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A role for rhodopsin in a signal transduction cascade that regulates membrane trafficking and photoreceptor polarity

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Abstract

This review summarizes the most recent progress in the understanding of the role of rhodopsin C-terminal domain in the regulation of intracellular trafficking and photoreceptor morphogenesis. A proposed cascade of molecular interactions, initiated by the rhodopsin C-terminal sequence VXPX-COOH during trafficking from the Golgi/TGN in retinal photoreceptors, is relayed by the small GTPase ARF4 to the downstream effectors. One of the candidates for an ARF4 effector is the ARF-GAP ASAP1, which may function as a subunit of, or form a novel protein coat involved in trafficking from the TGN and in cytoskeletal remodeling, whose assembly is regulated by the binding of ARF4 to rhodopsin, and whose function is essential for the polarized trafficking toward the ROS.

Keywords: Rhodopsin; Photoreceptor; Retinal degeneration

1. Rhodopsin trafficking to the ROS

The rod outer segment (ROS)[1], a photoreceptor-specific organelle elaborated from a modified primary cilium, encloses a stack of approximately 1000 flattened disk membranes with 10^4–10^6 molecules of rhodopsin/disk. In this environment rhodopsin functions as the light receptor, which activates the phototransduction cascade resulting in membrane hyperpolarization and inhibition of synaptic transmission (Burns & Arshavsky, 2005; Chen, 2005; Ridge, Abdulaev, Sousa, & Palczewski, 2003). Like other membrane proteins, rhodopsin is synthesized in the ER, and further modified in the Golgi. These biosynthetic organelles are localized in the cell body, the rod inner segment (RIS), thus necessitating the long-distance movement of newly synthesized rhodopsin on post-Golgi membranes toward ROS. They fuse with the plasma membrane surrounding the connecting cilium through which rhodopsin is delivered to the ROS.

Vectorial transport of rhodopsin on vesicles was first described in the 1980s (Besharse & Pfenninger, 1980; Defoe & Besharse, 1985; Papermaster, Schneider, & Besharse, 1985; Papermaster, Schneider, Defoe, & Besharse, 1986). Post-Golgi vesicles were subsequently isolated and their characterization was initiated in the 1990s (Deretic & Papermaster, 1991). As recent studies using live microscopy in a variety of cell types revealed that post-Golgi carriers are large pleiomorphic structures, rather than the small vesicles, as previously believed (Hirschberg et al., 1998; Toomre, Steyer, Keller, Almers, & Simons, 2000), we re-named post-Golgi vesicles rhodopsin transport carriers (RTCs). Molecular interactions depicted in Fig. 1 are essential for the generation of, and incorporation of rhodopsin into, RTCs that are properly targeted and rendered competent for fusion with the RIS plasma membrane.

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[1] *Abbreviations used:* adRP, autosomal dominant retinitis pigmentosa; ARF, ADP-ribosylation factor; RIS, rod inner segment(s); ROS, rod outer segment(s); RTC(s), rhodopsin transport carrier(s); TGN, trans-Golgi network.

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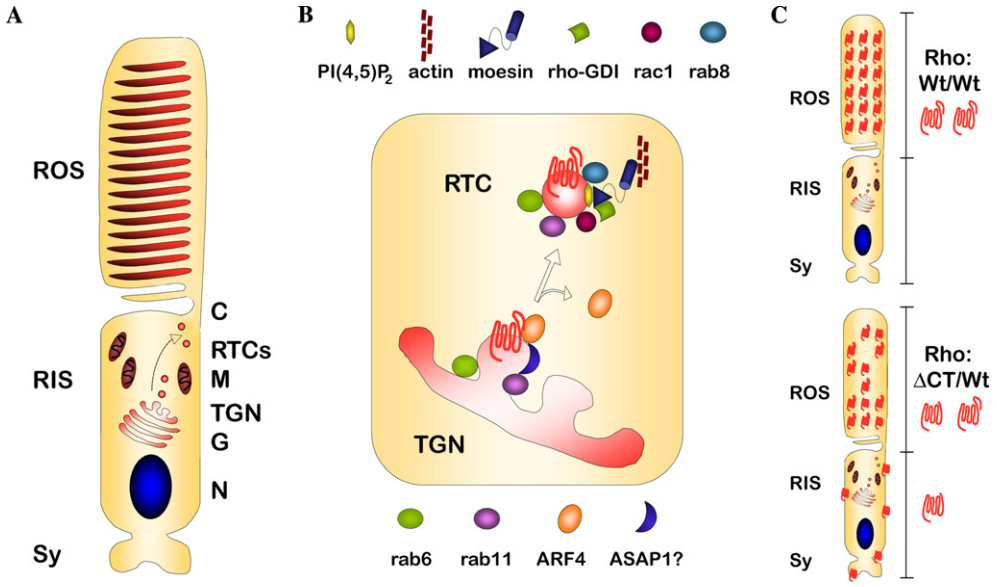

