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Molecular complexes that direct rhodopsin transport to primary cilia

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Abstract

Rhodopsin is a key molecular constituent of photoreceptor cells, yet understanding of how it regulates photoreceptor membrane trafficking and biogenesis of light-sensing organelles, the rod outer segments (ROS) is only beginning to emerge. Recently identified sequence of well-orchestrated molecular interactions of rhodopsin with the functional networks of Arf and Rab GTPases at multiple stages of intracellular targeting fits well into the complex framework of the biogenesis and maintenance of primary cilia, of which the ROS is one example. This review will discuss the latest progress in dissecting the molecular complexes that coordinate rhodopsin incorporation into ciliary-targeted carriers with the recruitment and activation of membrane tethering complexes and regulators of fusion with the periciliary plasma membrane. In addition to revealing the fundamental principals of ciliary membrane renewal, recent advances also provide molecular insight into the ways by which disruptions of the exquisitely orchestrated interactions lead to cilia dysfunction and result in human retinal dystrophies and syndromic diseases that affect multiple organs, including the eyes.

Keywords

Rhodopsin; Trafficking; Cilium; Arfs; Rabs

1. Introduction

Identifying physiological functions of human retinopathy-associated proteins is a long-term goal towards the treatment of blinding diseases. Rhodopsin is the main feature of retinal rod photoreceptors that plays an essential role not only in evoking visual signals, but also in shaping the necessary morphology of photoreceptor cells for the specific signaling processes (Arshavsky & Burns, 2012; Burns & Arshavsky, 2005; Deretic, 2006; Deretic & Wang, 2012; Palczewski, 2012). The phototransduction cascade that propagates visual excitation takes place in the light sensing organelle, the photoreceptor rod outer segment (ROS), which does not form in the absence of rhodopsin (Humphries et al, 1997). The last decade has seen

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a remarkable progress in our understanding of the functional effects of rhodopsin mutations and brought us closer to using this information to devise potential therapies for retinal dystrophies caused by rhodopsin mutations (Mendes et al, 2005; Rakoczy et al, 2011). However, much needs to be learned about the physiological roles of rhodopsin beyond its regulation of the phototransduction cascade in photoreceptor cells. For instance, mutations in the C-terminal domain of rhodopsin cause some of the most severe forms of Autosomal Dominant Retinitis Pigmentosa (ADRP) (Berson et al, 2002; Bessant et al, 1999). This suggests that photoreceptor cells have particularly low tolerance for injuries caused by this category of rhodopsin mutants, even in the presence of generally sufficient quantities of wild type rhodopsin. Moreover, rhodopsin variants that contain mutations within the C-terminal domain can function as light receptors, but fail to rescue ROS morphogenesis in rhodopsin knockout mice that otherwise lack ROS (Concepcion & Chen, 2010; Concepcion et al, 2002). So what is wrong with the photoreceptor cells expressing rhodopsin carrying the C-terminal ADRP mutations? A broad search for answers to this question has recently brought to light a number of physiological interactions that act in concert to localize rhodopsin efficiently and exclusively to the ROS.

2. The origin of the rod outer segment (ROS)

2.1. ROS is a modified primary cilium

The ROS is a light-sensing organelle that arises through a distinctive conversion of the plasma membrane of the sensory cilium, also called the primary cilium. Primary, or non-motile cilia are specialized projections that are found on the cell membranes of almost all eukaryotic cell types where they function to capture a wide range of extracellular signals (Singla & Reiter, 2006). They are exquisitely organized to enclose sensory receptors that are specifically targeted to, and highly concentrated in, the ciliary membranes. Because of the exceptional unidirectional membrane flow, rhodopsin trafficking represents an extreme case of ciliary receptor targeting. The recent appreciation of the high conservation of intracellular trafficking complexes that direct membrane delivery to specific intracellular destinations has provided new insight into the molecular mechanisms of ciliary membrane targeting. For example, it facilitated elucidation of the molecular mechanisms of a broad range of human diseases collectively known as ciliopathies, which are caused by dysfunctional formation and dysfunction of primary cilia that contribute to both retinal degeneration and cystic kidneys and are often associated with obesity, polydactyly and sensory impairments (Arts et al, 2007; Blacque & Leroux, 2006; Cui et al, 2011; Fliegauf et al, 2007; Gerdes et al, 2009; Otto et al, 2005; Wiens et al, 2010).

