Crag Is a GEF for Rab11 Required for Rhodopsin Trafficking and Maintenance of Adult Photoreceptor Cells

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Abstract

Rhodopsins (Rhs) are light sensors, and Rh1 is the major Rh in the Drosophila photoreceptor rhabdomere membrane. Upon photoactivation, a fraction of Rh1 is internalized and degraded, but it remains unclear how the rhabdomeric Rh1 pool is replenished and what molecular players are involved. Here, we show that Crag, a DENN protein, is a guanine nucleotide exchange factor for Rab11 that is required for the homeostasis of Rh1 upon light exposure. The absence of Crag causes a light-induced accumulation of cytoplasmic Rh1, and loss of Crag or Rab11 leads to a similar photoreceptor degeneration in adult flies. Furthermore, the defects associated with loss of Crag can be partially rescued with a constitutive active form of Rab11. We propose that upon light stimulation, Crag is required for trafficking of Rh from the trans-Golgi network to rhabdomere membranes via a Rab11-dependent vesicular transport.

Introduction

The Drosophila phototransduction pathway has been extensively studied [1], and most known members of the pathway were isolated in forward genetics screens by means of electroretinogram (ERG) and phototactic assays [2–6]. Since vision is not an essential sense, previous screens were performed on homozygous viable mutants. This strategy was highly successful and led to the characterization of numerous proteins that play a critical role in light responses. However, the phototransduction cascade is likely to also rely on components that are shared with other processes that are essential for viability. We therefore initiated a large mosaic ERG screen of lethal mutations on the X chromosome. Here, we report the characterization of Calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein (Crag).

The Crag gene was first identified in a biochemical screen for photoreceptor Calmodulin (CaM)–binding proteins [7]. Crag contains a CaM binding site and interacts with CaM in a calcium-dependent manner [7,8]. Mutations in the Crag gene were later shown to affect the epithelial architecture and polarized localization of basement membrane proteins including Perlecain, Laminin, and Collagen IV [8]. Based on sequence homology, the N-terminus of Crag contains three conserved domains: uDENN, DENN, and dDENN, and belongs to the DENN (differentially expressed in neoplastic and normal cells) protein superfamily [9]. The first DENN family member was identified in a screen for variable mRNA expression in neoplastic cells [10], and 18 genes encoding DENN domain proteins are present in the human genome. DENND4A, DENND4B, and DENND4C are the human homologs of Crag, and their cellular function in mammalian systems has not been characterized. Many DENN-domain-containing proteins have been found to interact directly and function as guanine nucleotide exchange factors (GEFs) of
Drosophila photoreceptor cells. Rh1 is the major Rh in depolarization of photoreceptor cells [1]. Besides its sensory role, the transient receptor potential (TRP) channel and leads to the through a G-protein-coupled cascade that triggers the opening of active form, metarhodopsin (metaRh), which in turn signals photon (580 nm), Rh1 undergoes a conformational change to an present in R1–R6 photoreceptor cells. Upon absorption of a
Rab11's important cellular functions, no GEF for Rab11 has been port in epithelial cells and neurons [25–27]. However, despite growth [24]. Rab11 has been shown to bind to a subunit of the transport from the TGN to the plasma membrane [19]. In various Rab proteins [11]. The DENN/MADD protein was found to be a GEF for Rab3 and Rab27 [12,13], whereas Connecden was found to be a GEF for Rab35 [14]. Furthermore, a genome-wide survey revealed that most DENN proteins are GEFs [15]. However, none of the DENN proteins were identified as GEFs for Rab11. Rabs are localized to distinct intracellular membranes [16,17], switch between the inactive (GDP-bound) and active (GTP-bound) conformational state, and respond to various signaling cues. In the active state, Rab proteins interact with their effectors and regulate vesicle trafficking at numerous different steps [16]. GEFs bind to inactive Rabs and facilitate the exchange of GDP for GTP, thereby activating the Rabs. Rab11 has been shown to affect many cellular processes. It mediates protein recycling by regulating membrane transport from recycling endosomes [18]. It is present in the trans-Golgi network (TGN) and post-Golgi vesicles, where it is required for membrane transport from the TGN to the plasma membrane [19]. In polarized MDCK cells, Rab11 is required for apical recycling and basolateral-to-apical transcytosis of immunoglobulin receptors [20–22]. In motile cells, Rab11 is required for transport of integrin to the leading edge [23]. During cellularization of Drosophila embryos, Rab11 is required for basolateral membrane growth [24]. Rab11 has been shown to bind to a subunit of the exocyst complex, Sec15, which regulates polarized vesicle transport in epithelial cells and neurons [25–27]. However, despite Rab11’s important cellular functions, no GEF for Rab11 has been identified to date. Rhodopsins (Rhs) are light sensors in Drosophila and vertebrate photoreceptor cells. Rh1 is the major Rh in Drosophila and is present in R1–R6 photoreceptor cells. Upon absorption of a photon (580 nm), Rh1 undergoes a conformational change to an active form, metarhodopsin (metaRh), which in turn signals through a G-protein-coupled cascade that triggers the opening of the transient receptor potential (TRP) channel and leads to the depolarization of photoreceptor cells [1]. Besides its sensory role, Rh1 is required to form a rhabdomere terminal web, a meshwork of F-Actin cables, which is proposed to play a supporting role in the highly stacked rhabdomeric membranes [28,29]. A complete loss of Rh1 causes a collapse of the rhabdomere membrane at ~90% of pupal development [30]. During development of photoreceptors, Rh1 is synthesized and matures in the endoplasmic reticulum, after which it is transported to the rhabdomeres via the Golgi. Impairment of its maturation process leads to severe photoreceptor degeneration [31,32]. Rab11 has been shown to be required for the post-Golgi trafficking of Rh1 to the apical rhabdomere membrane during the development of the photoreceptors. Rab11 colocalizes with Rh1 in the sub-rhabdomere region in vesicles, and reducing Rab11 activity causes defects in rhabdomere morphogenesis and accumulation of Rh1-positive vesicles in the cytosol [33,34]. However, a role for Rab11 in adult photoreceptor cells and the phototransduction pathway has not been documented. In adult flies, the conversion of metaRh1 back to Rh1 upon light exposure mainly occurs on the rhabdomere membrane upon absorption of a second photon (580 nm) [35]. In addition, some Rh1 is endocytosed and degraded through a lysosomal pathway, to scavenge spontaneously activated or phosphorylated metaRh, thereby preventing photoreceptor degeneration [34,36]. As a consequence, newly synthesized Rh1 is delivered back to rhabdomeres to maintain Rh1 homeostasis as well as the overall rhabdomere morphology. However, it is unclear how this process is regulated in response to light exposure. Our data indicate that Crag and Rab11 play an essential role in the regulated transport of Rh1 to the rhabdomere membrane upon light stimulation and Ca2+ influx.