Fig. 1. Molecular interactions essential for the generation of, and incorporation of rhodopsin into, RTCs. (A) Rhodopsin transport carriers (RTCs) move vectorially (dashed arrow) from the Golgi (G)/TGN, through the ellipsoid region filled with mitochondria (M), to the connecting cilium (C), where they fuse with the plasma membrane of the RIS. Newly synthesized rhodopsin is then delivered to the ROS. N, nucleus; Sy, synapse. (B) RTC budding from the TGN is regulated by the small GTPases rab6 and rab11 (Deretic et al., 1996). Rhodopsin C-terminal binds to, and recruits ARF4 to the TGN membrane and this process is essential for RTC budding (Deretic et al., 2005). ARF-GAP ASAP1 is currently investigated as a candidate for the novel coat protein that regulates RTC budding. RTC fusion at the connecting cilium is regulated by PI(4, 5)P_2, actin, moesin, rho-GDI, and the GTPases rac1 and rab8 (Deretic et al., 1995; Deretic et al., 2004; Moritz et al., 2001). Mutations in rab8 inhibit RTC fusion, cause accumulation of RTCs and retinal degeneration. (C) In healthy polarized photoreceptors, expressing two copies of the Wt rhodopsin gene, rhodopsin is sorted in the TGN, incorporated into RTCs, delivered to, and retained in the ROS. In animal models of adRP caused by rhodopsin C-terminal mutations Wt rhodopsin retains its normal subcellular localization in the ROS. By contrast, rhodopsin in which the sorting signal is ablated or mutated, loses its capacity to be recognized by, and to interact with the appropriate sorting machinery at the TGN. Consequentially, mutant rhodopsin follows the "bulk flow" of the membrane arriving randomly to the ROS, RIS and the synapse.

2. Rhodopsin targeting signal VXPX-COOH is a hot spot for adRP mutations

Convergent evidence points to the four highly conserved carboxyl terminal amino acids of rhodopsin as the most important determinant for its incorporation into RTCs, correct intracellular targeting and ROS localization (Concepcion, Mendez, & Chen, 2002; Deretic, Schmerl, Hargrave, Arendt, & McDowell, 1998; Tam, Moritz, Hurd, & Papermaster, 2000). Our studies using the retinal cell-free system that reconstitutes RTC budding from the central sorting organelle of the cell, the trans-Golgi network (TGN), identified the last five amino acids of rhodopsin as the essential targeting sequence (Deretic et al., 1998). In this assay, RTC budding was inhibited by a monoclonal anti-rhodopsin antibody 11D5, whose antigenic site is within the five C-terminal amino acids, and by synthetic peptides corresponding to the C-terminus of rhodopsin (Deretic, Puleo Scheppke, & Trippe, 1996; Deretic et al., 1998). Collectively, recent studies from several groups point to the minimal essential targeting sequence, VXPX-COOH, which interacts with the trafficking machinery thereby assuring correct delivery of rhodopsin (Deretic, 2004).

The integrity of the rhodopsin C-terminal targeting signal VXPX-COOH is crucial for photoreceptor survival. Mutations that affect this highly conserved motif cause particularly severe forms of the neurodegenerative disease autosomal dominant retinitis pigmentosa (adRP), with a significantly faster rate of progression than the adRP caused by other rhodopsin mutations (Berson, Rosner, Weigel-DiFranco, Dryja, & Sandberg, 2002). The severity of the disease allele suggests that the rhodopsin C-terminal domain participates in important cellular processes distinct from phototransduction. The accessibility of this domain is crucial for macromolecular interactions and a Ter349Glu mutation, which is predicted to add an additional 51 amino acids to the C-terminus, renders rhodopsin dysfunctional and causes one of the most severe adRP phenotypes described to date (Bessant et al., 1999). A newly described Ter349Gln adRP variant, which is predicted to add an additional segment of 58 amino acids, is highly likely to be pathogenic as well (Sullivan et al., 2006). V345, A346 and P347 are the sites of C-terminal adRP mutations involving single amino acid substitutions (http://www.retina-international.com/sci-news/rhomut.htm), highlighting their importance for photoreceptor viability.