To fulfill the specialized role in light capture and propagation of visual signals, rhodopsin and associated proteins involved in phototransduction are sequestered in the light-sensing membranes of the ROS. The connection between the ROS and the cell body, or the rod inner segment (RIS), is often called the connecting cilium. Although this is topographically correct considering photoreceptor morphology, it is functionally inaccurate as the entire ROS is in fact a sensory cilium. ROS has no biosynthetic organelles, thus rhodopsin is synthesized in the RIS and delivered to the ROS on membranous carriers that can be readily detected in amphibian photoreceptors in the immediate proximity of the cilia (Fig 1A). A comparison between rod photoreceptor “connecting” cilia and the green algae flagella reveals great structural similarities (Rosenbaum et al, 1999)(Fig 1B and C). Early studies of rhodopsin trafficking have determined that the rhodopsin-bearing vesicles deliver the newly synthesized protein to the base of the cilia (Papermaster et al, 1985; Papermaster et al, 1986) (Fig 1D), and that they fuse with a specialized domain elaborated by the RIS plasma membrane, the Periciliary Ridge Complex (Peters et al, 1983)(PRC, Fig 1E). These studies were originally performed on amphibians because of their extensive ROS membrane turnover compared to mammalian photoreceptors. *Xenopus laevis* and *Rana Berlandieri*

photoreceptors synthesize and transport $\sim 3 \mu\text{m}^2$ and $\sim 1.5 \mu\text{m}^2$ of membrane per minute, respectively, vs. $0.1 \mu\text{m}^2$ of membrane per minute synthesized by rat photoreceptors (Besharse, 1986). Additionally, due to their larger size, light-sensing membranes in amphibians contain 6×10^4 molecules of rhodopsin vs. 2000 molecules of rhodopsin in rats. Although mammalian ROS membrane turnover is much slower, they also have a region that is functionally equivalent to the frog PRC, which is called the periciliary membrane complex (PMC) (Maerker et al, 2008; Yang et al, 2010).

Early studies using GFP-fusion proteins, to address general principals of intracellular trafficking in living cells, indicated that post-Golgi transport involves formation of large complex membrane intermediates, rather than small vesicles, that often stretch into tubules as they move along the cytoskeletal elements (Hirschberg et al, 1998). Similarly, conventional EM analysis revealed that the membranes that carry rhodopsin are not small vesicles of defined size and shape (Deretic & Papermaster, 1991). These rather pleiomorphic structures are thus more accurately named rhodopsin transport carriers (RTCs) (Deretic, 2006). On their way to the cilium RTCs have to meet the challenge of the infinitesimally small fusion area combined with an extremely high membrane influx, which requires a supremely regulated process that enables them to deliver rhodopsin to the exact periciliary location in the photoreceptor cell and nowhere else. This task is further complicated by the densely packed mitochondria (m, Fig 1A and D) that crowd the RTC path to the cilium. Even more obstacles lie at the entrance to the cilium, which is also called the “ciliary gate”. To better understand the selective membrane targeting to and trafficking through the cilia, we need to fully appreciate their structure and function.

2.2. Organization of primary cilia

Following cell division, cilia are elaborated from the centrosome, or the basal body (BB), which is also the microtubule-organizing center (MTOC). The centrosome contains a centriole pair consisting of one modified (mother) centriole that acquires accessory structures such as the pericentriolar material (PCM) and appendages and becomes MTOC competent, and one unmodified (daughter) centriole that does not (Bettencourt-Dias & Glover, 2007) (Fig 1F and G). During ciliogenesis the mother centriole elaborates the axoneme containing a cylindrical array of nine doublet microtubules (MTs). Doublet MTs surround either the two singlet MTs (structure 9C2), as in *Chlamydomonas* flagella (Fig 1H), or zero MTs (structure 9C0), as in rod photoreceptors and other non-motile cilia. Microtubules are connected to the plasma membrane through dense proteinaceous structures that were first discovered in *Chlamydomonas* flagellum and named Intraflagellar Transport complexes (IFTs), and subsequently identified in photoreceptor cilia (arrows in Fig 1B and C) (Rosenbaum et al, 1999). Within the last decade, the conserved function of IFT complexes in photoreceptor ciliogenesis has been firmly established (Baker et al, 2003; Bhowmick et al, 2009; Hudak et al, 2010; Insinna & Besharse, 2008; Krock et al, 2009; Krock & Perkins, 2008; Luby-Phelps et al, 2008; Lunt et al, 2009; Omori et al, 2008; Pazour et al, 2002; Sedmak & Wolfrum, 2010; Sukumaran & Perkins, 2009; Zhao & Malicki, 2011). The distal part of the photoreceptor cilium is greatly enlarged to accommodate the highly structured and tightly packed stacks of light sensing disk membranes (Fig 1A, C and D). The “connecting” cilium of rod photoreceptors corresponds to the transitional zone of the primary cilia and flagella. The transitional zone is very narrow, approximately 300 nm, and filled with doublet microtubules that underlie the ciliary plasma membrane, to which they are connected through Y-shaped cross-linkers that form a unique organization termed the ciliary necklace (Besharse et al, 1985; Gilula & Satir, 1972; Horst et al, 1987).