Results

Mutations in XE10 Cause ERG Defects and Affect the Crag Gene

To isolate novel genes that are involved in visual transduction, we performed an F3 forward genetic screen on the Drosophila X chromosome [37]. Mutations were induced using low concentrations (7.5–15 mM) of ethylmethane sulfonate on an FRT-containing X chromosome, and 33,887 stocks were screened for lethal mutations. A collection of 5,859 X-linked lethal mutations was established, and mutant clones in the eye were generated with 

\[8\] inserted in the first exon of the crag gene [41] inserted in the first exon of the Crag gene that fails to complement all three XE10 alleles for larval lethality (Figure 1C). The XE10 mutations also fail to complement a previously isolated null allele of Crag. Crag [42] [43] [8]. A Crag genomic construct rescues the lethality and phenotypes associated with all XE10 mutations (blue in Figure 1D), showing...
Figure 1. XE10 mutant photoreceptors exhibit abnormal ERG, and XE10 corresponds to Crag. (A) Representative ERG traces of 3-wk-old wild-type control flies (Ctrl; y w FRT19Aiso) and flies with XE10 mutant clones. "On" and "off" transients are marked by red circles. (B) Quantification and statistics of ERG amplitude. Five ERG traces were measured for each genotype. An asterisk indicates a p-value less than 0.05. (C) XE10 alleles fail to complement a lethal insertion, which is inserted in the first exon of Crag. The red dotted lines mark the region of overlap of the deficiencies that fail to complement the XE10 mutations. (D) Structure of the Crag gene. A 12-kb genomic rescue construct [8] that covers 2 kb upstream and 1.5 kb downstream of the Crag coding region rescues the XE10 alleles. (E) Lethal phase analysis of Crag alleles. In the upper four lines, lethal phases of transheterozygous animals were tested. For genomic rescue experiment, heterozygous female flies were crossed with males bearing the genomic...
that we have identified novel alleles of Crag. We identified a missense mutation in CragA (C1371S), a nonsense mutation in CragB (R798STOP), and a point mutation affecting a splice donor site in CragC (Figure 1F). Lethal phase analyses indicate that the A and C alleles are severe loss-of-function or null alleles, whereas D is a hypomorphic allele (Figure 1E). We observe no immunoreactivity in CragC clones of L3 larval eye imaginal discs using an antibody recognizing Crag, whereas in wild-type cells, the antibody reveals cytoplasmic punctae (Figure 1G). Crag is a homolog of the human DENND4A, DENND4B, and DENND4C proteins. Expression of a UAS-human DENND4A construct using the ubiquitous daughterless-GAL4 driver rescues the lethality caused by loss of Crag (Figure 1E), indicating that Crag and DENND4A have conserved functions.

**Crag Mutant Photoreceptors Exhibit Activity-Dependent Photoreceptor Degeneration**

Since Crag mutant clones exhibit reduced ERG amplitude in 3-wk-old flies, we first examined whether the development of the visual system is affected by Crag mutations. To assess the morphology of photoreceptors, we performed cross-section and transmission electron microscopy (TEM) analysis of the eyes of newly eclosed flies. The data show that Crag mutant photoreceptors have normal rhabdomere morphology at day 1 (Figure 2B), indicating that the photoreceptors develop properly. To examine axonal targeting of the photoreceptors, we immunohistochemically stained the terminals of R7 and R8 in the medulla for Chaoptin, a photoreceptor-membrane-specific protein [42]. As shown in Figure S1, the two-layer projection patterns of R7 and R8 photoreceptors in the medulla in control and Crag mutant cells are indistinguishable. We then performed TEM in the lamina to determine whether R1–R6 photoreceptors target properly. Crag mutant photoreceptors form cartridges with the normal complement of photoreceptor terminals and synapses (Figure S2A–S2D). These data indicate that the photoreceptors display proper axonal guidance and synapse formation. Hence, the aberrant ERGs of Crag mutant clones are not likely due to developmental defects.

To determine whether Crag mutations cause photoreceptor degeneration, flies were kept either in a 12-h on/off light (~1,800 Lux) cycle or in constant darkness, and ERGs were recorded at different time points. Flies carrying eye-specific clones of the CragA or CragC/J101 null alleles (Figure S3A and S3B) exhibit normal ERG responses at day 1 when kept in the dark, indicating that the photoreceptors develop and function properly (Figure 2A, 2C, and 2D). To further examine whether phototransduction is affected by Crag mutations in dark-raised flies, we performed intracellular recording of single photoreceptor cells. The data show that Crag mutant photoreceptors exhibit normal response to light stimulation when compared to controls (Figure S3C–S3E).

However, both the ERG amplitude and the size of on-off transients become smaller with age when the flies are reared in a 12-h on/off light cycle, whereas the ERGs of wild-type controls are unaffected in aged flies (Figure 2A, 2C, and 2D). Interestingly, Crag mutant clones still exhibit a normal ERG response when kept in the dark for 2 wk. The phenotypes of aged flies kept in a 12-h on/off light cycle are fully rescued by the genomic rescue construct. Since loss of Crag causes a light-dependent degeneration, we hypothesized that Crag in photoreceptor cells may play a role in phototransduction. Upon light stimulation, Rh1 undergoes a conformational change to metaRh, which consequently triggers the opening of TRP channels through G-protein-coupled signaling and leads to the depolarization of photoreceptor cells [1]. InaD contains five PDZ domains and serves as a scaffold protein to allow many players in the pathway to form a signaling complex [43–45]. We performed immunostainings of major phototransduction proteins with a whole mount protocol to assess whether Crag is required for their subcellular distribution. Rh1, TRP, and InaD are all properly localized in Crag mutant photoreceptors in flies kept in dark, as the staining patterns are similar to those of wild-type cells (Figure 3A). These data are in agreement with the functional data, as Crag mutant clones exhibit normal ERG responses in newly eclosed flies or flies kept in darkness for several weeks. Upon 5 d of exposure to a light/dark cycle, these proteins are still properly localized in control photoreceptors. However, in Crag mutant cells, Rh1 is massively accumulated in the cytosol (Figure 3B). In contrast, a cytosolic accumulation of TRP and InaD is not observed (Figure 3B). Notably, with the whole mount staining protocol, a crescent shaped distribution pattern of these proteins is observed at the base of the rhabdomeres, which is consistent with previous studies [36,46–48]. In contrast, a uniform distribution of these proteins in the rhabdomeres has been observed in immunostainings of thin sections [49–52]. We therefore also performed immunostainings in cross-sections of photoreceptors and observed a rhabdomeric localization of Rh1.
InaD, and TRP in flies kept in dark (Figure 3C). Similarly, the data also show that light stimulation induces a cytosolic accumulation of Rh1, but not TRP and InaD, in Crag mutant cells (Figure 3D). The difference in localization between the two protocols is probably due to a different accessibility of the antibodies, since rhabdomeres are highly packed membrane stacks. These data suggest that Crag is involved in Rh1 transport upon light stimulation but not during photoreceptor development. Since Rh1 is a transmembrane protein, its accumulation in the cytosol of Crag mutant cells upon light stimulation would imply an accumulation of membrane structures. We therefore examined the ultrastructure of the light-stimulated photoreceptor cells by TEM. After 5 d of 12-h on/off light stimulation, Crag mutant cells exhibit a massive accumulation of vesicles in the cytosol (25.2±8.4 in wild type; 186.8±21.3 in Crag mutants: n=10, p<0.001) when compared to controls (Figure 3E and 3F). The accumulation of

![Figure 2. Mutations in Crag lead to a light-dependent photoreceptor degeneration.](image)

