Crumbs regulates rhodopsin transport by interacting with and stabilizing myosin V

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The evolutionarily conserved Crumbs (Crb) complex is crucial for photoreceptor morphogenesis and homeostasis. Loss of Crb results in light-dependent retinal degeneration, which is prevented by feeding mutant flies carotenoid-deficient medium. This suggests a defect in rhodopsin 1 (Rh1) processing, transport, and/or signaling, causing degeneration; however, the molecular mechanism of this remained elusive. In this paper, we show that myosin V (MyoV) coimmunoprecipitated with the Crb complex and that loss of crb led to severe reduction in MyoV levels, which could be rescued by proteasomal inhibition.

Introduction

The transmembrane protein Crumbs (Crb) plays a crucial role in regulating photoreceptor cell (PRC) morphogenesis (Izaddoost et al., 2002; Pellikka et al., 2002) and in protecting PRCs from light-dependent degeneration (Johnson et al., 2002), the latter function being conserved between all core members of the Crb complex. Crb was first identified as an apical determinant in Drosophila melanogaster embryonic epithelia, where it is required for the maintenance of apicobasal polarity (Tepass et al., 1990; Wodarz et al., 1993, 1995; Tepass and Knust, 1993; Grawe et al., 1996; Tepass, 1996). The highly conserved intracellular domain of Crb recruits a core plasma membrane–associated protein scaffolding complex composed of the membrane-associated guanylate kinase protein Stardust (Sdt) and the PDZ domain–containing proteins DPatJ and DLin7 (Bulgakova and Knust, 2009). In several tissues, stabilization and localization of all four components of the Crb complex members are interdependent (Richard et al., 2006a; Bachmann et al., 2008); for example, in adult sdt mutant PRCs, Crb protein levels are dramatically reduced, and DPatJ and DLin7 are mislocalized (Bulgakova et al., 2008).

The role of Crb in the retina is evolutionarily conserved, as mutations in the human Crb homologue CRB1 result in retinitis pigmentosa and Leber’s congenital amaurosis, both inherited retinopathies characterized by degeneration of PRCs and the gradual loss of vision (Richard et al., 2006b; den Hollander et al., 2008). Initial studies in flies showed that feeding larvae and flies a vitamin A (carotenoid)–depleted medium prevented the light-dependent degeneration of crb (Johnson et al., 2002), sdt (Berger et al., 2007), and DLin7 mutant PRCs (Bachmann et al., 2008). In the absence of vitamin A, the levels of rhodopsin 1 (Rh1), the key light-sensing pigment in photoreceptors, is reduced by ∼97% (Nichols and Pak, 1985). These experiments indicated that degeneration in these mutants is somehow Rh1 dependent, but the molecular mechanisms were not known.

Rh1 is a crucial component of the Drosophila phototransduction cascade (Borst, 2009), and there is a vast body of literature documenting the degeneration that occurs upon disruption of its synthesis or maturation (Kumar and Ready, 1995; Rosenbaum et al., 2006; Wang and Montell, 2007; Griciuc et al., 2010;
Wang et al., 2010), light-dependent internalization (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Wang and Montell, 2007; Griciuc et al., 2010), or degradation (Chinchore et al., 2009). One major conclusion of all of these studies is that PRCs are exquisitely sensitive to perturbations in Rh1 and that any such impairment leads to retinal degeneration. Indeed, the pivotal role of Rh1 homeostasis in maintaining retinal integrity is also conserved in humans, as mutations in Rh1 alone account for >25% of autosomal dominant retinitis pigmentosa cases (Kennan et al., 2005).

To perform its function in the phototransduction cascade, mature Rh1 needs to be transported to the rhabdome, the microvilli-based light-sensing organelle of the fly, analogous to the vertebrate photoreceptor outer segment. One of the proteins known to be crucial for this transport step is the actin-dependent motor protein myosin V (MyoV), which, in conjunction with Rab11 and dRip11, mediates the post-Golgi transport of Rh1 to the rhabdome (Satoh et al., 2005; Li et al., 2007). In the absence of any of these proteins, Rh1 is retained within the cell body, and very little is seen entering the rhabdome. MyoV is a member of the unconventional myosin family, which, unlike the conventional myosins, do not participate in filament formation and contractile force generation (Woolner and Bement, 2009). Instead, the unconventional myosins use their F-actin binding ability to transport organelles and secretory granules along F-actin tracks (for example, pigment granules in Xenopus laevis melanophores by myosin 5; Rodionov et al., 1998; Rogers and Gelfand, 1998). In addition, the unconventional myosins have recently been shown to be involved in a range of activities such as dynamic membrane tethering of endosomes and membrane-associated proteins, the organization of microtubule and actin-based structures, and the retrograde flow of F-actin in filopodia, microvilli, and stereocilia (Woolner and Bement, 2009).