The C-terminal rhodopsin mutations responsible for adRP were suggested to inhibit its interaction with Tctex-1, a ubiquitous light chain of the molecular motor cytoplasmic dynein (Tai, Chuang, Bode, Wolfrum, & Sung, 1999), but, taken together, studies by the same group revealed that the rhodopsin VXPX-COOH sequence is completely dispensable for Tctex-1 binding (Chuang & Sung, 1998; Tai, Chuang, & Sung, 2001). Thus, it remains controversial if the

proposed rhodopsin–Tctex-1 interaction is in fact related to rhodopsin targeting through the VXPX-COOH sorting signal, whose disruption in rod photoreceptors leads to pathological changes in animal models and in patients with adRP.

3. The NPXXY and the FR motifs are potential regulators of rhodopsin trafficking

In addition to the VXPX-COOH sequence, rhodopsin contains at least two putative sorting signals whose function has not been explored (Fig. 2). First, it contains the NPXXY motif in the cytoplasmic domain of the seventh transmembrane helix, known in other G-protein coupled receptors (GPCRs) as the binding site for ADP-ribosylation factor (ARF) GTPases (Mitchell et al., 1998; Robertson et al., 2003). ARFs are important regulators of membrane trafficking responsible for the recruitment of coat proteins, lipid modifying enzymes and actin cytoskeleton regulators, cooperatively affecting membrane curvature and sorting of cargo molecules into membrane carriers (Donaldson & Jackson, 2000; Nie, Hirsch, & Randazzo, 2003). Second, the cytoplasmic helix H8 of rhodopsin contains a highly conserved ciliary targeting signal, the FR motif (Corbit et al., 2005). Therefore, even rhodopsin with impaired or deleted primary targeting sequence VXPX-COOH retains some information directing its subcellular localization. The existence of the secondary sorting signal, recently implicated in ciliary targeting, underscores the importance of dissecting the hierarchy of signals responsible for the delivery of rhodopsin to ROS.

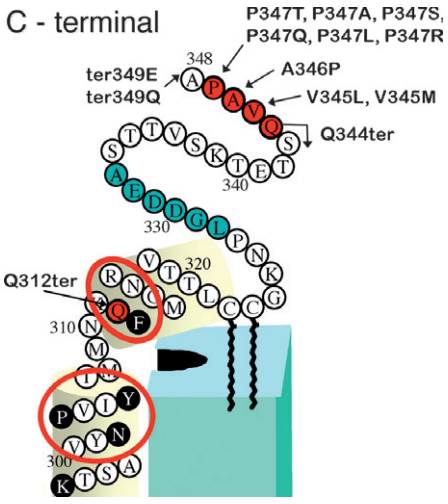

Fig. 2. Schematic representation of the rhodopsin C-terminal domain (modified from (Palczewski et al., 2000), where key residues were shown in black filled circles and residues not modeled in the crystal structure in green filled circles). The VXPX C-terminal sequence is a hot spot for mutations causing adRP, indicated in red, and listed in the figure. Likewise, changes in the termination codon, predicted to add additional amino acids to the C-terminus, lead to adRP. The C-terminal sequence is the antigenic site of the inhibitory mAb 11D5 (Deretic et al., 2005). Two additional domains, possibly involved in interaction with ARF GTPases (helix 7), and in ciliary targeting (helix 8), are circled in red. Truncation mutation disrupting the putative ciliary targeting sequence also results in adRP.