(A) ERG traces of wild-type control (y w FRT19A iso) and Crag mutant clones in flies raised in a 12-h on/off light cycle (L/D), or in constant light, or in the dark at different ages. Note that both the depolarization amplitude and the size of “on” and “off” transients become smaller upon light/dark exposure in Crag mutants but are not affected in wild-type controls. Constant light triggers a more severe reduction of ERG amplitude in Crag mutant cells. These defects are strictly light-dependent and can be rescued by the genomic rescue construct. (B) TEM of ommatidia cross-sections. Rhabdomere structures are recognized by their high electron density. Note that the aged Crag mutant photoreceptor cells are largely disrupted (lower right). Scale bar, 1 µM. (C) Quantification of ERG depolarization amplitudes shown in (A). Ten ERG traces were measured for each genotype at each time point. An asterisk indicates a p-value less than 0.05; “n.s.” indicates a p-value greater than 0.05. (D) Quantification of ERG off-transient amplitudes shown in (A). Ten ERG traces were measured for each genotype at each time point. (E) Quantification of rhabdomere areas shown in (C). Rhabdomere area in Crag mutant photoreceptors is largely reduced after 2 wk of light exposure. The rhabdomeres were outlined and their sizes were calculated using Image J software. Ten ommatidia from different cross-sections were analyzed for each genotype.

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Figure 3. Rh1 accumulates in the cytosol of Crag mutant photoreceptor cells upon light stimulation. (A) Whole mount staining of photoreceptors of flies raised in the dark. Note that Rh1, InaD, and TRP all exhibit crescent shaped patterns at the base of the rhabdomeres in both genotypes. Rhabdomeres are labeled by phalloidin staining of Actin. Scale bar, 5 μM. (B) Same staining as (A) in control (Ctrl) and Crag mutant flies upon 5 d of light/dark (L/D) exposure. Note the cytosolic accumulation of Rh1 in Crag mutant photoreceptors. (C) Cross-sections of control (yw FRT19Aiso) and Crag mutant photoreceptors in flies raised in dark were stained with antibody against Rh1, InaD, and TRP. All markers examined exhibit a normal distribution in both genotypes. Note that all the proteins are uniformly distributed in the rhabdomeres. Scale bar, 2 μM. (D) Same staining as
Rh1 as well as vesicles in the cytosol indicates a defect in vesicular trafficking of Rh1 in Crag mutant cells.

Crag Is Required for the Transport of Newly Synthesized Rh1 to the Rhabdomeres during Light Stimulation

During photoreceptor development, Rh1 is delivered into rhabdomeres through a Rab11-mediated vesicle transport [34]. In adult flies, a subpopulation of metaRh is endocytosed and degraded [36,46], and this Rh1 loss should be replenished with newly synthesized Rh1 to maintain homeostasis. The accumulation of vesicles in Crag mutant photoreceptors could be due to an increase in endocytosis, a decrease in the clearance of the endocytosed vesicles, or a failure to secrete newly synthesized vesicles. With white light stimulation, Rh1 is internalized at a very slow rate, as determined by Western blots and immunostainings. Therefore, we kept flies for a 6-h period in blue light to determine which aspect of Rh1 trafficking is affected by Crag. Blue light converts Rh1 to metaRh, and metaRh requires orange light (580 nm) to be converted back into Rh1. In the absence of orange light, blue light triggers massive endocytosis and degradation of metaRh [33]. Western blot data show that exposure for 6 h to blue light leads to a similar decrease of Rh1 in both wild-type and Crag mutant cells (Figure 4B, upper panel), indicating that endocytosis and degradation of metaRh is not significantly affected in Crag mutant photoreceptors. Moreover, upon 24 h of recovery in the dark, there is a significant increase in Rh1 levels in both wild-type and Crag mutant cells, indicating that the de novo synthesis of Rh1 is also not affected in Crag mutant cells. Previous studies have shown that internalized Rh1 may form insoluble aggregates that are not detectable in Western blot [36]. We therefore homogenized fly heads in SDS buffer containing 1 M urea and performed dot blots to assess total Rh1 levels. The data confirm that Rh1 is indeed degraded upon 6 h of blue light stimulation in both wild-type and Crag mutant cells (Figure 4B, lower panel). In summary, Crag does not appear to affect endocytosis, degradation, or synthesis of Rh1.

We further examined Rh1 dynamics upon blue light stimulation with the whole mount staining protocol. As shown in Figure 4C, 6 h of blue light stimulation triggers massive endocytosis of Rh1 in both wild-type and Crag mutant photoreceptors. After 18 h of recovery in the dark, cytosolic Rh1 is reduced and the crescent shaped pattern is re-formed in the wild-type photoreceptors, whereas in Crag mutant photoreceptors, Rh1 remains accumulated in the cytosol. Furthermore, the accumulation of Rh1 in Crag mutants persists even after 36 h of recovery.

Blue light exposure drives photoreceptors into prolonged depolarizing afterpotential (PDA) [35]. To test whether the accumulation of Rh1 in Crag mutant cells is caused by a PDA, we exposed the flies to blue light followed by orange light for 20 min to terminate the PDA. We then kept the flies for 18 h in the dark and examined the Rh1 distribution (Figure S3C). Rh1 still accumulates in the cytosol of Crag mutant photoreceptors but not in controls, indicating that Rh1 localization defects are not caused by PDA.

We next performed TEM analysis of photoreceptors during the course of the blue light treatment and the recovery (Figure S6). The data indicate that electron densities in the cytosol correlate with the Rh1 levels in the cytosol. Interestingly, after 36 h of dark recovery, the rhabdomeres start to break down in Crag mutant cells, as revealed by Actin staining and TEM analyses (Figure S6). The data suggest that persistent accumulation of Rh1 in the cytosol leads to the breakdown of rhabdomeres and the degeneration of photoreceptor cells.

Since the degradation of Rh1 is unaffected in Crag mutant cells, we hypothesized that the accumulation of Rh1 in the cytosol may be the result of a defect in transport of newly synthesized Rh1 to the rhabdomeres. We therefore stained the photoreceptors with a trans-Golgi marker, Peanut agglutinin [54], and observed an expansion of the TGN in Crag mutant photoreceptors (Figure 4D). Moreover, a portion of the accumulated Rh1 colocalizes with the trans-Golgi marker. These data, along with the vesicle accumulation observed by TEM, suggest that Crag is required for post-Golgi trafficking of Rh1 to the rhabdomeres. In summary, loss of Crag leads to accumulation of newly synthesized Rh1 in post-Golgi vesicles, a breakdown of rhabdomeres, and, eventually, photoreceptor degeneration.