Early studies in Drosophila embryos identified key domains in Crb that are vital for its function. The intracellular domain of Crb is crucial for its role in maintaining embryonic epithelial polarity, as a transgene encoding a truncated Crb protein lacking the extracellular domain is sufficient to suppress the embryonic crb mutant phenotypes to the same extent as full-length Crb (Wodarz et al., 1995). Interestingly, the ability of the truncated transgene to rescue requires the PDZ-interacting motif present at the very C terminus of Crb (Klebes and Knust, 2000). Therefore, the function of Crb in the embryo is dependent on its ability to interact with the cytosolic components of the Crb complex and is independent of the extracellular domain. The case is somewhat more complex in the eye, as two different mutant phenotypes are observed. As for embryonic epithelia, to rescue the morphological defects observed in the crb mutant retinas, the extracellular domain appears dispensable (Richard et al., 2009). On the contrary, rescue of the light-dependent degeneration observed in the absence of Crb requires the extracellular domain (Johnson et al., 2002). Indeed, the importance of the extracellular domain in preventing retinal degeneration seems to be conserved, as the vast majority of the mapped CRB1 mutations (including amino acid substitutions and in-frame deletions) that lead to retinopathies lie within the extracellular domain (den Hollander et al., 2004). These studies show that Crb performs its different functions through different domains and therefore most likely through different molecular mechanisms.

**Results**

**MyoV interacts with the Crb complex**

We reasoned that identification of novel interaction partners of Crb and the Crb complex would provide clues to understand the molecular mechanisms behind the light-dependent degeneration that occurs in the absence of any member of the Crb complex. To identify novel interactors, we used antibodies raised against different members of the Crb complex in an attempt to immunoprecipitate (IP) the entire complex and proteins associated with it. When Drosophila head lysate was incubated with antibodies raised against DPatJ or Sdt, we were able to coIP the other members of the complex (Fig. 1 A). Mass spectroscopic analysis of both IPs and a negative control IP using normal rabbit IgG confirmed that all Crb complex members were present in Sdt and DPatJ IPs, demonstrating the specificity of the IPs (Fig. 1 B). In addition, several putative interactors were coprecipitated. One potential interactor with an established role in the retina was the unconventional myosin MyoV. The mass spectroscopy analysis was verified by Western blotting (WB; Fig. 1 A), which confirmed that MyoV specifically coIPs with both Sdt and DPatJ, thus demonstrating that the Crb complex can interact with MyoV.

**MyoV is dramatically reduced and partially mislocalized in Crb mutant retinas**

To investigate the effect that loss of the Crb complex might have on MyoV, we induced the formation of mosaic eyes containing large crb mutant clones, using the functionally null mutant crb1IA22. Analysis of the protein levels of these adult retinas by WB revealed a marked reduction of MyoV protein in the absence of Crb protein (Fig. 1 C). Loss of MyoV protein was reproducible, and quantification by densitometry indicated that only ~10% of MyoV protein remains in crb1IA22 retinas when compared with wild-type (WT) levels (Fig. 1 D). These data suggest that Crb is required for the stabilization of MyoV in PRCs.

Consistent with published data (Li et al., 2007), Crb still localizes to the stalk membrane in MyoV mutant tissue (Fig. S1 A); therefore, MyoV is not required to transport Crb or members of the Crb complex to the apical membrane. A previous study into the role of MyoV in Drosophila PRCs showed that endogenous MyoV localizes to the rhabdome base, an area abutting the stalk membrane and previously identified as the site of the rhabdome terminal web, an actin-rich structure that protrudes from the rhabdome into the cell body (Fig. 1 E; Li et al., 2007; Xia and Ready, 2011). To identify the localization of the remaining MyoV in crb mutant photoreceptors, we generated small clones of crb1IA22 in the retina, allowing us to image mutant and WT tissue adjacent to one another. Staining such retinal sections (from 2–4-d-old adult flies) for MyoV confirms what we observe in the Western blot data, as very little signal can be detected in the mutant tissue (Fig. 2 A). The remaining MyoV localizes to the rhabdome base and sometimes to the stalk membrane (Fig. 2 A, arrowheads), the latter being seldom, if ever, observed in WT tissue.
The actin terminal web is thought to provide the tracks along which MyoV transports its Rh1 payload. Therefore, we tested the integrity of this structure in crb mutant tissue to ensure that the loss of MyoV we observed is not a secondary effect caused by a loss of the actin terminal web. To test this, we expressed the F-actin-binding domain of moesin in a crb11A22 small clone background (Fig. S2). Despite the mosaic expression of the transgene, using this method, we could show that the F-actin tracks at the base of the rhabdomere were present in both WT and crb11A22 photoreceptors (Fig. S2, arrows). Therefore, we are confident that the loss of MyoV in crb11A22 tissue is not a result of morphological defects in the rhabdomere terminal web.

The finding that the residual MyoV is partially mislocalized led us to ask whether the Crb complex is required not only for protein stability but also for restricting MyoV to the rhabdomere base, preventing it from spreading to the stalk region of the apical membrane. To investigate this, we overexpressed a GFP-tagged MyoV (generated previously and shown to be functional; Krauss et al., 2009) in a crb11A22 small clone background. Overexpression of upstream activating sequence (UAS)–MyoV-GFP using Rhl-Gal4 drives expression of the transgene in the outer photoreceptors (R1–6) only. Most interestingly, staining for GFP (to detect the transgene-encoded protein only) or for MyoV (to detect both transgene-encoded and endogenous protein) showed that in otherwise WT PRCs, MyoV-GFP was well expressed (compare cells marked with arrows in Fig. 2 A with Fig. 2 C). However, in crb11A22 clones, the levels of MyoV-GFP and endogenous MyoV were dramatically reduced (Fig. 2, B and C). This reduction is similar to that seen when crb mutant eyes are stained for endogenous MyoV (Fig. 2 A). This suggests that the mechanism by which the Crb complex is controlling MyoV stability is tight enough to reduce MyoV protein levels even upon overexpression. In addition, these data rule out the possibility that Crb is directly controlling MyoV gene expression, as the MyoV-GFP is expressed under the control of an exogenous promoter.