4. Rhodopsin mislocalization, not the absence of rhodopsin in the ROS, is the likely cause of adRP caused by the C-terminal mutations

Phototransduction is not disrupted in animal models of rhodopsin adRP mutants affecting the VXPX-COOH sequence (Osawa & Weiss, 1994; Weiss et al., 1995). Rather, the defective recognition of rhodopsin bearing the C-terminal adRP mutations results in mistargeting due to abnormal incorporation of mutant rhodopsin into RTCs, likely leading to pathological changes in photoreceptor cells in patients with adRP. This was documented in transgenic mice (Li, Snyder, Olsson, & Dryja, 1996; Sung, Makino, Baylor, & Nathans, 1994), as well as in transgenic rats and pigs carrying adRP mutations in the sorting signal in rhodopsin, or transgenic frogs carrying the same mutations in GFP-rhodopsin-C-terminal fusion proteins, where the absence of the correct sorting information resulted in mistargeting to the RIS plasma membrane and the synapse in vivo (Green, Menz, LaVail, & Flannery, 2000; Li et al., 1998; Tam et al., 2000). In the S334ter rhodopsin transgenic rat model of adRP mutant rhodopsin represents only ~10% of the total, yet the RIS plasma membrane labeling is 20-fold higher than in wild-type rats, and the rate of cell death directly correlates with the severity of missorting of mutant rhodopsin (Green et al., 2000). This mechanism is different from the pathology caused by the folding-deficient rhodopsin N-terminal mutants, which induce aggregation and degradation by the ubiquitin proteasome system and affect the maturation of the wild-type (Wt) rhodopsin (Rajan & Kopito, 2005; Saliba, Munro, Luthert, & Cheetham, 2002). The Wt rhodopsin is unaffected by the folding-proficient C-terminal adRP mutants and retains its correct localization in all animal models reported to date. The potential effect of mutant rhodopsin lacking the C-terminal sorting signal on the localization of other putative membrane or soluble cargo normally carried by RTCs is presently unknown.

Rhodopsin bearing the C-terminal adRP mutations is impaired in its interactions with the sorting machinery and is likely to incorporate indiscriminately, by "bulk flow", into post-Golgi carriers destined for different subcellular domains, not only into RTCs. This results in the random delivery of mutant rhodopsin to all photoreceptor domains actively receiving new membrane, including the ROS, as illustrated in Fig. 1C. This untargeted and unregulated delivery clearly favors the ROS, due to the exceptionally high magnitude of new membrane addition. When randomly delivered to the ROS, C-terminal mutants are not expected to cause significant disruption, since they retain light-dependent function and do not interfere with Wt rhodopsin. However, mislocalization of even a fraction of rhodopsin, which represents 90% of newly synthesized protein in photoreceptor cells, is likely detrimental to the function

of other photoreceptor membranes, normally devoid of rhodopsin. Ectopic expression of the large quantity of mutant rhodopsin is likely to significantly alter the physical properties, the composition and the function of affected membranes. The injury caused by the continuous delivery of mutant rhodopsin to non-native membranes is further compounded by the absence of the mechanism for its elimination, since sequestration and endocytosis, the mechanisms utilized for the removal of other GPCRs, are not employed by vertebrate rhodopsin, which is normally removed only through ROS disk shedding and phagocytosis by retinal pigment epithelial (RPE) cells.

Accumulation of mutant rhodopsin triggers interactions that lead to pathological changes in photoreceptor cells, which are followed by apoptotic cell death and retinal degeneration. All rhodopsin C-terminal adRP mutants mislocalize in photoreceptor cells, and it is this mislocalization, rather than the absence of rhodopsin in the ROS, that is the likely cause of the disease. It has been proposed that the light activation of the mislocalized mutant rhodopsin causes retinal degeneration, but recent studies clearly demonstrate that retinal degeneration is independent of light activation (Tam, Xie, Oprian, & Moritz, 2006). A more likely cause of rod cell death is the membrane crowding, particularly at the synapse, caused by the build-up of mislocalized mutant rhodopsin. Fusion of synaptic vesicles with the plasma membrane is tightly coupled to endocytosis of vesicle constituents that are continuously recycled for further use (Ryan, 2006). This task is particularly challenging for neurons firing for sustained periods, such as rod photoreceptors. The accumulation of mutant rhodopsin likely interferes with the dynamic remodeling of the synapse and causes neurite outgrowth in adRP patients and in animal models (Li, Kljavin, & Milam, 1995; Li et al., 1998; Tam et al., 2006), suggesting a dominant negative effect on synaptic membrane turnover that may underlie the dominant inheritance of adRP.