Knockdown of Rab11 in Adult Eyes Causes Photoreceptor Degeneration

Crag possesses three DENN domains and may serve as a GEF for a Rab protein. To identify its potential target(s), we performed a screen using a collection of 31 Rab dominant negative (DN) transgenic lines [55] (Figure S7). To bypass the requirement of some Rabs for the development of photoreceptor cells, we used Rh1-GAL4, which is expressed late in photoreceptor formation and in adult photoreceptors, to drive expression of the Rab-DNs. To lower expression levels we also raised the flies at 18°C prior to eclosion. We then aged the flies in a 12-h light/dark cycle or darkness for 21 d and performed ERGs. Expression of only Rab11-DN (S25N) causes a light-dependent reduction of ERG amplitude in 3-wk-old flies exposed to light, similar to that in Crag mutants (Figures 5A, 5B, and S7). To further confirm that loss of Rab11 activity in the adult eye leads to a light-dependent photoreceptor degeneration, we expressed two Rab11 double-stranded RNA constructs [34,56] in the adult eye using Rh1-GAL4 and found that they also cause a reduction of ERG amplitude upon light stimulation (Figure 5A and 5B). In contrast, expression of wild-type or constitutively active (CA) Rab11 does not cause a phenotype (Figure 5A and 5B). Next, we performed TEM after light and dark exposure of photoreceptors with Rab11 knockdown. Light exposure disrupts the photoreceptor cell morphology in these cells, whereas dark incubation causes no obvious effects after 3 wk (Figure 5C). These data indicate that knockdown of Rab11 leads to a light-induced photoreceptor degeneration similar to that of loss of Crag. Hence, Rab11 is a potential target of Crag.

Crag Is a GEF for Rab11

To assess whether Crag and Rab11 physically interact, we generated tagged Crag (FLAG) and Rab11 (HA) expression constructs and first examined their protein localizations in Drosophila S2 cells. Crag colocalizes with Rab11 when both are co-expressed (Figure 6A). When Rab7 and Crag are co-expressed, the large overlapping punctae observed when Rab11 and Crag are co-expressed are not obvious (Figure 6B). We then performed co-immunoprecipitation (co-IP) using an antibody against HA to pull down Rab11, and found that Crag is co-precipitated with Rab11, indicating that Crag is a binding partner of Rab11 (Figure 6C).
Figure 4. Crag is required for Rh1 transport to the rhabdomeres upon light stimulation. (A) In adult flies, blue light triggers the conformational change of Rh to metaRh, whereas orange light is required for converting metaRh to Rh. In the presence of blue light and absence of orange light, metaRh is accumulated in the rhabdomere, which in turn triggers massive endocytosis and degradation of Rh. (B) Fly heads with control or CragC mutant clones in the eyes were collected from flies that were kept in the dark or in blue light for 6 h with or without 24 h of recovery. Western blots and dot blots were performed to detect total Rh1 and Actin (loading control) levels. Note that in both genotypes, the Rh1 level significantly decreased after 6 h of blue light treatment and was restored after 24 h of recovery in the dark. (C) Whole mount staining of Rh1 in photoreceptors kept in the dark, exposed to 6 h of blue light, or exposed to 6 h blue light with 18 or 36 h of recovery in the dark. 6 h of blue light treatment triggers massive endocytosis of Rh1 in both control and Crag mutant photoreceptors. Note the strong Rh1 staining in the cytosol. After
18 h of recovery, a crescent shaped pattern of Rh1 is reformed in controls but not in Crag mutant photoreceptors. Rh1 accumulation is persistent in Crag mutant cells after 36 h of recovery. Note that breakdown of rhabdomeres (indicated by red arrows) is observed in flies kept for 36 h in the dark but not in flies kept for 18 h in the dark after blue light exposure. Scale bar, 5 μM. (D) Immunostaining of the trans-Golgi compartment (Peanut agglutinin) and Rh1 in flies after 6 h of blue light exposure and 18 h of dark recovery. Note the enlargement of the trans-Golgi compartment and the colocalization between Rh1 and the trans-Golgi compartment (indicated by white arrowheads) in Crag mutant cells.

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Figure 5. Knockdown of Rab11 in adult eye causes light-dependent photoreceptor degeneration. (A) ERG traces of flies with expression of UAS-GFP, Rab11-DN(S25N), Rab11 double-stranded RNA constructs (Rab11 RNAi1 and Rab11 RNAi2), Rab11, Rab11-CA(Q70L), and Rab10-DN(T23N) driven by Rh1-GAL4 at day 1 and day 21 with or without 12-h on/off light exposure (L/D). Note that the ERG amplitudes are reduced in the flies with Rab11 knockdown and light exposure (red box). (B) Quantification of ERG depolarization amplitudes shown in (A). Five ERG traces were measured for each genotype at each time point. An asterisk indicates a p-value less than 0.05; “n.s.” indicates a p-value greater than 0.05. (C) TEM of photoreceptor cross-sections of flies expressing GFP, Rab11-DN, or Rab11 RNAi2 in photoreceptor cells after 21 d of incubation in light/dark cycle or in constant dark. Note that the rhabdomere morphology is disrupted in flies with Rab11 knockdown upon light exposure. Since Rh1-GAL4 drives expression only in R1–R6 photoreceptors, R7/R8 are better preserved than R1–R6. Scale bar, 1 μM.

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Figure 6. Crag interacts with Rab11 in vitro. (A) Immunostaining of S2 cells transfected with FLAG-tagged Crag (green) and HA-tagged Rab11 (red). Their expression regions largely overlap. Scale bar, 2 μM. (B) Immunostaining of S2 cells transfected with FLAG-tagged Crag and HA-tagged Rab7. No significant colocalization is observed. (C) S2 cells transfected with FLAG-tagged Crag and/or HA-tagged Rab11, Rab11-DN, or Rab11-CA were harvested and lysed. Anti-HA gel was used to pull down HA-tagged proteins. When Rab11 and Crag are co-transfected, Crag was also co-immunoprecipitated, as detected on Western blots, indicating that Crag binds to Rab11. Also note that Crag preferentially binds to the DN form of Rab11 rather than the CA form. (D) Quantification and statistics of binding affinity between Crag and the different forms of Rab11 shown in (A). The data are quantified using the Gel analysis function in Image J. Results from three independent experiments were analyzed. An asterisk indicates a p-value less than 0.05. (E) A scheme of different Crag deletion constructs and their binding affinity with Rab11 was tested by co-IPs. Crag segments
GEFs bind to the GDP-loaded Rabs to promote the release of GDP, whereas binding of GTP to Rabs diminishes the binding of the GEFs. Hence, GEFs have a higher affinity for GDP-bound forms of Rabs than for GTP-bound forms. We therefore performed co-IP between Crag and the DN (mostly GDP-bound) or CA (mostly GTP-bound) form of Rab11. The results show that Crag binds preferentially to the Rab11-DN, and weakly to Rab11-CA (Figure 6C and 6D). We next mapped the Rab11 binding domain of Crag by generating a series of deletion constructs. The constructs containing the three DENN domains co-immunoprecipitate with Rab11, whereas those lacking DENN domains do not co-immunoprecipitate with Rab11 (Figure 6E). These data suggest that Crag may be a GEF for Rab11.