**MyoV is degraded by the proteasome in crb mutant photoreceptors**

To investigate the cause of MyoV loss observed in crb mutant photoreceptors, we aimed to prevent the loss of MyoV by overexpression of a dominant-negative proteasome subunit Pros26I2B (Belote and Fortier, 2002). This resulted in a marked increase in MyoV staining (compare Fig. 2 A with Fig. 2 D). Quantification of MyoV fluorescence (normalized to WT MyoV fluorescence per ommatidium, as described in Materials and methods) showed that the reduction of MyoV seen in crb mutant photoreceptors is rescued to ~80% of that seen in WT ommatidia upon proteasome inhibition in comparison with ~50% in the absence of proteasomal inhibition (Fig. 2 E). These data suggest that crb stabilizes MyoV protein by protecting it from degradation by the proteasome.

In addition, from this experiment, we can assess the localization of the rescued MyoV in the absence of Crb. Although MyoV does accumulate apically, it does not adopt the regular crescent shape seen in WT tissue and appears in large clumps rather than localizing to the entire rhabdomere base (Fig. 2 D).

Therefore, it appears that Crb is not directly responsible for the apical localization of MyoV.

**MyoV fails to accumulate apically in crb mutant late pupal photoreceptors**

To further analyze the functional interaction between MyoV and Crb, we studied earlier stages of retinal development (schematically represented in Fig. 3 A). At early pupal stages, around 30–40% pupal development, we observed that MyoV distributed evenly throughout the cell, not accumulating at any particular
small extent (Fig. 3 C, arrows). In crb11A22 mutant photoreceptors, the apical accumulation is dramatically reduced (Fig. 3 C). From these data, we conclude that at early pupal stages, MyoV is independent of Crb, whereas at later stages, Crb is required for MyoV apical accumulation.

crb8F105 mutants partially maintain MyoV levels and localization

To further investigate the interaction between the Crb complex and MyoV, we analyzed a weaker crb allele, crbBF105, which contains a stop in the cytoplasmic domain and results in the production of a truncated protein lacking the C-terminal 23 subcellular location (Fig. 3 B). This MyoV distribution is unperturbed in early pupal crb11A22 mutant PRCs (Fig. 3 B, circled areas), suggesting that at early stages, before rhabdomere elongation, MyoV localization and levels are not Crb dependent. Expression of Rh1 starts at ~70% pupal development (Satoh et al., 2005); therefore, if the steady-state localization of MyoV at the rhabdomere base correlates with its role in Rh1 transport, such localization would only develop at late pupal stages. Indeed, in pharate adults (around 80–90% pupal development), MyoV starts to accumulate apically (Fig. 3 C). Interestingly, at this stage, MyoV decorates not only the rhabdomere base but is also seen at the stalk membrane, colocalizing with Crb to a small extent (Fig. 3 C, arrows). In crb11A22 mutant photoreceptors, the apical accumulation is dramatically reduced (Fig. 3 C). From these data, we conclude that at early pupal stages, MyoV is independent of Crb, whereas at later stages, Crb is required for MyoV apical accumulation.

**Fig. 2.** MyoV is reduced and mislocalized in Crb mutant photoreceptors. (A) Section through an adult retina harboring clones of crb11A22 tissue (identified by loss of Crb staining) stained for Crb, MyoV, and F-actin (to mark rhabdomeres). Arrowheads identify MyoV staining at the stalk membrane. White boxes are shown at higher magnifications in the bottom row. The arrow highlights WT MyoV staining to be compared with that in B. (B) Section through an adult retina harboring clones of crb11A22 (indicated by asterisks) and expressing MyoV:GFP under the control of the Rh1 driver (expressed in outer photoreceptors only). Stained for GFP, Crb, and F-actin (to mark rhabdomeres). The arrow highlights overexpression of MyoV, including increased cytoplasmic staining when compared with that in A. (C) Section through an adult retina harboring clones of crb11A22 (indicated by asterisks) and expressing MyoV:GFP under the control of an Rh1 driver (expressed in outer photoreceptors only). Stained for MyoV, Crb, and F-actin (to mark rhabdomeres). (D) Section through an adult retina harboring clones of crb11A22 (indicated by asterisks) and expressing Pros26'2B under the control of an Rh1 driver (expressed in outer photoreceptors only). Stained for MyoV, Crb, and F-actin (to mark rhabdomeres). Boxed areas are enlarged in the insets. [A–D] Bars, 5 µm. (E) Quantification of MyoV fluorescence WT and of crb11A22 ommatidia in the presence of absence of Pros26'2B expression. n indicates the number of ommatidia analyzed; error bars show SD (for more details, see Materials and methods). a.u., arbitrary unit.
Expression of Crb in S2R+ cells in the absence of Sdt recruits MyoV-GFP to the plasma membrane

To further investigate the possibility that Crb itself can interact with MyoV, we took advantage of the Drosophila S2R+ cell line that does not express endogenous crb or sdt (Fig. S4 A). This allowed us to express MyoV-GFP and without Crb in the system. Importantly, Crb expression in S2R+ cells does not result in the expression of Sdt (Fig. S4 B). When expressed alone, MyoV-GFP localizes to puncta distributed evenly throughout the cell (Fig. 4 C). Strikingly, upon coexpression with Crb, MyoV-GFP is recruited to the plasma membrane, where it colocalizes with Crb (Fig. 4, D and E). The ability of Crb to recruit MyoV-GFP to the plasma membrane in the absence of Sdt strongly argues that the interaction with MyoV is not depended on Crb acting as part of the Crb complex but rather on Crb itself.

