crb in wild-type (see also Fig. 3), is not detectable in mutant PRCs (C'''). Scale bar: 10 μm.

Fig. 3

Members of the Crb complex are expressed in ocelli

Immunostainings of longitudinal sections of wild-type ocelli, labelling F-actin (blue), neuronal membranes (anti-HRP, red) and Sdt (A-A''), *D*Lin-7 (B-B'') and *D*PATJ (C-C''), in green. Staining of Sdt, *D*Lin-7 and *D*PATJ is visible along the entire length of the rhabdomeres, similar as Crb. Scale bar: 10 μm.

Fig. 4

Immunogold localisation of Crb

Crb is localised to the stalk membrane of the ocelli (arrows). Note that in a longitudinal section the stalk membrane runs parallel to the rhabdom (R). Distal is up. Scale bar: 400 nm

Fig. 5.

***crb* regulates localisation of junctional and polarity markers in ocelli**.

Longitudinal sections through wild-type (A, C, E, G) and *crb* mutant (B, D, F, H) ocelli, labelling F-actin (blue). Green: Na^+/K^+-ATPase (A, B), *D*E–cadherin (C, D), aPKC (E, F), TRP (G, H). Red: *D*PATJ (A, B), *D*Lin-7 (C, D). Note that *D*E-cadherin, which is localised at the most distal and proximal ends of the rhabdomeres in wild-type (A), is scattered throughout the depth of the retina in the mutant (D). *D*PATJ and *D*Lin-7 cannot be detected in the mutant (B', D'), and Na^+/K^+-ATPase is mislocalised (compare A' and B'). TRP co-localises with F-actin in wild-type (G'') and mutant (H'') rhabdomeres. In all figures, distal is up and proximal down. Scale bar: 10 μm.

Fig. 6

Na^+/K^+-ATPase distribution suggests difference in apico-basal polarity organisation in PRCs of compound eyes and ocelli

(**A, B**) Transverse (A-A'') and longitudinal (B-B'') sections of compound eyes stained with F-actin highlight the rhabdomere (blue), Crb, which markes the stalk membrane (red) and Na^+-K^+-ATPase, marking the baso-lateral membrane (green). Note that the Na^+-K^+-ATPase staining parallels F-actin and Crb staining in B-B''. (**C**) Longitudinal section of an ocellus, stained with the same antibodies. Note that while F-actin and Crb staining ran parallel, Na^+-K^+-ATPase is restricted to membranes proximal to the rhabdomere. Scale bars: A-A'': 5 μm, B-B'': 20 μm, C-C'': 10 μm.

Fig. 7

Crb protects ocellar photoreceptors from light dependent degeneration

(**A, B**) Section through ocelli of wild-type (*w*) flies exposed to light for seven days. Note the nicely organised rhabdomeres and intact junctions. Cornea (c) and corneagenous cells (cc) are unaffected. Few light-induced holes (B) were occasionally detected. (**C, D**) Section through crb^{11A22} mutant ocelli of flies exposed to light for seven days. Note complete elimination of rhabdomere organisation and darkly stained cytoplasm, indicative of apoptic cells (asterisk). Note the devolution of the microvillar rhabdomere structure (R in D). (**E, F**) Section through crb^{8F105} mutant ocelli of flies exposed to light for seven days (F shows a higher magnification). Rhabdomeres are rather well organised, and no signs of cell death can be detected. Arrows point to multivesicular bodies in E and to electron dense material found between cells in F. Scale bars: 1.4 μm (A, B, C, E), 700 nm (D), 500 nm (F).

Fig. 8

Topology of PRCs of the compound eyes and ocelli

PRCs of the compound eye (top) rotate their apical surface by 90°, followed by a tremendous expansion of the apical surface, including the rhabdomere (blue), the stalk

membrane (membrane) and the *zonula adherens* (yellow), which now run perpendicular to the surface and parallel to baso-lateral Na^+/K^+-staining (green) (compare with Fig. 6B). Our data suggest that ocellar PRCs (bottom) expand their apical surface, probably in an asymmetric way, leaving the position of the adhesion belt unaffected. Therefore, only stalk membrane staining (red) from one side runs parallel to the rhabdomere (blue), while ZA markers (yellow) and Na^+/K^+-staining (green) are localised proximal to the rhabdomere (compare with Fig. 5C', 6C').