To determine whether Crag possesses GEF activity, we performed in vitro activity assays. Unfortunately, expression of the 180-kDa Crag protein in Escherichia coli produces insoluble protein in inclusion bodies. We therefore purified the protein using a baculovirus expression system in insect cells. For the GEF assay we preloaded the Rab proteins with fluorescence-labeled BODIPY-GDP, and then added excessive unlabeled GDP with or without the potential GEF and measured the release rate of BODIPY-GDP from the Rabs. Since the human homolog of Crag, DENND4A, was shown to exhibit GEF activity against Rab10 [15], we first performed the GEF assay with Rab10. As shown in Figure 6F, Crag strongly promotes GDP release from Rab10, indicating that Crag and DENND4A are functionally conserved and that the purified Crag protein is a GEF in vitro. However, Rab10 is not expressed in the adult eye [57], and expression of Rab10-DN does not cause a light-dependent degeneration (Figure 5). Hence, the degeneration phenotypes associated with Crag mutations are unlikely to be caused by defects in Rab10 activation.

Next, we performed the GEF assay for Crag against Rab11 and Rab5. As shown in Figure 6G and 6H, Crag indeed facilitates the GDP dissociation from Rab11 but not from Rab5. However, the kinetics of the reaction with Rab11 is slow compared to that of Crag against Rab10. We hypothesized that the slow kinetics is due to the molecular properties of purified Rab11. We therefore added 10 mM EDTA to GDP-preloaded Rab11 to examine its release kinetics. EDTA absorbs Mg^{2+} from the Rab proteins and induces a rapid release of GDP [38]. Although the kinetics for GDP release is significantly accelerated, it is relatively slow when compared to EDTA-triggered GDP release of Rab5 (Figure 6G and 6H). We concluded that the kinetics of Rab11 in vitro is generally slow. Since Crag binds to CaM in a calcium-dependent manner [7,8], and since cellular Ca^{2+} levels increase during photoactivation, we next examined whether CaM regulates Crag activity. In the presence of CaM and Ca^{2+}, the exchange rate is indeed increased (Figure 6G). Hence, it’s possible that Crag activity is enhanced during light stimulation upon a Ca^{2+} influx in photoreceptor cells. These data, together with our in vivo observations, indicate that Crag is a GEF for Rab11.

We next examined the interactions between Crag and Rab11 in vivo. We first performed immunostaining of Crag and Rab11. Crag and Rab11 colocalize in punctate structures (Figure 7A), indicating that they may physically interact in vivo. The subcellular localization of Rab proteins often depends on their activation status. When bound to GTP, Rab proteins bind to various effectors that help target the proteins to the proper membrane compartments. Hence, many GEFs regulate the subcellular localization of Rabs. To determine whether Crag is required for the proper subcellular localization of Rab11, we exposed the flies to 12 h of light stimulation and performed immunostaining of Rab11 and Rh1. In control photoreceptors, Rab11 colocalizes with Rh1 in numerous punctae, and many of the punctae are in close vicinity to the rhodopsomes, providing further evidence for a role for Rab11 in regulating Rh1 transport.

In Crag mutant photoreceptors, Rab11 exhibits a more diffuse pattern, less punctae are observed, and the punctae are rarely localized in the vicinity of rhodopsomes (Figure 7B). These data show that Crag is indeed required for the proper localization of Rab11 in adult photoreceptors.

To determine whether Rab11 functions downstream of Crag, we overexpressed a CA form of Rab11 (Rab11-CA), which does not require a GEF for its activation, in Crag mutant cells. If Crag functions as a GEF for Rab11, expression of Rab11-CA may rescue the defects associated with the loss of Crag. Hence, we aged the flies for 3 wk in a 12-h light/dark cycle and examined photoreceptor function with ERGs. As shown in Figure 7C–7E, Rab11-CA expression in Crag mutant cells partially rescues the ERG phenotypes. We also expressed Rab10-CA in the Crag clones and observed no significant rescue (data not shown). To further determine whether the Rab11-CA is able to also suppress the light-induced morphological defects of photoreceptors associated with the loss of Crag, we performed TEM. The data show that the rhodopsomes are much better preserved upon light stimulation in Crag mutant cells expressing Rab11-CA than in those expressing a GFP control (Figure 7F). In addition, we compared the time course of photoreceptor degeneration using ERG recordings in flies that contained Crag mutant clones and/or expressed a DN mutation for Rab11 in the eyes (Figure S8). The data show that no significant additive or synergistic effect is observed when Crag and Rab11 are impaired, suggesting that they are involved in the same pathway required for photoreceptor maintenance. In summary, these data indicate that Rab11 functions downstream of Crag genetically, and suggest that Crag regulates Rh1 transport via Rab11 in Drosophila photoreceptors.

**Discussion**

Here, we show that Crag is a novel GEF for Rab11 and that it is required for the post-Golgi transport of Rh1 to the rhodopsomes during light activation (Figures 7G and S10). This regulated transport of Rh1, which is independent of Rh1 transport during the development of the photoreceptors, replenishes the loss of Rh1 induced by light stimulation. Loss of Crag leads to accumulation of secretory vesicles in the cytosol of photoreceptor cells, and eventually leads to a light- and age-dependent photoreceptor degeneration.
Figure 7. Crag genetically interacts with Rab11 in vivo. (A) Immunostaining of Crag (green) and Rab11 (red) in cross-sections of wild-type photoreceptors upon 12 h of light stimulation. The proteins colocalize in punctuate structures (indicated by arrowheads). The rhabdomeres are marked by Actin staining (blue). Scale bar, 5 μM. (B) Immunostaining of Rab11 (red) and Rh1 (green) in photoreceptors of wild-type controls and Crag mutants upon 12 h of light stimulation. In controls, Rab11 is present in punctae that partially colocalize with Rh1 (indicated by arrowheads), and most of the punctae are close to the rhabdomeres; in Crag mutants, many fewer punctae of Rab11 are observed, and these punctae are not closely associated with rhabdomeres (indicated by arrows). Scale bar, 5 μM. (C) GFP or Rab11-CA were expressed in Crag mutant clones, and the flies were kept in a 12 hour light/dark cycle (L/D) for 3 wk along with controls (y w P{neoFRT}19Aiso with GFP expression in the photoreceptors). Representative
Crag Is Required to Maintain Rh1 Homeostasis upon Light Exposure

During development of photoreceptors, Rh1 and other phototransduction proteins are synthesized in the endoplasmic reticulum and transported to the rhabdomeres to build functional photoreceptors. Some molecular players, including Rab11 and XPORT, have been shown to play a role in this process [32,34]. Upon light activation Rh1 is converted to metaRh (Figure S10A). MetaRh is then converted back into Rh1 on rhabdomere membranes via absorption of another photon, allowing the maintenance of Rh1 levels in the rhabdomere [35]. In wild-type photoreceptors, a portion of metaRh is phosphorylated and endocytosed [46], and it has been proposed that internalization of metaRh promotes the clearance of dysfunctional proteins and serves as a proofreading mechanism (Figure S10B). Internalized Rh1 is then degraded through an endosomal/lysosomal pathway [36]. Obviously, the gradual loss of Rh1 in wild-type photoreceptors leads to the rhabdomeric pool. This is nicely illustrated with the loss of retinol dehydrogenase (RDH), which is required for the regeneration of rhabdomeric pool. This is nicely illustrated with the loss of retinol dehydrogenase (RDH) in adult photoreceptors, we exposed the flies to blue light to trigger massive endocytosis and degradation of Rh1, and then measured the new synthesis and transport of Rh1 back to the rhabdomeres over time. Crag is not required for the synthesis of Rh1. However, in Crag mutants, the newly synthesized Rh1 accumulates in the cytosol. We propose that Crag is required for the delivery of newly synthesized Rh1 to the rhabdomeres and that loss of Crag leads to a gradual reduction in the size of rhabdomeres and to degeneration of the photoreceptor cells (Figure S10C and S10D). Indeed, the time course and morphological features of degeneration associated with loss of Crag are very similar to the phenotypes observed in RDH mutants, further supporting that Crag is involved in the Rh1 synthesis/delivery pathway.