The recruitment of MyoV-GFP to the plasma membrane in S2R+ cells requires only the membrane-spanning region and first 14 amino acids of the intracellular domain of Crb

To further characterize the interaction between Crb and MyoV, we used the S2R+ system to express MyoV-GFP with various Crb truncations. First, to confirm the data acquired using the amino acids and, thus, the PDZ-binding motif (Wodarz et al., 1993). This truncated protein localizes to the stalk membrane and ectopically to the outermost membranes of the rhabdomere (Fig. S3 B). crb8F105 mutants photoreceptors display morphological defects, which are slightly less severe than those seen in crb11A22 mutants. Interestingly, despite the absence of the Sdt-binding ERLI motif in the truncated protein, crb8F105 mutant photoreceptors retain low levels of Sdt protein, which is, however, mislocalized (Fig. S3, A and B). Most importantly, crb8F105 mutant photoreceptors do not undergo light-dependent degeneration (Johnson et al., 2002). The localization of MyoV in crb8F105 mutant photoreceptors is similar to that seen in WT tissue (Fig. 4 A), showing enrichment at the rhabdomere base (Fig. 4 A, arrows) and also an accumulation in cytoplasmic puncta within the cell body. This partial maintenance of MyoV localization in crb8F105 mutant PRCs is concomitant with increased MyoV protein in crb8F105 mutants compared with crb11A22 mutants (Fig. 4 B) but still reduced levels compared with WT PRCs. The latter could be explained by the fact that truncated Crb protein is expressed to a slightly lower level than the WT Crb protein (Fig. 4 B). These data suggest that the stability of MyoV does not depend on an intact Crb complex; however, the presence of residual levels of Sdt in the crb8F105 mutants leaves open the possibility that Sdt is able to stabilize MyoV in these photoreceptors, despite being mislocalized.
**In Drosophila PRCs, Crb overexpression does not lead to the mislocalization of MyoV**

Although the results from the S2R+ experiments allowed us to narrow down the portion of Crb required for interaction with MyoV, they also showed that in this system, Crb could ectopically recruit MyoV to the plasma membrane. Despite the extremely simplified nature of the S2R+ system in comparison with PRCs and the fact that in crb mutant photoreceptors, the residual MyoV localization is predominantly WT, we tested whether or not Crb is capable of performing the same function in PRCs. Using Rh1-Gal4, we expressed either full-length Crb (Crb\(^{FL}\)) or a construct of Crb in which the extracellular domain of Crb has been replaced by a Flag tag (Crb\(^{FL\_tag}\)). Although both of these transgene-encoded proteins localize ectopically to the rhabdomere base and basolateral membranes, as previously described (Richard et al., 2009), neither is able to recruit MyoV to these sites (Fig. 5, E and F). Therefore, we conclude that the S2R+ experiments can be used only as a basic method to analyze the domains for the Crb–MyoV interaction and that the physiologically relevant role of this interaction in the adult photoreceptor is not one of recruitment/localization but rather of stabilization.

**Rhodopsin transport is defective in crb mutant PRCs**

As previously shown (Li et al., 2007), MyoV mutants exhibit defects in Rh1 transport to the rhabdomere (Fig. S1 A). Interestingly, this phenotype depended heavily on the MyoV allele used. Severe defects in Rh1 transport were only seen with null mutations, whereas hypomorphic alleles displayed normal steady-state Rh1 staining (Li et al., 2007). The authors concluded from this that only minimal MyoV activity is sufficient for Rh1 transport.

Therefore, we tested the localization of Rh1 in eyes containing crb\(^{11A22}\) small clones. The WT steady-state localization of Rh1 is light dependent; in the dark, it fills the entire rhabdomere, whereas upon light exposure, Rh1 is restricted to a crescent shape at the lower half of the rhabdomere (Satoh and Ready, 2005). Despite the enlarged rhabdomeres seen in crb\(^{11A22}\) photoreceptors, the normal crescent of Rh1 is still detectable (Fig. 6 A, arrows), suggesting that the residual MyoV present in crb\(^{11A22}\) cells is sufficient to transport Rh1. As staining of Rh1 in very young adult flies represents the steady state after very little exposure to the night/day cycling, we left crb\(^{11A22}\) mosaic–eyed flies in normal night/day conditions for 20 d posteclosion (dpe), reasoning that subtle defects in Rh1 transport might accumulate over time. In crb\(^{11A22}\) photoreceptors kept under these conditions, Rh1 staining shows an accumulation in large punctae in the cytoplasm (Fig. 6 B, arrowheads), which is seldom observed in neighboring WT tissue. The accumulation of Rh1 within PRC bodies is closely linked to degeneration (Satoh and Ready, 2005; Chinchore et al., 2009). Therefore, we tested whether
crb1A22 mutants show signs of degeneration at 20 dpe under normal night/day conditions. Electron micrographs of crb1A22 mutant PRCs at 3 dpe show no defect in the ultrastructure of the rhabdomeres; microvilli are intact and tightly packed, as in neighboring WT cells (Fig. 6 C). At 20 dpe, however, nearly all crb1A22 mutant ommatidia contain rhabdomeres that exhibit features of disintegration (Fig. 6 D); packing of microvilli is not as tight as in neighboring WT cells, and there is an increased loss of microvillar material into the interrhabdomeral space, which is rarely seen in WT rhabdomeres (Fig. 6 D) nor in w0 mutants (Fig. S1 B). Together, these data indicate that crb mutant photoreceptors display subtle defects in Rh1 localization and, over time, start displaying signs of degeneration.