Figure 1

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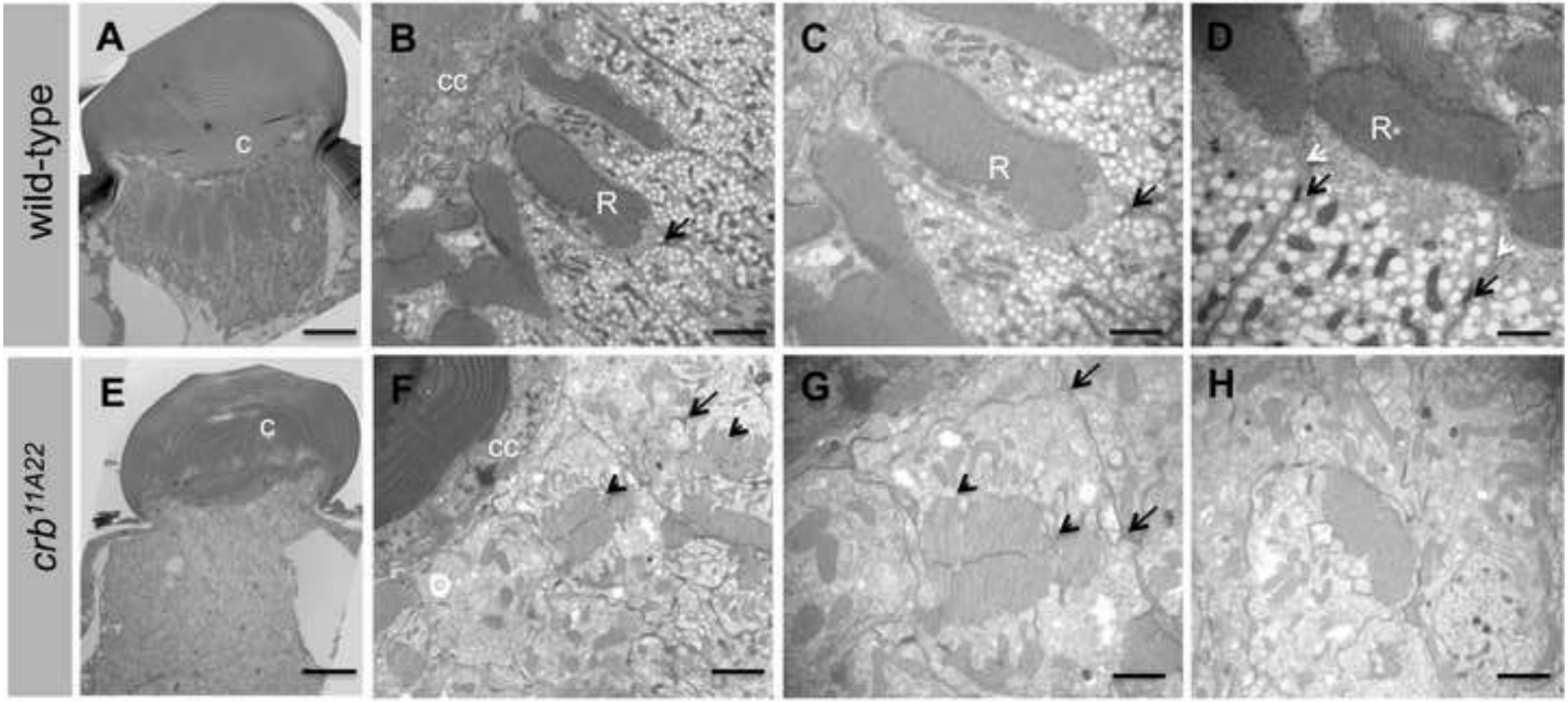