The basal body is connected to the ciliary membrane via transition fibers at the base of the cilium (Fig 1H). Notably, transition fibers are actually wing-like sheets that obstruct most of the space leaving only 60 nm openings at the ciliary base (Anderson, 1972). This geometry

led Nachury and colleagues to rule out the possibility of vesicular traffic through the ciliary pores, because the majority of known transport vesicles exceed 60 nm (Nachury et al, 2010). Furthermore, the ciliary pore complex has been likened to the nuclear pore complex, because the components of the nuclear import machinery, including the Ran GTPase, importins and nucleoporins were found to regulate ciliary entry (Dishinger et al, 2010; Kee et al, 2012). The obstruction of the proximal end of the basal body, together with the densely packed transition fibers and transition zone protein complexes makes the entry into the cilia a highly selective process, leading to naming of the cilia as the “privileged” compartments (Rosenbaum & Witman, 2002). However, this barrier does not generally restrict the entry of soluble proteins into the photoreceptor cilia. Recent studies show that the concentration of GFP monomers, dimers, and trimers expressed transgenically in frog rods is regulated by steric volume exclusion, which is primarily dictated by the ROS architecture and the constrained spaces between the stacks of light sensing disk membranes (Calvert et al, 2010; Najafi et al, 2012). Nevertheless, these model soluble proteins have no interacting partners in the ROS and the ciliary entry of the resident soluble ROS proteins may be under more complex regulation.

2.3. The ciliary gate

The ciliary membrane is topologically continuous with the plasma membrane, yet the diffusion of membrane proteins across the ciliary base is restricted by the periciliary diffusion barrier, or the ciliary gate. The cilia-mediated signaling entails transport of specific membrane components to the base of the cilia followed by the selective admission through the ciliary gate (Christensen et al, 2007; Emmer et al, 2010; Leroux, 2007; Nachury et al, 2010; Rosenbaum & Witman, 2002). The formation of the periciliary diffusion barrier involves specific lipid ordering, septin rings and the NPHP-JBTS-MKS ciliopathy complexes located at the transition zone, which are linked into networks that build and maintain primary cilia (Chih et al, 2012; Craige et al, 2010; Garcia-Gonzalo et al, 2011; Garcia-Gonzalo & Reiter, 2012; Hu et al, 2010; Nachury et al, 2010; Sang et al, 2011; van Reeuwijk et al, 2011; Vieira et al, 2006; Williams et al, 2011). Consistent with the role in ciliary morphogenesis, mutations in NPHP-JBTS-MKS transition zone proteins cause nephronophthisis (NPHP), Joubert syndrome (JBTS) and Meckel syndrome (MKS). In retinal photoreceptors, NPHP2, NPHP5 and MKS1-related protein B9d2 interact with IFT complexes to support the ciliary transport of rhodopsin (Zhao & Malicki, 2011). Taken together these insights strongly suggest that to gain access to the privileged ciliary compartment, rhodopsin needs a “password” to get through the ciliary gate. As it turns out, the rhodopsin sequence encodes more than one password and these passwords are conserved among other ciliary sensory receptors.