The accumulation of Rh1 within the cell body of 20-dpe crb1A22 mutant photoreceptors might be caused by defects in synthesis, transport to the rhabdomere, or recycling. As MyoV was shown to be required for the post-Golgi transport of Rh1 to the rhabdomere, we tested the role of crb in the movement of Rh1 through the secretory pathway using a pulse-chase assay that takes advantage of the highly complex Rh1 biogenesis (Satoh et al., 1997). Flies raised on carotenoid-free media synthesize little or no Rh1 protein, as the chromophore is absent. Feeding these animals with all-trans-retinal and maintaining them in the dark results in the production of Rh1 protein containing all-trans-retinal, which is retained within the ER. Exposure of these flies to a pulse of blue light isomerizes the chromophore to 11-cis-retinal, which allows Rh1 to be transported through the secretory pathway and into the rhabdomere (depicted schematically in Fig. 7 A; Satoh et al., 1997). Performing this assay on flies harboring crb1A22 retinal clones shows that at early time points 20 and 40 min after blue light pulse, there is little difference in the transport between the mutant and WT tissue (Fig. 7, B and C).

Figure 5. The interaction between Crb and MyoV requires only the transmembrane-spanning and first 14 amino acids of the cytoplasmic domain. (A and B) S2R+ cells transfected with MyoV-GFP and Crb8F105 and plated on concanavalin A. A cell expressing MyoV-GFP only is shown in A as a control. (C and D) S2R+ cells coexpressing MyoV-GFP and Crb intracytosolic (C) or Crb intramembrane (D) and plated on concanavalin A, showing some recruitment of MyoV-GFP to the plasma membrane, where it colocalizes with Crb intracytosolic. (E and F) Sections through adult retinas expressing Crb intracytosolic (E) or Crb intramembrane (F) under the control of the Rh1 promoter, stained for MyoV, F-actin, and Crb (E) or Flag (F). Bars, 5 µm.
However, at later stages 60, 80, and 120 min after pulse, there is a clear delay in transport to the rhabdomere in crb11A22 cells, with Rh1 remaining within the cell body of mutant photoreceptors when most has already reached the rhabdomere of WT cells (Fig. 6, B and C). This is consistent with the role of MyoV in post-Golgi transport of Rh1 to the rhabdomere, as early time points during which Rh1 is transported from ER to Golgi appear unaffected. Thus, we conclude that in the absence of crb, transport of Rh1 to the rhabdomere is delayed, and we propose that this is a result of the reduction of MyoV in these photoreceptors.

**Discussion**

The role of the Crb complex in polarity is well studied, but the mechanism behind its ability to prevent light-dependent retinal degeneration is poorly understood. Some insight into the latter came from studies reporting that feeding flies a vitamin A (carotenoid)–depleted medium prevented the light-dependent degeneration of crb, (Johnson et al., 2002), sdt (Berger et al., 2007), and DLin7 mutant PRCs (Bachmann et al., 2008). These data suggested that degeneration in Crb complex mutants involves Rh1; however, the molecular mechanism behind this remained unknown. Here, we provide the missing link by showing that the Crb complex interacts with MyoV, an unconventional myosin, which has an established role in the transport of Rh1 to the rhabdomere. We show that MyoV levels are reduced by ~90% in crb mutant retinas, which can be largely rescued by inhibition of the proteasome, and that Rh1 transport is defective in crb mutant PRCs. Therefore, we propose that the Crb complex protects against light-dependent degeneration by interacting with and maintaining MyoV levels, thereby ensuring proper Rh1 transport to the rhabdomere.

Blocking proteasome activity also allowed us to assess the localization of MyoV in the absence of Crb. We observed apical localization of MyoV; however, rather than adopting the WT localization that spans the entire rhabdomere base, the rescued MyoV was seen in large clumps, which only partially covered the base of the rhabdomere. The steady-state WT localization of MyoV reflects its role in transporting Rh1 from the cell body to the rhabdomere base. Therefore, these large accumulations may suggest that some level of MyoV degradation is also important for maintaining efficient transport by the total pool of MyoV. Thus, the levels of MyoV and its ability to transport Rh1 to the rhabdomere base may depend on external cues (e.g., light), which alter the balance between stabilization and degradation.

We show that IPs of both Sdt and DPATd contain the respective other members of the Crb complex and that together with these, MyoV is precipitated specifically. The strong reduction in MyoV protein we see in crb mutant photoreceptors raises the question of whether stability of MyoV is dependent on Crb itself or on the integrity of the Crb complex. As loss of Crb results in the loss of Sdt (Fig. S3; Bachmann et al., 2008) and the delocalization of DPATd and DLin7 (Richard et al., 2006a; Bachmann et al., 2008), the data obtained using crb11A22 mutants can be used to analyze the role of the Crb complex. Data obtained from crbcrf105 mutants, however, show that the integrity of the Crb complex is not required for the crb-dependent stabilization of MyoV. This was further supported by experiments in S2R+ cells that showed Crb alone, in the absence of Sdt, can recruit MyoV-GFP to the plasma membrane, suggesting that the interaction we observe between Crb and MyoV is not mediated by any of the other core components of the Crb complex. As we detected the interaction by IP, the possibility remains that the interaction between Crb and MyoV is mediated by another still unknown protein.