Mishra et al., Fig. 1

Figure 2

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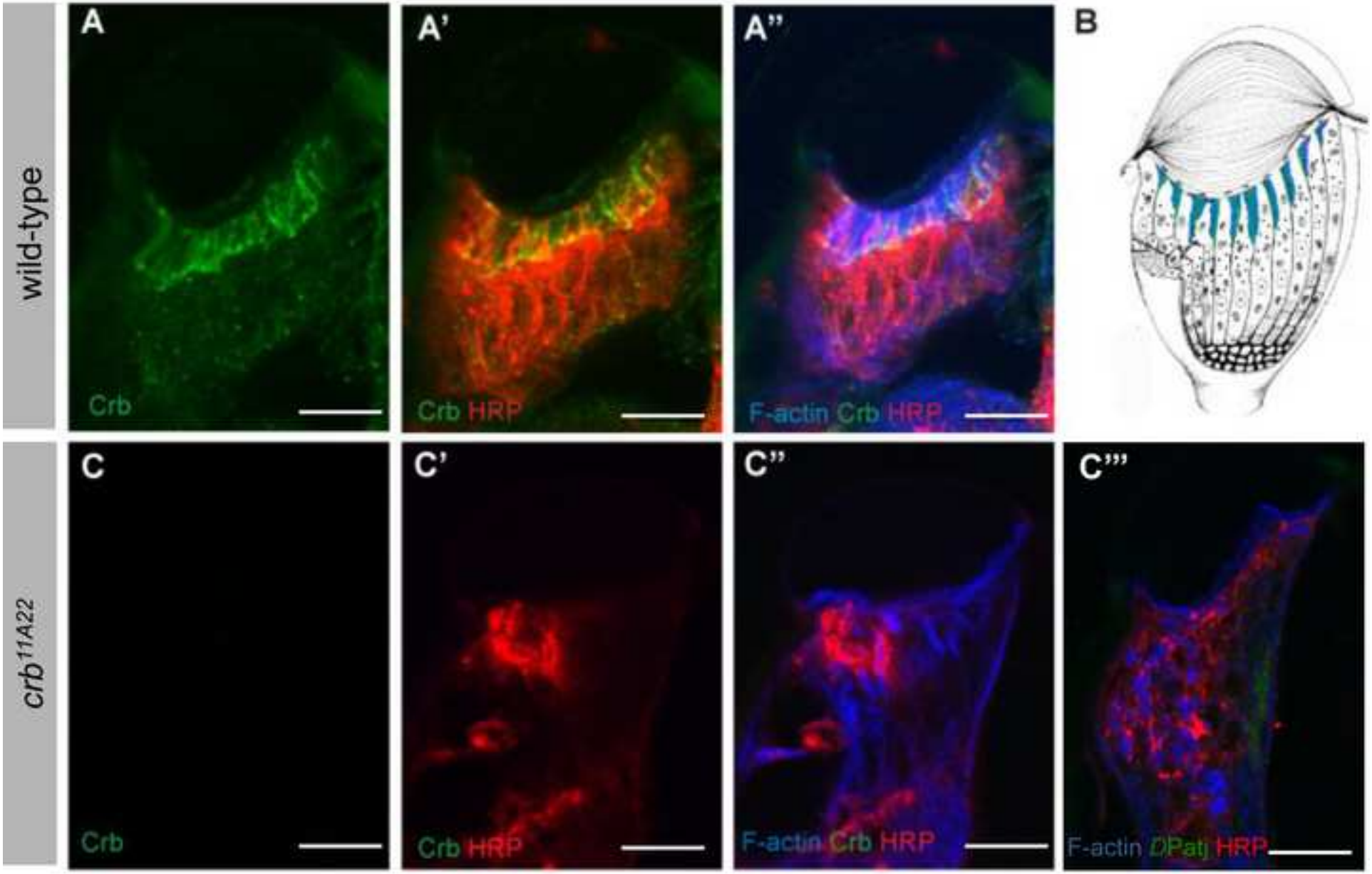