Crag Is a GEF for Rab11

Rab11 has been implicated in various intracellular membrane trafficking processes. Its diverse functions in different membrane compartments are mediated through its downstream effectors in a context-specific manner; many of these functions have been identified in previous studies [60]. However, GEFs for Rab11 in any context have not yet been identified. Our in vivo and in vitro data provide compelling evidence that Crag is a GEF for Rab11. First, in Drosophila S2 cells, Crag colocalizes and physically interacts with Rab11. Second, Crag preferably binds to the GDP-bound form of Rab11, and the DENN domains are required for binding. Third, Crag is required for the proper localization of Rab11 in photoreceptors upon light stimulation. Fourth, loss of Crag or Rab11 leads to a similar light-induced photoreceptor degeneration. Fifth, expression of Rab11-CA partially rescues the degeneration caused by Crag mutations. Finally, an in vitro GEF assay shows that Crag facilitates the release of GDP from Rab11. It has been previously established that Rab11 is essential for photoreceptor cell development and Rh1 transport during pupal stages [34]. However both rhabdomere morphology and Rh1 localization are normal in Crag clones in newly eclosed flies. Similarly, initial deposition of TRP is also not affected by Crag mutations, in agreement with previous findings that Rh1 and TRP are co-transported to the rhabdomeres during their development [32]. Interestingly, cytosolic localization of TRP is not observed in Crag mutant photoreceptor cells exposed to light, suggesting that during light stimulation, Rh1 and TRP dynamics are distinct. Indeed, internalization of TRP upon light stimulation has not been reported in previous studies. Our data therefore indicate that other GEFs must exist for Rab11 during photoreceptor development, and that Crag is specifically required for Rab11 GDP/GTP exchange during light activation in adult flies. In addition, Crag may function as a GEF for Rab10 in other processes and cells, such as polarized deposition of basement membrane proteins in follicle cells.

The biochemical assay shows that the kinetics of Crag GEF activity is slow when compared to the GEF activity of other DENN-domain-containing proteins such as the Rab35 GEF [14]. Rab11 exhibits GEF activity against Rab10 with much faster kinetics than against Rab11, indicating that the slow kinetics may be due to properties of Rab11. This is further supported by the slow kinetics of EDTA that triggers GDP release of Rab11. It’s possible that the GDP/GTP exchange of Rab11 requires other cofactors besides its GEF, as, for example, documented for Rab6 [61,62].

CaM is a ubiquitously expressed calcium sensor [63]. In the Drosophila photoreceptor cells, photoactivation leads to influx of Ca$^{2+}$ and activation of CaM. It has been shown that CaM is required for the termination of the photoreceptor in several steps, including TRP inactivation and conformational change of metaRh [59,64]. Crag contains a CaM binding site and interacts with CaM in a calcium-dependent manner [7,8]. In our in vitro GEF assay, the presence of CaM and Ca$^{2+}$ indeed enhances the GEF activity of Crag. Hence, it is possible that a light-induced increase of intracellular Ca$^{2+}$ level enhances Crag activity via CaM binding. The activation of Crag/Rab11 then may serve to replenish rhabdomeric Rh1, whose loss is also induced by light stimulation.
DENND4A and Human Photoreceptor Degeneration

In vertebrate rod cells, polarized transport of Rh is mediated by post-Golgi vesicles that bud from the TGN and fuse with the base of the outer segment [65, 66]. Rab11 has been detected on rhodopsin-bearing post-Golgi vesicles in photoreceptors [67, 68]; however, it has not yet been shown that Rab11 is required for Rh trafficking. DENND4 proteins are highly similar to Crag. Here we showed that expression of the UAS–human DENND4A construct not only rescues the lethality but also rescues the light-induced photoreceptor degeneration caused by loss of Crag (Figure S9), showing that the molecular function of DENND4A is also conserved. Moreover, three different subtypes of Usher syndrome, an inherited condition characterized by hearing loss and progressive vision loss, have been mapped to the vicinity of the DENND4A locus at 15q22.31 [69–71]. Hence, DENND4A may also function through Rab11 in human photoreceptors, and loss of DENND4A may lead to photoreceptor degeneration.

Materials and Methods

Fly Strains and Genetics

Mutagenesis. ~8,600 y w P(neoFRT)19A iso rescued male flies were treated with low concentration of ethylmethane sulfonate (7.5–15 mM) to induce mutations. Flies were starved for 12 h followed by feeding with ethylmethane sulfonate-laced sucrose solution for 15 h. The treated males were crossed with Df(1)Ja27/FM7c, Kr-GAL4 UAS-GFP [hereafter abbreviated as Kr-GFP] females. The F1 females y w mut* P(neoFRT)19A/FM7c, Kr-GFP were individually crossed with FM7c, Kr-GAL4 UAS-GFP/TM6B flies to establish stocks and then screened for lethal mutations. 5,859 stocks bearing lethal mutations were established. Female y w mut* P(neoFRT)19A/FM7c, Kr-GFP flies from these stocks were crossed with cl(1) P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males to establish clones, and then screened for lethal mutations. 5,859 stocks bearing lethal mutations were established. Female y w mut* P(neoFRT)19A/FM7c, Kr-GFP flies from these stocks were crossed with cl(1) P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males to generate flies with homozygous mutant clones in the eye. These flies were aged for 3 wk, and ERGs were performed on the mutant patches marked by a loss of w*, as described previously [38].

Mapping of the XE10 group. Female y w mut* P(neoFRT)19A/FM7c, Kr-GFP flies were crossed to a set of large duplications (~1–2 Mb) covering the X chromosome [39] to roughly map the lethality and generate rescued males. Mutations rescued by the same duplication were then crossed inter se to establish complementation groups. For the XE10 group, the alleles were rescued by Df(1)Ja619 (7D–8B3). We then crossed the XE10 alleles with deficiencies within this region, and found that Df(1)BSC627 and Df(1)BSC592 fail to complement all XE10 alleles. These Dfs share a ~120-kb interval, and a insertion PBac(WH)CG12659[2000] in CG12737 fail to complement all XE10 alleles. CG12737 corresponds to Crag, and a null Crag1,000 allele was obtained from Trudi Schupbach [8]. Crag1,000 fails to complement all XE10 alleles.