Interestingly, loss of MyoV in crb11A22 mutants cannot be overcome by overexpression of a MyoV transgene, which is expressed under the control of an exogenous system, the UAS/Gal4 system. This demonstrates that Crb is required to maintain MyoV stability posttranscriptionally. We investigated this further by inhibiting proteasomal degradation and observed a marked increase of MyoV staining in crb mutant photoreceptors compared with controls. These findings support our previous conclusion that the
interaction between Crb and MyoV is stabilizing the latter by protecting it from degradation by the proteasome.

crb is known to have two main functions in the eye, one during development of the retina to ensure correct morphogenesis of the PRCs (Izaddoost et al., 2002; Pellikka et al., 2002) and the other to prevent degeneration of the adult eye in constant light (Johnson et al., 2002). Here, we show that MyoV does not show a polarized distribution at early pupal stages nor is its localization perturbed by loss of Crb in early stages, the time at which morphogenetic defects in crb mutants start (Izaddoost et al., 2002; Pellikka et al., 2002), suggesting that the interaction between Crb and MyoV is not required for proper morphogenesis to occur. This is supported by reports that MyoV-null mutant adults display only mild morphological defects, which are distinct from those observed in crb mutants (Li et al., 2007; Satoh et al., 2008).

The finding that MyoV fails to start accumulating apically in crb mutant cells during late pupal stages after Rh1 expression starts corroborates the conclusion that the Crb–MyoV interaction is required for the second role of Crb in the retina, preventing light-dependent degeneration. It is also plausible that the steady-state localization of MyoV seen in the adult is largely the result of its role in Rh1 transport to the rhabdomere, as MyoV is seen evenly distributed throughout the cell before Rh1 expression starts. This assumption is supported by published data showing that the localization of MyoV in the adult is light dependent (Satoh et al., 2008) and therefore reflects the status of Rh1 activation and transport. Fittingly, the apical accumulation of MyoV at later pupal stages coincides with increased MyoV staining and increased colocalization of MyoV with Crb.

We tested the effect that loss of Crb has on Rh1 and demonstrated that in normal 12-h light/12-h dark conditions, defects in Rh1 staining are only seen in old flies. This is suggestive of a subtle defect in Rh1 transport that is only visible at steady state if allowed to accumulate over time or if the system is under stress (i.e., constant light). As it was reported that only minimal MyoV activity is required for proper Rh1 localization (Li et al., 2007), it is probable that the remaining 10% of MyoV seen in crb mutants is sufficient for Rh1 transport in young flies, but, over time, the effect of this deficiency accumulates, resulting in the retention of Rh1-positive punctae in the cell body. Together with our results from the Rh1 pulse-chase assay, we conclude that in crb mutant tissue, Rh1 transport to the rhabdomere is delayed and that the cumulative effect of this delayed transport leads to the accumulation of Rh1 within the cell body, which is associated with a gradual deterioration of the rhabdomeres.

Figure 7. Crb mutant PRCs display slower Rh1 transport kinetics. (A) Schematic of the Rh1 pulse-chase experiment. Red dots symbolize immature Rh1 containing all-trans-retinal. Blue dots symbolize mature Rh1 containing 11-cis-retinal (for a detailed description, see Results and Materials and methods). (B and C) Rh1 pulse-chase experiment. Sections through adult retinas containing crb11A22 clones from flies that were fed on carotenoid-free medium from egg to adult, given all-trans-retinal for 2 d in the dark, and then pulsed with CFP-filtered light for 10 min and returned to the dark for the times indicated. Sections were stained for Rh1 and F-actin. Borders between WT and crb11A22 (crb) tissue are outlined in white. The structure of the eye is not as well preserved as those in Fig. 5 A because the flies are fixed whole to avoid exposure to light before fixation. Bars, 5 µm.
Previous findings have shown that Crb-mediated protection against light-dependent retinal degeneration is not solely dependent on the ability of Crb to assemble and integrate into the Crb complex (Johnson et al., 2002). Photoreceptors of crb\textsuperscript{8F105} mutants, which express a Crb protein lacking the Sdt-interacting ERLI motif, do not undergo light-dependent degeneration. This observation is in agreement with the findings we present here that MyoV is retained in Crb\textsuperscript{8F105} mutant photoreceptors. In addition, overexpression of a Crb transgene encoding the transmembrane and intracellular domains was not able to rescue the light-dependent degeneration observed in crb\textsuperscript{11A22} mutants (Johnson et al., 2002). Interestingly, this membrane-tethered intracellular domain–encoding transgene does rescue the morphogenetic defects observed in both crb\textsuperscript{8F105} and crb\textsuperscript{11A22} mutants (Richard et al., 2009). Therefore, the two roles of Crb in the retina—photoreceptor morphogenesis and maintenance—appear to occur through distinct mechanisms. Correct morphogenesis seems to necessitate the assembly of the Crb complex through the Crb ERLI motif. In contrast, Crb-mediated protection against light-dependent degeneration and stabilization of MyoV does not require an intact Crb complex. How do these finding correlate with reports of light-dependent retinal degeneration in other members of the Crb complex? We propose that in sdt and DPatT mutants, it is the concomitant loss of Crb that is responsible for the degeneration phenotype rather than the loss of an intact Crb complex itself.