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Figure 3

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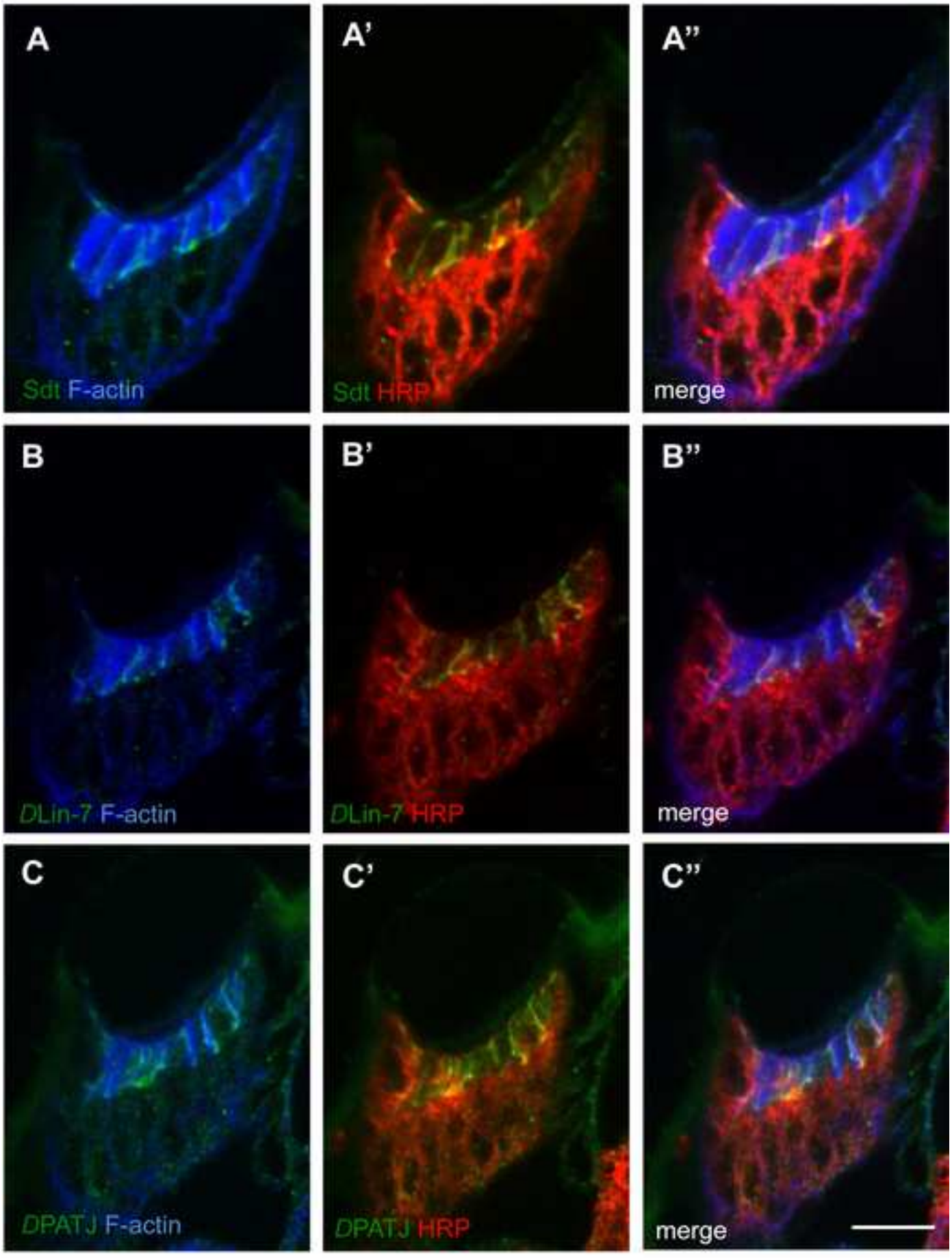