For ERG, Chaoptin staining, and TEM experiments, we crossed y w Crag P(neoFRT)19A/FM7c, Kr-GFP females with cl(1) P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males. Mutant clones correspond to white patches. Flies in which more than 95% of the eyes is mutant are typically analyzed. For controls, homozygous y w P(neoFRT)19A/FM7c females were crossed with the cl(1) P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males to produce clones. For immunostaining in the L3 larval eye imaginal discs, y w Crag1 P(neoFRT)19A/FM7c, Kr-GFP female flies were crossed with cl(1) Ubi-GFP P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males, and L3 female larva that lacked Kr-GFP expression were selected. For immunostaining in photoreceptors and the blue light experiments, y w cl(1) P(neoFRT)19A/Dp(1;Y)y iso; ey-FLP males were used to eliminate pigment from the eye. Mutant clones can be recognized, as they exhibit a subtle roughness. To express Rab11-CA in Crag clones, y w Crag P(neoFRT)19A/FM7c, P(UASp-YFP.Rab11.Q70L)CG13895 females were crossed with P(GMR-hid), y w P(neoFRT)19A/FM7c, P(GAL4-cy.H), P(UAS-FLP.D), P {rh1-GAL4} males. For controls, y w Crag P(neoFRT)19A/FM7c, P(UAS-GFP) and y w P(neoFRT)19A/FM7c, P(UAS-GFP) females were crossed with the same males.

Electroretinograms

ERG recordings were performed as previously described [38]. In brief, adult flies were glued to a glass slide, a recording probe was placed on the surface of the eye, and a reference probe was inserted in the thorax. A 1-s flash of white light was given, and the response was recorded.

Single-Photoreceptor Recording

Single-photoreceptor recordings were performed as previously described [72]. In brief, a small hole was cut on the cornea of the eye, and the hole was scaled by Vaseline. A reference probe was placed at the back of the head, and a recording probe was inserted into the retina through the previously cut hole. The membrane potential was monitored by AXONCLAMP-2B (Axon Instruments). When the membrane potential dropped to below ~60 mV, the fly was given a 10-ms white light stimulation (white LED, 7,000 mcd, super bright LEDs), and the response was recorded.

Transmission Electron Microscopy

Electron microscopy of the photoreceptor and the lamina was performed as described [73]. In brief, fly heads were dissected and fixed at 4°C in 4% paraformaldehyde, 2% glutaraldehyde, 0.1 M sodium cacodylate, and 0.005% CaCl2 (pH 7.2) overnight, postfixed in 1% OsO4 for 1 h, dehydrated in ethanol and propylene oxide, and then embedded in Embed 812 resin (Electron Microscopy Sciences). Thin sections (~50 nm) of the photoreceptor and lamina were stained in 4% uranyl acetate and 2.5% lead nitrate, and TEM images were captured using a transmission electron microscope (model 1010, JEOL) with a digital camera (US1000, Gatan). For quantification, the sizes of rhabdomeres are determined in Image J.

Immunostaining of the Photoreceptor Cells

For cross-sections, fly heads were bisected, fixed in 4% paraformaldehyde for 3 h, dehydrated in acetone, embedded in LR white resin (Polysciences), and sectioned. Immunostaining was performed as described by [51]. For whole mount staining of fly heads, heads were fixed in 4% formaldehyde upon removal of the proboscis. The photoreceptors were dissected and fixed for 15 min. Standard immunostaining procedures were then performed, as previously described [74]. Images were obtained with a Zeiss LSM 710 confocal microscope. Antibodies were as follows: rat anti-Crag [8], 1:200; mouse monoclonal anti-Rab11 (BD Biosciences), 1:20; rabbit anti-Rab11 [34], 1:1,000; mouse anti-Rh1 [75], 1:50; rabbit anti-TRP [76], 1:100; rabbit anti-InaD [45], 1:200; rabbit anti-Arr2 [52], 1:200; biotin-conjugated Peanut agglutinin (Vector Labs), 1:1,000; Alexa 488–conjugated phalloidin (Invitrogen), 1:200; and Alexa 405–, Alexa 488–, Cy3–, or Cy5–conjugated secondary antibodies (Jackson ImmunoResearch), 1:200.

Blue Light Treatment and Rh1 Detection

Flies were kept in a box with a blue LED (465 nm) light panel (~1,000 lux) for 6 h with or without 24 h recovery in dim white light. For Western blots, fly heads were separated, homogenized,
and incubated with SDS loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue) for 20 min at room temperature. For dot blots, fly heads were homogenized in SDS buffer with 1 M urea, as previously described [36]. Sample buffer was applied to natural cellulose membrane and air dried, followed by antibody detection. A polyclonal rabbit anti-Rh1 antibody (1:2,000) was used to detect Rh1 [34].

S2 Cell Transfection, Immunostaining, and Co-Immunoprecipitation

S2 cells were cultured in Schneider’s media with 10% fetal bovine serum at room temperature and transfected using Lipofectin LTX (Invitrogen). For immunostaining, mouse anti-FLAG (M2, Sigma) and rat anti-HA (Roche) antibodies were used to detect Crag (FLAG) and Rab (HA) proteins. For co-IP, cells were harvested 40 h after infection and lysed with lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and Complete Protease Inhibitor Cocktail Tablets [Roche]). For comparing the binding affinity of Rab11-DN and Rab11-CA with Crag, EDTA was removed from the lysis buffer. Then the cell lysates were incubated with anti-HA affinity gel (Sigma) for 3 h in lysis buffer at 4°C. The anti-HA gel was pelleted and analyzed by Western blot using anti-HA (Roche), 1:1,000, followed by HRP-conjugated secondary antibodies (Jackson Immunoresearch), 1:5,000.

Protein Expression and Purification

Crag cDNA tagged with GST (GE Healthcare) was cloned into a pOPINX vector [77]. The construct was transfected into SF9 cells (Invitrogen), along with Bsu36I-digested BacPAK6 DNA. Recombined viruses were harvested from the medium and amplified with a second round of infection. To express Crag protein, Hi-5 cells (Invitrogen) were infected with the virus and cultured for 40 h before they were harvested and lysed. Crag protein was then purified from the cell lysates with glutathione sepharose 4B (GE Healthcare). Rab5, Rab10, Rab11, and CaM proteins were generated using the same protocol.

GEF Assay

GEF assays were performed as described previously [61]. For preloading with GDP, Rab5, Rab10, and Rab11 were incubated in 110 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.8 mM DTT, 0.005% Triton X-100, and 50 mM BODIPY-GDP (Invitrogen) for 60 min at 30°C. 10 mM MgCl2 was then added to stop the reaction. 0.1 μM preloaded Rab proteins were then incubated with or without 0.05 μM Crag in a 110 mM NaCl, 50 mM Tris-HCl (pH 8.0), 12 mM MgCl2, 0.8 mM DTT, and 2 mM GDP solution. To test whether CaM regulates Crag function, 0.05 μM CaM and 1 mM CaCl2 were added to the above reaction. As a positive control, preloaded Rab11 was incubated with 10 mM EDTA in the above solution. The fluorescence intensity was recorded automatically by a FLUOSTar OPTIMA plate reader (BMG Labtech) every 30 s over a 2-h period.