The absence of endogenous Crb and Sdt from S2R+ cells made them particularly useful to identify the regions of Crb required for its interaction with MyoV, which we determined to include the membrane-spanning and first 14 amino acids of the cytoplasmic domain. However, the readout for this interaction—the recruitment of MyoV-GFP to the plasma membrane—may not reflect the purpose of this interaction in vivo, particularly considering the highly polarized and functionally specialized nature of PRC. Indeed, the finding that the majority of the residual MyoV in crb mutant photoreceptors localizes to the rhabdomere base suggests that in photoreceptors, the role of the Crb–MyoV interaction is primarily to stabilize MyoV and not to recruit it to the rhabdomere base. In addition, the ability of Crb lacking the extracellular domain to recruit MyoV to the plasma membrane of S2R+ cells but not to rescue light-dependent degeneration suggests that S2R+ cells lack many qualities (morphology, protein expression, and functionality) of PRCs. Considering the requirement of the extracellular domain (discussed in the Introduction), it is possible that in the context of a light-sensing photoreceptor, Crb responds to a stimulus that is transmitted via the extracellular domain, which then initiates the interaction with and/or the stabilization of MyoV. This hypothesis is an intriguing one, as the only function of Crb that requires its extracellular domain is its role in preventing light-dependent degeneration, and, to date, no known partner of the extracellular domain has been identified.

We propose a model in which the interaction between Crb and MyoV stabilizes the latter, maintaining a complete Rh1 transport cycle. In crb mutants, this cycle is slowed down at the MyoV-dependent stage of delivery to the rhabdomere. Whereas in normal light/dark conditions the effect of this is minimal, upon exposure to constant light, Rh1 accumulates in the cell body, suggesting that the rate of removal from the rhabdomere (as a result of constant activation) exceeds the rate of delivery to the rhabdomere. As previously discussed, photoreceptors are extremely sensitive to perturbations in the phototransduction cascade, and it has been well documented that mutations that affect the synthesis, delivery, and recycling of Rh1 lead to degeneration. Together with previously published data showing the rescue of Crb-dependent retinal degeneration in the absence of vitamin A, this strongly supports our model that the accumulation of Rh1 in the cell body as a result of a deficiency of Rh1 transport in crb mutants leads to degeneration.

These data provide for the first time a molecular mechanism for the light-dependent degeneration observed in crb mutant animals. Recent findings that myoVI\textit{a} mutant mice display light-dependent degeneration as a result of defects in rod protein translocation (Peng et al., 2011) suggest that the efficient transport of opsins by myosins is crucial to prevent degeneration across species. Therefore, it will be intriguing to see whether the mechanism we identified here is conserved and whether human photoreceptors from patients with CRB1 mutations also display reduced myosin levels and delays in Rh1 transport.

Materials and methods

\textit{Drosophila} genetics

Large crb mutant clones were generated by crossing male FRT82B crb\textsuperscript{1422}/TM6B (Wodarz et al., 1995) or FRT82B crb\textsuperscript{105}/TM6B flies to ey\textit{FLP}; FRT82B[C3]\textit{w} virgin. Small clones were generated by crossing yw ey\textit{FLP}; FRT82B[neo\textit{w}] virgin to w;FRT82B crb\textsuperscript{1422}/TM6B or FRT82B crb\textsuperscript{105}/TM6B males. Crb overexpression was achieved by crossing Rh1 \textit{Gal4} (Tabuchi et al., 2000) virgin to UAS Crb\textsuperscript{106\textit{Imag}} [line Crb\textsuperscript{w24}] previously described in Wodarz et al. (1995) or UAS Crb\textsuperscript{flg\textit{apo}} (Richard et al., 2009) males. MyoV clones were generated using yw hs\textit{FLP}/FRT42B ubiGFP virgin crossed to w;FRT42B[MyoV\textsuperscript{106\textit{Imag}}] (a gift from D. Ready, Purdue University, West Lafayette, IN). Heat shock was performed on first instar larvae at 37°C for 2 h. UASMyoV\textit{GFP}/TM6B was a gift from A. Ephrussi (European Molecular Biology Laboratory, Heidelberg, Germany; Krauss et al., 2009). UASPros26/2B\textit{Flies} were obtained from the Bloomington Drosophila Stock Center at Indiana University.