Mishra et al., Fig. 3

Figure 4

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Figure 5

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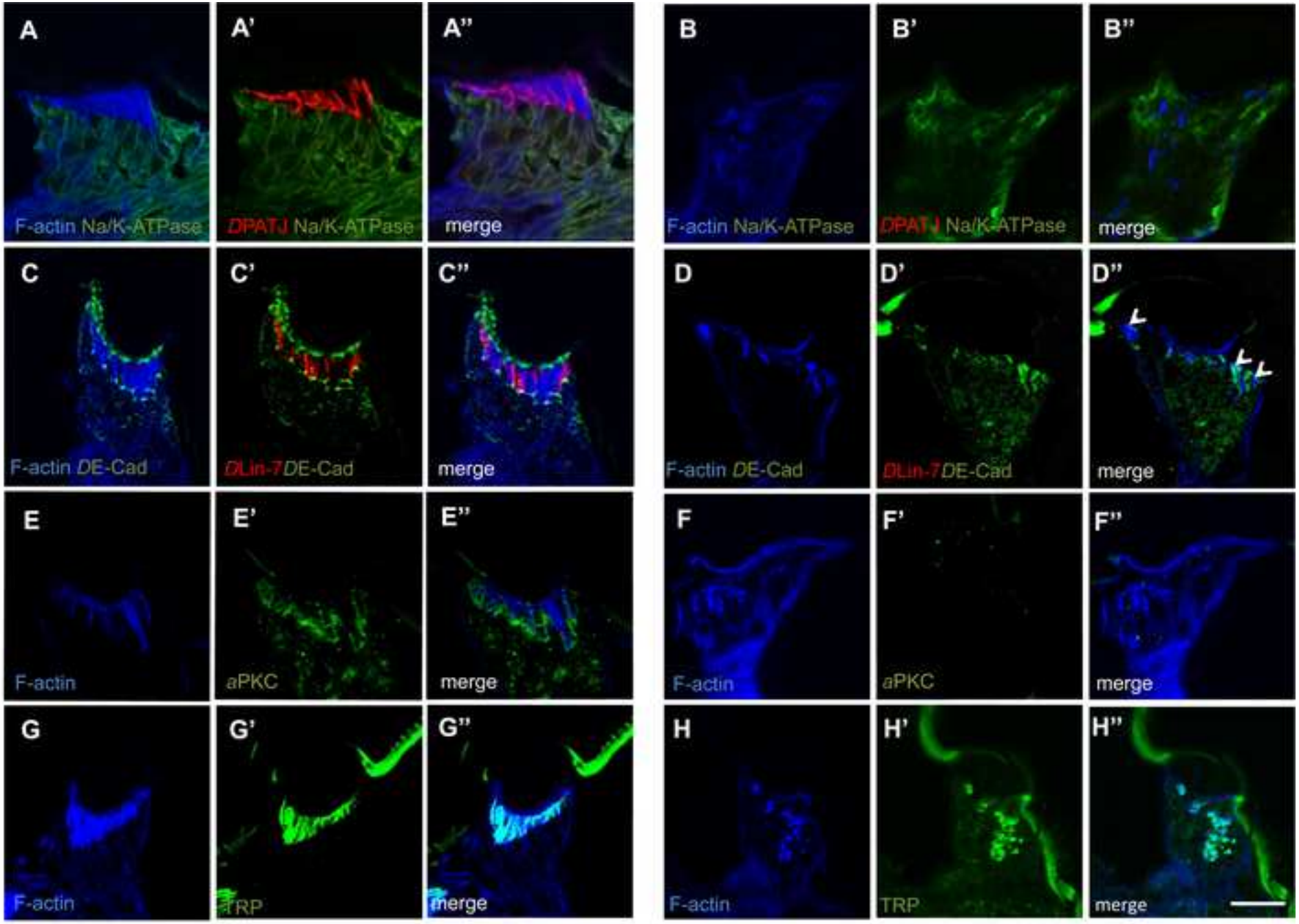