Supporting Information

Figure S1 Crag mutant photoreceptors target their axons and synapses properly to the medulla, but exhibit subtle defects in ommatidial organization. Crag mutant R7 and R5 photoreceptors target properly to the medulla, y w FRT19A (control), y w Crag P[neoFRT]19A/FM7c, ki-GFP, or y w Crag P[neoFRT]19A/FM7c, ki-GFP female flies were crossed with cl(1) P[neoFRT]19A/Dp(1;Y)Xv+ v+ (3); cy-FLP males. Progenies with large mutant eye patches (>95%) were assayed. 1-d-old fly brains were dissected and stained with Chaoptin antibody (mAb24B10, Developmental Studies Hybridoma Bank). Arrows point to the R7 terminals, and arrowheads point to R5 terminals. No significant differences between the two Crag alleles and the controls were observed. (TIF)

Figure S2 Crag mutant R1–R6 photoreceptors target properly to the lamina, but the terminal portions of the photoreceptors are affected in aged flies upon light exposure. (A and B) TEM of lamina sections in 1-d-old flies of control y w FRT19A or Crag mutant clones shows that Crag mutant R1–R6 photoreceptors target properly to the lamina. Each photoreceptor terminal is outlined by a red circle. Typical terminal structures were observed in Crag mutant clones, including normal capitate projections, active zones, synaptic vesicles, and mitochondria. Scale bar, 1 μM. (C and D) Average number of terminals per cartridge (C) and average number of capitate projections per terminal (D) were calculated, n = 10. There are no significant differences between control and Crag alleles. (E and F) TEM of lamina cross-sections in flies cultured for 2 wk with 12-h on/off light exposure. Red cycles outline the wild-type R1–R6 photoreceptor terminals in (E). However, in Crag mutants, the intracellular structures of photoreceptor terminals are not recognizable (red arrows in [F]). (TIF)

Figure S3 Phototransduction is not affected in dark-raised Crag mutant photoreceptors. (A) Representative pictures of control mosaic eyes used for ERG experiments. y w FRT19A females were crossed with cl(1) P[neoFRT]19A/Dp(1;Y)Xv+ v+ (3); cy-FLP males to generate homozygous clones in the eye. y w FRT19A photoreceptors are marked by white patches. (B) Representative pictures of mosaic eyes that contain homozygous Crag mutant photoreceptors, which are marked by white patches. The corneas of the eyes with Crag mutant clones show some roughness; however, the function of these photoreceptor cells is not affected, as determined by ERG and single-cell recordings. (C) Intracellular recordings of single photoreceptors in response to a brief light stimulus (10 ms). 2-d-old flies raised in the dark were used for the experiments. Each trace is the average of 20 repetitive recordings of the same photoreceptor. Note that control and Crag mutant photoreceptors exhibit similar responses to light. (D and E) Average depolarization amplitude (D) and average decay time (E) were calculated for the single-photoreceptor recordings, n = 6. The decay time was measured from the peak to the 80% repolarization point. There are no significant differences between control and Crag alleles. (TIF)

Figure S4 Rh1 levels gradually decrease in Crag mutant clones in flies raised in light/dark cycle. Fly heads with control or Crag mutant clones in the eyes were dissected from flies that were kept in the dark or in light/dark cycle at different stages. Western blots were performed to detect total Rh1 and Actin (loading control) levels. In Crag mutant clones, the Rh1 levels gradually decrease when the flies are aged in light/dark cycle but not when they are kept in the dark. (TIF)

Figure S5 Blue light triggers a much more severe internalization of Rh1 than white light, and accumulation of Rh1 in Crag mutant photoreceptors is not due to PDA. (A) Western blot of Rh1 and Actin of fly heads dissected...
from flies that were kept in the dark or after 6 h in white light. Note that the Rh1 levels are not significantly altered after 6 h of white light exposure. (B) Whole mount immunostaining of Rh1 in control and Cragmutant photoreceptors exposed to 6 h of white light. Internalization of Rh1 is not obvious in both genotypes when compared to blue-light-triggered endocytosis of Rh1 (see Figure 4C). (C) Whole mount immunostaining of Rh1 in control and Cragmutant photoreceptors kept for 6 h in blue light, 20 min in orange light, and 18 h in the dark. Orange light exposure was used to terminate the PDA caused by blue light exposure. Note that Rh1 accumulates in the cytosol in this paradigm.

Figure S56 TEM of photoreceptor cross-sections of flies exposed to blue light. TEM analyses of control and Cragmutant photoreceptors kept in the dark, exposed to 6 h blue light, or exposed to 6 h blue light with 15 or 36 h recovery in the dark. Note that the electron density of the cytosol correlates with the Rh1 levels in the cytosol (see Figure 4C for immunostaining results). Also note that the rhabdomeres start to break down after 6 h of blue light stimulation and 36 h of dark incubation in Cragmutant photoreceptors. Scale bar, 2 µm.

Figure S57 Screen for potential targets of Crag: the Drosophila Rab proteins. 31 DN Rab transgenes were examined. Rh1-GAL4 was used to drive their expression in the adult eye. ERGs were performed at day 1 and after 21 d of culture in a light/dark cycle or in complete darkness. Expression of Rab1-DN and Rab5-DN leads to a reduction of ERG amplitude in light/dark and dark conditions. Expression of Rab11-DN leads to a reduction of ERG amplitude only when flies are treated with light. No significant differences were observed when other DN-Rabs were expressed in either light condition in our screen.

Figure S58 Mutations in Crag and Rab11 lead to a similar time course of photoreceptor degeneration. (A) Representative ERG traces at different stages in the light/dark cycle of flies of the following genotypes: Rh1-GAL4, UAS-GFP (control); Cragmutant clones with Rh1-GAL4, UAS-GFP (Crag-GFP); Rh1-GAL4, UAS-Rab11-S25N (Rab11-DN); and Cragmutant clones with Rh1-GAL4, UAS-Rab11-S25N (Crag-GFP+Rab11-DN). Note that the ERG amplitudes are gradually decreased when Crag and/or Rab11 function is impaired. (B) Quantification of the ERG depolarization amplitudes shown in (A). Ten ERG traces were measured for each genotype at each time point. Note that when Crag and Rab11 function are both impaired, the degeneration rate of the photoreceptors is not significantly enhanced.

Figure S59 Expression of DENND4A, the human homolog of Crag, rescues the ERG defects and photoreceptor degeneration associated with Crag loss of function. (A) ERG traces of wild-type controls (y w FRT19A), Crag mutants, and Cragmutant flies rescued by expression of DENND4A with da-GAL4. These flies were cultured in a 12-h on/off light cycle for 14 d. Consistent with previous data, the ERG depolarization amplitude is reduced in Crag mutants but is unaffected in both wild-type controls and Cragmutant flies rescued by expression of DENND4A. (B) Quantification of the ERG depolarization amplitudes shown in (A). Ten ERG traces were measured for each genotype. (C) TEM of photoreceptor cross-sections of Cragmutant cells with or without DENND4A expression upon 14 d of light/dark exposure. Rhabdomere structures are severely disrupted in Cragmutant cells but not in Cragmutant cells expressing DENND4A. Scale bar, 1 µm. (D) Quantification of the rhabdomere areas shown in (C). Rhabdome area is significantly smaller in Cragmutant photoreceptors than in Cragmutant photoreceptors expressing DENND4A. Ten ommatidia from different cross-sections were analyzed for each genotype.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: BX HJB. Performed the experiments: BX VB MJ KZ HS WLC TL GD SY. Wrote the paper: BX HJB. YQL GGN. Analyzed the data: BX YQL HJB. Contributed reagents/materials/analysis tools: MJ LD SY. Wrote the paper: BX HJB.

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