3. Rhodopsin trafficking to the primary cilium

3.1. The rhodopsin transport carriers (RTCs)

The unique topological organization of photoreceptor cells, as illustrated in Fig 2A, facilitates isolation and characterization of specific membranes that carry rhodopsin to the cilia and the ROS. Because the volume of ROS membrane trafficking is much higher in amphibians than in mammals (Besharse, 1986), frogs offer a unique model where biochemical and morphological data can be correlated in a single experimental system for the study of the basic mechanisms underlying photoreceptor membrane biogenesis. Subcellular fractionation of radiolabeled frog retinas to detect and identify proteins that associate with newly synthesized rhodopsin during trafficking through the RIS has been fruitful in revealing the identity of important regulators of rhodopsin trafficking such as the small GTPases Rab6, Rab8 and Rab11 (Deretic et al, 1995; Deretic & Papermaster, 1993; Deretic et al, 1996). The photoreceptor Golgi and the trans-Golgi network (TGN)

membranes as well as the rhodopsin transport carriers (RTCs) can be prepared following sequential centrifugation and fractionation steps on sucrose density gradients (Deretic & Mazelova, 2009)(Fig 2B). Characterization of these membranes has expanded our understanding of the regulators of rhodopsin targeting to the cilia. Establishment of the retinal cell-free system that reconstitutes rhodopsin trafficking in vitro permitted identification of the ciliary sorting signal of rhodopsin, its interacting protein Arf4 and the ciliary targeting complex organized by Arf4 (Deretic et al, 1998; Deretic et al, 2005; Mazelova et al, 2009a). Most recent studies using this system established that rhodopsin co-immunoprecipitates from the Golgi/TGN membranes with the small GTPases Arf4 and Rab11, and the Arf GAP ASAP1 (Fig 2C) (Wang et al, 2012). These findings support the notion that these proteins are a part of the ciliary targeting complex that recognizes rhodopsin at the TGN (Mazelova et al, 2009a; Wang et al, 2012). Collectively, these studies expanded fundamental understanding of the intersection between rhodopsin trafficking, ciliary targeting and ROS morphogenesis and permitted identification of a more common mechanism involved in ciliary targeting, shared by rhodopsin and other sensory receptors. They showed that rhodopsin is not just a passive cargo that is sorted into post-Golgi carriers but that it actively recruits the sorting machinery and regulates its trafficking, a concept that has not yet been proven for other sensory membrane proteins.

3.2. The rhodopsin VxPx ciliary targeting signal

The rhodopsin VxPx ciliary targeting signal (CTS) directly binds the small GTPase Arf4 to direct rhodopsin to the primary cilia (Deretic et al, 2005). CTS VxPx is highly conserved among ciliary sensory receptors, including polycystins 1 and 2 and the cyclic nucleotide-gated olfactory channel CNGB1b subunit (Deretic et al, 1998; Deretic et al, 2005; Geng et al, 2006; Jenkins et al, 2006; Ward et al, 2011), as depicted in Fig 2D. Numerous mutations affecting the CTS VxPx cause the most severe forms of ADRP. Modeling of ADRP mutations in transgenic animals has unequivocally confirmed the crucial role of this signal in the delivery of rhodopsin to the ROS. Transgenic mice, frogs, rats and pigs carrying mutations in the VxPx domain all develop retinal degeneration that is preceded by mislocalization of rhodopsin to multiple cellular compartments, including the photoreceptor synapse, which is normally completely devoid of rhodopsin (Concepcion & Chen, 2010; Concepcion et al, 2002; Green et al, 2000; Lee & Flannery, 2007; Li et al, 1996; Li et al, 1998; Ng et al, 2008; Sommer et al, 2011; Tam et al, 2000). The presence of mislocalized rhodopsin likely causes retinal degeneration in transgenic rods through the impairment of normal cellular functions that depend on the functional compartmentalization of photoreceptor cells. Notably, light activation of rhodopsin is not required for these detrimental effects (Tam et al, 2006).

In addition to rhodopsin, the only other ROS constituent that has a reported functional VxPx targeting signal is retinol dehydrogenase, a membrane-associated protein (Luo et al, 2004). In contrast, other ROS membrane components rely on different targeting signals and/or mechanisms. For example, the targeting of cyclic nucleotide-gated (CNG) channels that localize exclusively to the ROS plasma membrane requires interaction with ankyrin-G (Kizhatil et al, 2009). Targeting of peripherin/rds to ROS requires its C terminus, and particularly the valine at position 332, although the protein that recognizes this motif has not been identified (Salinas et al, 2013; Tam et al, 2004). Notably, NPHP and MKS proteins that regulate the ciliary transport of rhodopsin are not involved in the transport of peripherin/rds (Zhao & Malicki, 2011). Guanylyl cyclase 1 (GC1) appears to have a disperse targeting signal and may be cotransported to ROS with other proteins, possibly including rhodopsin (Bhowmick et al, 2009; Karan et al, 2011). Readers are referred to the recent comprehensive review (Pearing et al, 2013) for further information on sorting, targeting and trafficking of other ROS proteins in photoreceptor cells.