Mishra et al., Fig. 5

Figure 6

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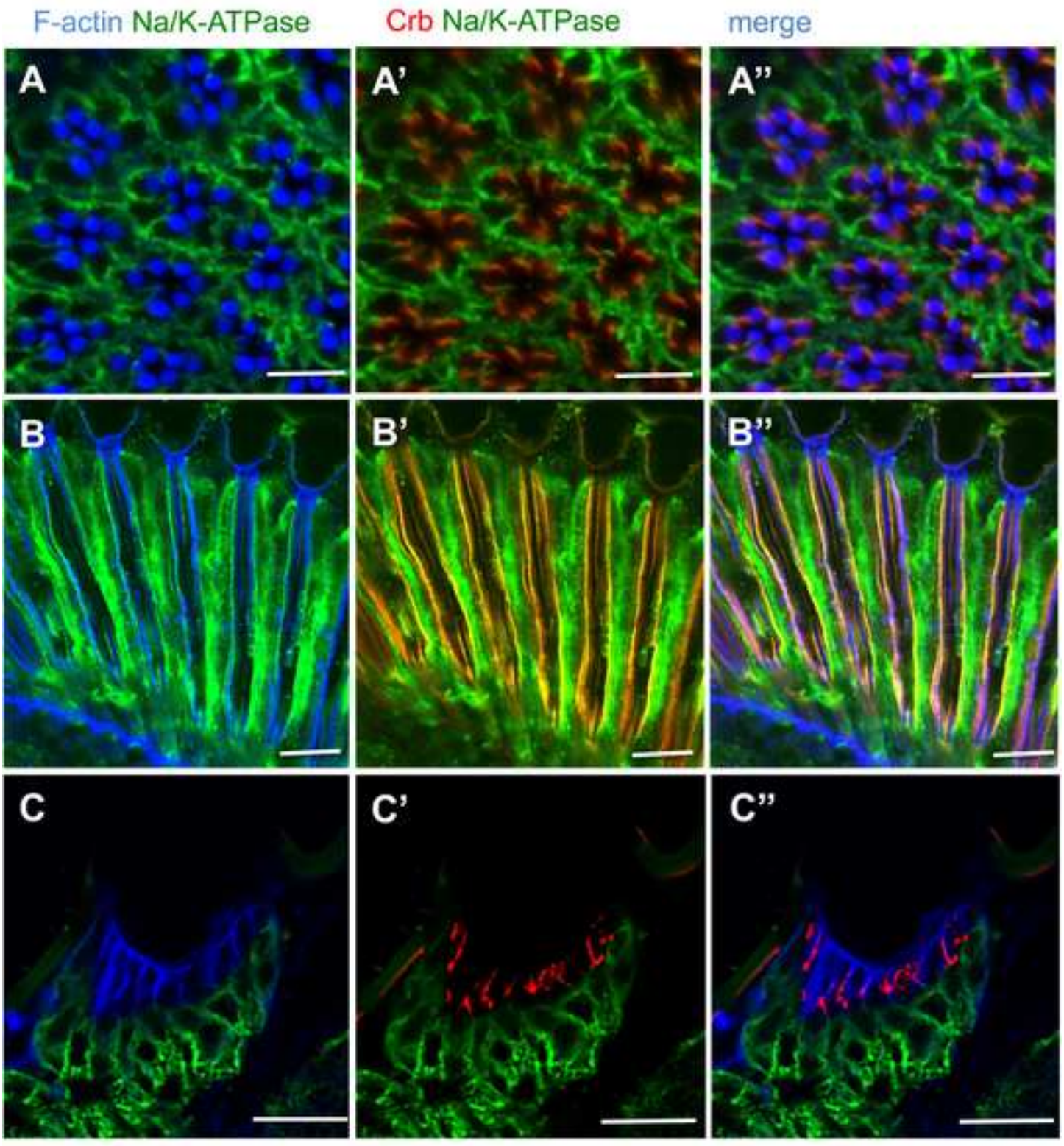