5. Rhodopsin C-terminus also regulates photoreceptor morphogenesis

Expression of rhodopsin is required for photoreceptor morphogenesis, since photoreceptors lacking rhodopsin fail to elaborate light-sensitive membranes and degenerate in humans and in animal models (Humphries et al., 1997; Rosenfeld et al., 1992). Rhodopsin replacement restores normal retinal morphology (McNally et al., 1999). However, replacement of mouse rhodopsin with GFP-C-terminally-tagged human rhodopsin causes defects in photoreceptor morphogenesis and retinal degeneration, (Chan, Bradley, Wensel, & Wilson, 2004), partially mimicking the Ter349Glu adRP mutation, which affects the availability of the rhodopsin C-terminal targeting sequence for molecular interactions. Conversely, transgenic mice expressing truncated rhodopsin missing the C-terminal domain, on the rhodopsin-null background, do not form ROS (Concepcion et al., 2002). Expression of mouse S-opsin, with conserved targeting sequence VXPX-COOH, on the same background, restores ROS morphogenesis (Shi, Concepcion, & Chen, 2004). Therefore, the recognition of the C-terminal targeting sequence not only directs intracellular trafficking, but also triggers the onset of ROS morphogenesis. The molecular mechanisms by which the rhodopsin C-terminal targeting motif initiates ROS morphogenesis are not clear at present, but the small GTPases and their interacting proteins are emerging as candidate regulatory molecules involved in these processes.

6. Rhodopsin C-terminus regulates trafficking by direct binding to the small GTPase ARF4

Our recent study documents direct and specific functional association between the small GTPase ARF4 and the rhodopsin targeting sequence, VXPX-COOH, which is essential for its sorting into RTCs and its polarized trafficking (Deretic et al., 2005). The molecular machinery underlying regulation of membrane traffic by ARF GTPases has been elucidated for the class I ARFs (ARFs 1–3), but the function of class II ARFs (ARFs 4 and 5) remains largely obscure. In their GTP-bound form ARFs bind to membranes to recruit coat proteins. Subsequent GTP-hydrolysis assisted by GTPase-activating proteins, ARF-GAPs, is coupled to cargo sorting, formation of transport carriers and concomitant release of ARF-GDP from the membranes. Recent study suggests that a cooperation of two ARFs at the same site may be a common feature in their regulation of membrane trafficking (Volpicelli-Daley, Li, Zhang, & Kahn, 2005). We have established that ARF4 regulates RTC budding from the Golgi/TGN by direct binding to rhodopsin C-terminus. It remains to be determined if ARF4 cooperates with a class I ARF, which may bind to the NPXXY motif.

The exclusion of ARF4 from the ROS rules out a role for ARF4 in the visual transduction cascade. The intracellular distribution of ARF4 in photoreceptors is consistent with its interaction with rhodopsin during RTC membrane budding, indicating that the Golgi/TGN is the site of interaction of newly synthesized rhodopsin and ARF4. Further, the anti-ARF4 antibody and the peptide corresponding to the α helix 3 of ARF4, which was used to generate the anti-ARF4 antibody, inhibit RTC budding in the in vitro assay to the same extent as the anti-rhodopsin mAb 11D5 and the rhodopsin C-terminal peptide (Deretic et al., 2005). Thus, the effects of blocking ARF4 action are functionally equivalent to the effects of blocking the rhodopsin C-terminal sorting signal. By extension, our data suggest that the failure of rhodopsin C-terminal adRP mutants to bind to ARF4 results in their omission from RTCs, and missorting to the RIS plasma membrane and synapse, as seen in animal models.

7. Rhodopsin-ARF4 interaction may provide spatio-temporal control of the ARF4-GTPase reaction

Our data also raise a possibility that the α helix 3 of ARF4 is involved in the direct interaction with the rhodopsin

C-terminus. The inhibitory anti-ARF4 antibody likely blocks the access of the rhodopsin C-terminal tail, and of the coat proteins to ARF4. Bound anti-ARF4 antibody could prevent GTP hydrolysis and cause longer residency time of ARF4 on the Golgi membranes, thus inhibiting budding of RTCs. In preliminary experiments we have detected an increase in membrane association of ARF4 following incubation with anti-ARF4 antibody. The membrane residency time of ARF4 on the Golgi/TGN could be controlled by the rhodopsin C-terminus, which may regulate the access of an unknown ARF-GAP to ARF4. Thus, rhodopsin, possibly in conjunction with the TGN coat proteins may regulate the ARF4-GTPase reaction, which is necessary for RTC budding.