Antibodies

Antibodies were used at the following concentrations for WB or immunofluorescence (IF): rat anti-Crb 2.8 [1:2,000; WB and IF; Tepass et al., 1990], rabbit anti-Sdt [1:1,000; WB; Berger et al., 2007], rat anti-tubulin [1:4,000; WB; Abd Serote et al., 2007], rabbit anti-\textit{Pat} [1:3,000; WB; Richard et al., 2006a], rabbit anti-Lin7 [1:2,000; WB; Bachmann et al., 2004], rabbit anti-Rh1 [1:1,000; IF; a gift from D. Ready], rabbit anti-GFP [1:1,000; IF; Invitrogen], mouse anti-Myc [9E10 supernatant; 1:75; IF; Developmental Studies Hybridoma Bank], mouse anti-Flag (M2; 1:1,000; IF; Sigma-Aldrich), and normal rabbit IgG (Santa Cruz Biotechnology, Inc.). Rabbit anti-MyoV was generated as previously described (Li et al., 2007) and purified as described in Poch and Cory (2009). In brief, rabbit antiserum was raised against the peptide CGGEDIELPSHLNLDEFLTKI conjugated to keyhole limpet haemocyanin (Charles River). Serum from the final bleed was clarified by centrifugation at 25,000 g for 30 min, and the supernatant was supplemented with 1x TBS (0.5 M NaCl, 20 mM Tris, pH 8.0, and 0.1% Tween 20). This was then purified using an affinity column containing the peptide coupled to epoxy–Sepharose 6B (GE Healthcare). Clarified serum was passed over the column twice, and then the column was washed with TBS until the flow-through had an OD\textsubscript{280 nm} < 0.01. Antibodies were then eluted using 0.2 M glycine and neutralized with Tris-HCl, pH 8.0.

\textit{Immunoprecipitation}

\textit{Drosophila} heads were lysed in a homogenizer with the following lysis buffer: 50 mM Tris, pH 8.0, 0.5% Triton X-100, 130 mM NaCl, 1 µg/ml leupeptin, 250 µg/ml Pefabloc, 2 µg/ml aprotonin, and 1 µg/ml pepstatin.
The lysate was left on ice for 30 min. Antibody was added and then clarified by centrifugation. 6 mg total protein was used per IP. Antibody was added and incubated at 4°C for 1 h. Then, 50 μl protein G agarose (GE Healthcare) per IP was added to the lysate antibody mixture and left to rotate again at 4°C for 3 h. The beads were then washed with lysis buffer six times and then boiled with loading buffer for 5 min at 105°C and analyzed by conventional SDS-PAGE. Mass spectrometry (MS) was then performed as described in the next section.

Preparation of samples for MS
After protein separation, SDS gels were stained with Coomassie brilliant blue R250, and entire gel lanes were cut in 20–25 slices. Each gel slice was further cut into 1 × 1-mm cubes and in-gel digested with trypsin as described in Shevchenko et al. (2006). In brief, gel pieces were rinsed with 200 μl of water, shrunken with 200 μl acetonitrile, destained by adding 100 μl of 100 mM ammonium bicarbonate followed by 200 μl acetonitrile, and finally shrunken with acetonitrile. Then, gel pieces were incubated overnight at 37°C in 13 ng/μl of modified trypsin (Promega) in 10 mM ammonium bicarbonate and 10% acetonitrile. Peptides were extracted with 50 μl of 5% formic acid and 50% acetonitrile, and pooled extracts were dried down in a vacuum centrifuge. Peptides were redisolved in 15 μl of 5% formic acid and analyzed by liquid chromatography tandem MS (LC MS/MS).

Protein identification by LC MS/MS
LC/MS/MS analysis was performed on an Ultimate 3000 Nano LC System (Dionex) interfaced online to a linear ion trap LTQ mass spectrometer (Thermo Fisher Scientific), as described in Shevchenko et al. (2008). The mobile phase was 0.1% formic acid in water (solvent A) and 100% of acetonitrile (solvent B). Peptide mixtures were separated using an 80-min gradient from 5 to 100% of solvent B. In the data-dependent acquisition cycle, the three most abundant precursor ions detected in the full MS survey scan (m/z range of 350–1,700) were isolated and fragmented. MS/MS fragmentation was triggered by a minimum signal intensity threshold of 500 counts. m/z of fragmented precursors was dynamically excluded for another 60 s. Acquired spectra were converted to Mascot generic format and then searched against Drosophila sequences in the National Center for Biotechnology Information protein databases by MascOT software (v. 2.4.04; Matrix Science) installed on a local server. Database searching settings were set as mass tolerance ±0.5 Da for precursor and fragment ions, respectively. Variable modifications were set as propionamide (C), N-acetylation (protein N terminus), and oxidation (M). Enzyme settings were set as trypsin, with one missed cleavage allowed. All protein hits matching with at least two peptides having a peptide ion score ≥30 were then manually evaluated.

Preparation of heads and retinas and WB
Drosophila heads were collected on dry ice and mashed with a pestle before the addition of lysis buffer and further mashing. Drosophila retinas were dissected in ice-cold PBS before addition of SDS loading buffer and mashing with a pestle. Lysates were incubated on ice for 30 min and clarified by centrifugation. Supernatants were collected and boiled with a standard SDS loading buffer and processed for SDS-PAGE and western transfer using standard laboratory procedures.

Immunohistochemistry and rhodopsin-trafficking assay
Pupal and adult eyes were prepared as previously described (Richard et al., 2006b). In brief, adult heads were removed, bisected, and fixed immediately with 8% PFA, 15% picric acid, and 75 mM Pipes, pH 7.4, for 20 min at room temperature, washed with PBS, pH 7.2, and cryopreserved by immersion in 30% glycerol containing 20% 1× phosphate buffer, pH 7.4, followed by fixation in 1% OsO4, 72% glutaraldehyde and then 2% OsO4. After dehydration, eyes were embedded in Araldite, and 0.1-μm ultrathin sections were contrasted and analyzed with a Tecnai 12 BIO2WIN (FEI Company) and photographed with a digital camera (F214A; TemCam).

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