Mishra et al., Fig. 6

Figure 7

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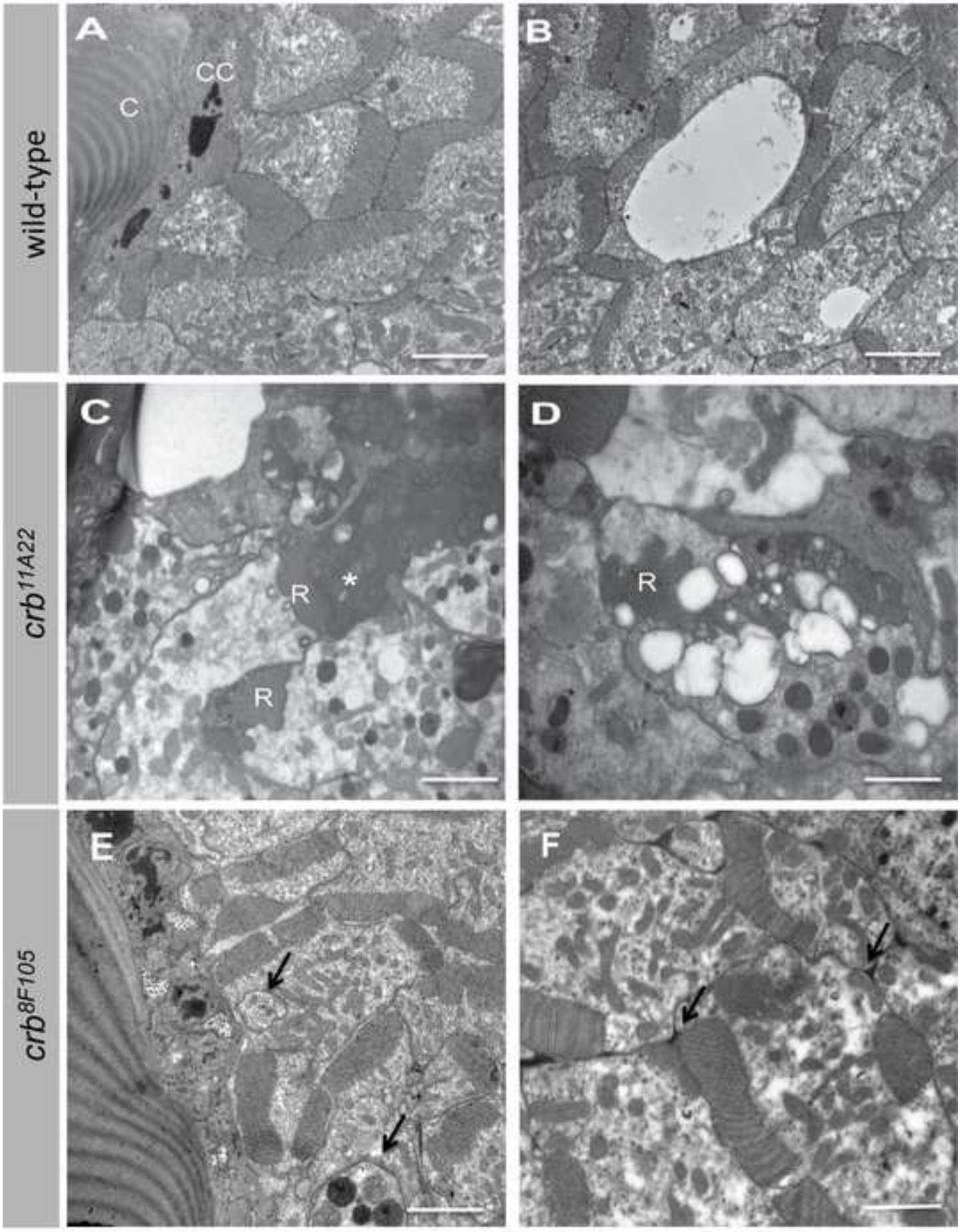