8. ARF-GAP ASAP1 is a strong candidate for the novel protein coat recruited by ARF4 and involved in rhodopsin trafficking and cytoskeletal remodeling

To identify the ARF-GAP that acts on ARF4, and to explore the involvement of ARF-GAPs in rhodopsin trafficking, we have determined that photoreceptor cells express high levels of an ARF-GAP ASAP1 (Astuto-Gribble, Mazelova, Ransom, Inoue, Randazzo & Deretic, manuscript in preparation). ASAP1 is a member of a family of multifunctional proteins that regulate membrane traffic and actin remodeling (Brown et al., 1998; Che et al., 2005; Randazzo & Hirsch, 2004). Our preliminary data show that ARF4 cooperates with previously identified small GTPase rab11 (Deretic et al., 1996) to control membrane trafficking toward ROS. ARF4 and rab11 likely exert control over each other through arfophilins, the multifunctional regulatory ARF/rab11 binding proteins (Meyers & Prekeris, 2002; Shin, Couvillon, & Exton, 2001; Shin, Ross, Mihai, & Exton, 1999). ARF4 is released from the membrane during RTC budding, ASAP1 dissociates after budding, while rab11 remains associated with RTCs. This sequence of events suggests that ASAP1 may function as a subunit of, or form a novel protein coat recruited by ARF4, regulated by rab11, and involved in rhodopsin trafficking from the TGN. ASAP1 contains numerous protein–protein and protein–lipid interaction domains (Nie et al., 2006; Nie & Randazzo, 2006; Randazzo & Hirsch, 2004). Thus, ASAP1 recruited to the Golgi/TGN by the rhodopsin/ARF4 interaction, may act as a signaling platform for large molecular complexes, including cytoskeleton regulators. The coordinated macromolecular assembly may insure the generation of fusion-competent RTCs, depicted in Fig. 1B, by linking RTC budding with the recruitment of the molecular machinery, including lipid modifying enzymes, moesin and the GTPases rac1 and rab8 (Deretic et al., 2004), necessary for actin polymerization and subsequent RTC fusion.

The cascade of interactions initiated by the recognition of the rhodopsin C-terminal targeting sequence upon its expression in photoreceptor cells is likely to regulate not only rhodopsin trafficking but also to shape and organize the TGN, promote polarized membrane transport through reorganization of membrane cytoskeleton, and direct the trafficking of molecular components involved in photoreceptor morphogenesis and the development of ROS.

9. Neurodegenerative diseases are often caused by the breakdown of membrane–cytoskeleton interactions

Terminally differentiated neuronal cells are highly susceptible to trafficking defects, which frequently cause neurodegenerative disorders. The number of proteins involved in rhodopsin trafficking affected by disease-causing mutations is continuously growing. Our studies identified the small GTPase rab8 as an essential component of the machinery that is involved in RTC docking and fusion with the plasma membrane (Deretic et al., 1995; Moritz et al., 2001). Experimental manipulation of rab8-dependent pathways, through the introduction of mutant rab8 into photoreceptors of transgenic frogs, causes severe retinal degeneration (Moritz et al., 2001). Interestingly, rab8 interacting proteins Optineurin and Huntingtin, are linked to neurodegenerative diseases primary open angle glaucoma (POAG) and Huntington's disease, respectively (Faber et al., 1998; Hattula & Peranen, 2000; Rezaie et al., 2002; Sahlender et al., 2005). Rab8, Optineurin and Huntingtin are collectively involved in the linkage of membrane organelles to the cytoskeleton (Sahlender et al., 2005), suggesting that the breakdown of this linkage may be a common theme in retinal degeneration and in other neurodegenerative diseases. In that light, elucidation of the function of rhodopsin in membrane trafficking and photoreceptor morphogenesis through the recruitment of ARF4, and the cytoskeletal regulator ARF-GAP ASAP1, may shed new light on the mechanism of retinal degeneration caused by carboxyl-terminal adRP mutations in rhodopsin.

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