3.3. The rhodopsin FR ciliary targeting signal

The second CTS of rhodopsin is comprised of amino acids phenylalanine and arginine (FR) within the cytoplasmic helix 8 of rhodopsin. In addition to rhodopsin, this so-called CTS FR is present in ciliary-targeted GPCRs such as Smoothed and SSTR3 (Corbit et al, 2005) (Fig 2D). The CTS FR has recently been identified as a recognition motif for the Arf GTPase activating (GAP) protein ASAP1, an Arf4 regulatory protein that is a component of the ciliary targeting complex (Wang et al, 2012). ADRP mutations also affect the cytoplasmic helix 8 of rhodopsin, but the direct involvement of the FR motif in its ciliary targeting in rod photoreceptors still awaits proof. Nonetheless, this signal appears essential for the delivery to primary cilia of mouse kidney IMCD3 cells, of a fusion protein comprised of bovine rhodopsin and eGFP, followed by the eight C-terminal amino acids of rhodopsin containing the VxPx motif (Rh-GFP-VxPx). In contrast to the ciliary targeted Rh-GFP-VxPx, the fusion protein in which the CTS FR is replaced with alanines, designated [FR-AA]Rh-GFP-VxPx, does not interact with ASAP1 and consequently does not localize to cilia (Wang et al, 2012).

It is important to note that the recent discoveries of the conserved ciliary targeting signals, and conserved protein complexes with which they interact, made possible the use of ciliated epithelial cells for the studies of ciliary trafficking of rhodopsin. This model system, though artificial, has greatly facilitated identification of targeting motifs and of regulatory factors through the use of siRNA that cannot be transfected into mature photoreceptors. However, although the ciliary targeting machinery is conserved, caution must be taken in the studies of rhodopsin trafficking in epithelial cells in culture, particularly if they have not elaborated primary cilia. This is exemplified by the over expression of rhodopsin in epithelial MDCK cells that resulted in its targeting to the apical plasma membrane, instead of primary cilia (Chuang & Sung, 1998). Moreover, the insight that the promulgated apical localization of rhodopsin in MDCK cells is non-physiological is highlighted by reports demonstrating that IMCD3 and hTERT-RPE1 cells expressing low levels of Rh-GFP-VxPx do, in fact, recapitulate the specific and restricted ciliary localization of rhodopsin (Trivedi et al, 2012; Wang et al, 2012). The selective ciliary localization of Rh-GFP-VxPx indicates that in these epithelial cells the endogenous Arf4 and ASAP1, the crucial regulators of ciliary transport, correctly recognize the rhodopsin VxPx and FR targeting signals.

4. Regulators of rhodopsin trafficking to the primary cilium

4.1. The Arf family of GTPases

Intracellular membrane transport is regulated by the small GTPases of the Rab and Arf families, which function as molecular switches and play a central role in organizing membrane trafficking and directed delivery of membrane cargo to the site of its function. Arf4 belongs to the Arf family of small GTPases that includes Arf, Arf-like (Arl) and Sar proteins (Kahn et al, 2006). In general, Arfs regulate membrane trafficking, lipid metabolism, organelle morphology and cytoskeleton dynamics (Donaldson, 2005; Kahn et al, 2006)(Fig 3). They normally function through the activation-inactivation cycles regulated by Arf guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In their GTP-bound conformation Arfs are membrane associated as a result of the N-terminal “myristoyl switch” that tightly couples activation and membrane binding. Due to the intrinsically low rate of GTP hydrolysis by Arfs, Arf GAPs are critical for their inactivation and are often incorporated into protein coats (Lee et al, 2004). The downstream effectors of Arfs in membrane targeting include protein adaptors and the coat complexes such as COPI, COPII, GGA and AP1–4 that shape the membranes in preparation for budding of distinct transport carriers (Donaldson & Jackson, 2011; Kahn et al, 2006). The mammalian Arfs consist of six isoforms (Arf1–Arf6). Arfs1–5 are associated with the Golgi