Mishra et al., Fig. 6

Figure 8

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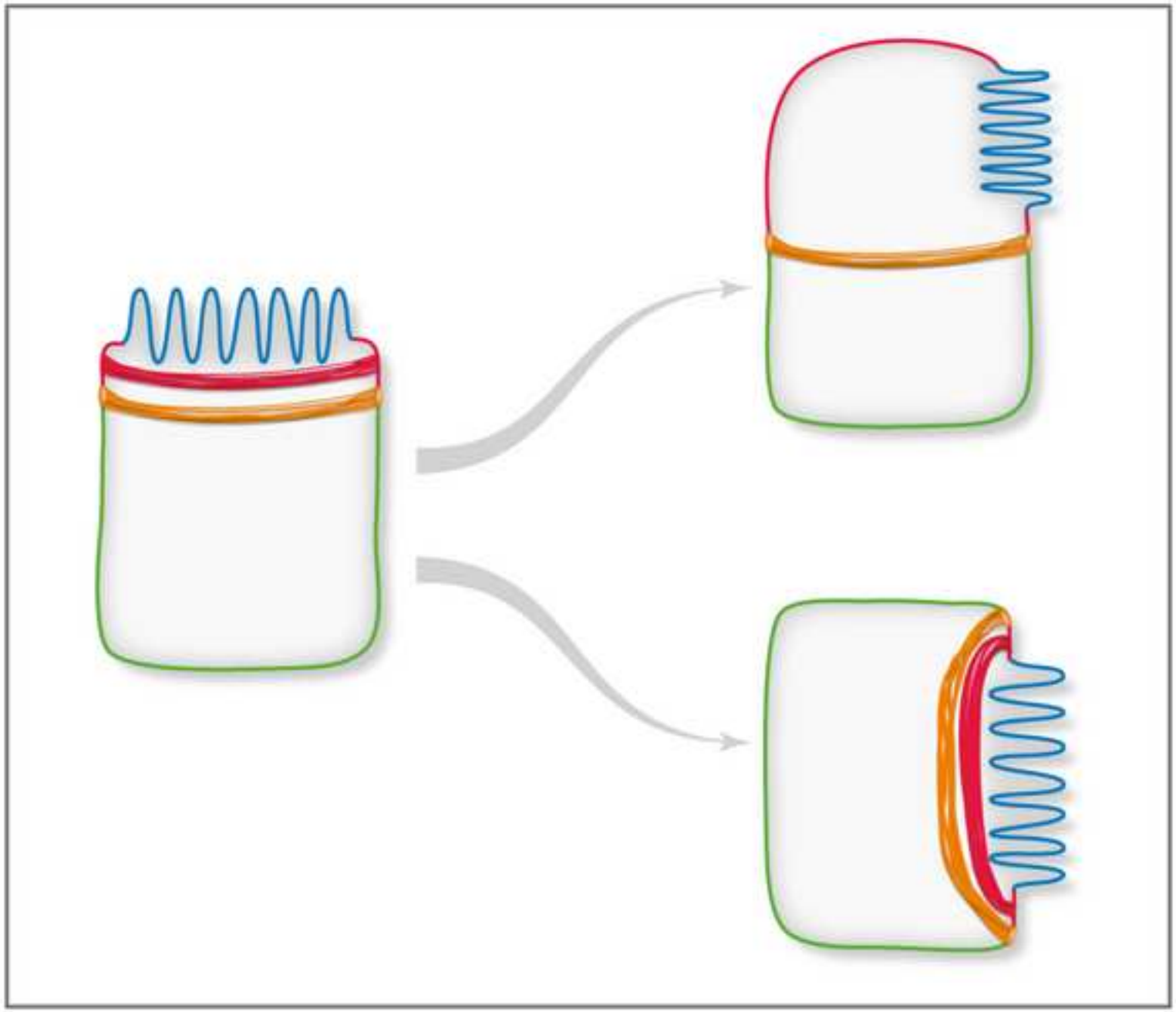

Mishra et al., Fig. 8