and Arf6 functions at the plasma membrane. In addition to the broad function in the Golgi, Arf4, Arl3, Arl6 and Arl13b are also strongly implicated in specialized membrane transport, particularly in membrane targeting to primary cilia (Donaldson & Jackson, 2011; Mazelova et al, 2009a; Nachury et al, 2007)(Fig 3). In addition to the Arf4-regulated ciliary targeting complex, the BBSome complex comprised of seven highly conserved proteins affected by mutations causing the Bardet-Biedl syndrome is also involved in ciliary targeting as an effector of Arl6 (Jin et al, 2010; Nachury et al, 2007). Furthermore, Arl3 and Arl13b are involved in ciliogenesis as well, and their impaired function is responsible for Retinitis Pigmentosa 2 (RP2) and Joubert syndrome, respectively. RP2 protein is a GAP for Arl3 that regulates the delivery of lipidated proteins to the ROS by releasing the ciliary cargo from the lipid moiety-binding protein UNC119 (Ismail et al, 2012; Schwarz et al, 2012; Veltel et al, 2008; Wright et al, 2011; Zhang et al, 2011). Intriguingly, Arl13b is a part of a separate network that regulates ciliary targeting of lipid-modified proteins, which is affected in JBTS and NPHP (Humbert et al, 2012). Thus, even the limited number of ubiquitous regulatory proteins such as Arfs can regulate very specific cellular processes through interactions with a particular set of regulators and effectors.

4.2. The Arf4-based ciliary targeting complex

The small GTPases Arf4 and Rab11, the Arf GAP ASAP1 and the Rab11-Arf interacting protein FIP3 comprise the targeting complex that first recognizes the ciliary targeting motifs of rhodopsin at the TGN (Mazelova et al, 2009a; Wang et al, 2012). A model for the sequential formation of the ciliary targeting complex was generated by combining the known crystal structures and the available information on the sites of various protein-protein interactions (Barrowman et al, 2010; Burguete et al, 2008; Dong et al, 2007; Eathiraj et al, 2006; Ismail et al, 2010; King et al, 2012; Munson & Novick, 2006; Palczewski et al, 2000; Zhu et al, 2007). As illustrated in Fig 4, this model suggests that the molecular interactions taking place en route to cilia likely proceed in the following order: Arf4 is activated by the known Arf-GEF GBF1 (Lowery et al, 2013; Szul et al, 2007), which colocalizes with Arf4 at the photoreceptor Golgi (Wang and Deretic, manuscript in preparation). At the TGN, activated Arf4 interacts directly with the rhodopsin VxPx CTS (Deretic et al, 2005), initiating Stage I of the assembly of the rhodopsin ciliary targeting complex. ASAP1 then recognizes the FR CTS of rhodopsin and forms a complex with activated Arf4 and rhodopsin (Wang et al, 2012). ASAP1 selectively binds Rab11a and the Rab11-Arf effector FIP3. While mediating GTP-hydrolysis on Arf4, ASAP1 likely induces membrane deformation through its BAR domain. Stage I is completed by the proofreading of the rhodopsin CTSs through GTP hydrolysis on Arf4 by ASAP1, which is expected to be assisted by FIP3. At this point rhodopsin is approved for incorporation into RTCs. However, the nascent RTCs still do not contain the full information on their cellular destination, which is necessary to navigate their way to the cilium. This information is provided in Stage II of the complex assembly (Fig 4). Following GTP hydrolysis, inactivation and dissociation of Arf4, ASAP1 and Rab11a remain associated at the TGN where they recruit Rab8 and its GEF Rabin8 (Wang et al, 2012). At this point the nascent RTCs are endowed with the cellular address, because in its active conformation Rab8 regulates the final stages of polarized membrane traffic, fusion of ciliary-targeted carriers and ciliogenesis (Bryant et al, 2010; Deretic et al, 1995; Moritz et al, 2001; Murga-Zamalloa et al, 2010; Nachury et al, 2007; Wang et al, 2012; Yoshimura et al, 2007). Activation of Rab8 by Rabin8 likely takes place during budding of RTCs, which renders them competent for fusion with the periciliary plasma membrane. Multiple lines of experimental evidence support this sequence of events, as already discussed and further explained below.

The Arf GAP ASAP1 is the centerpiece of the Arf4-based ciliary targeting complex. ASAP1 is a crucial GAP and an effector of Arf4 that couples the GTP hydrolysis on Arf4 with

rhodopsin incorporation into nascent RTCs. The effector function of ASAP1 essentially regulates rhodopsin trafficking, as additional Arf GAPs can substitute for ASAP1 and hydrolyze GTP on Arf4, but they cannot fulfill the effector function of ASAP1 (Mazelova et al, 2009a). It is now clear that the inability of other Arf GAPs to substitute for the effector function of ASAP1 is partially due to the direct interaction of ASAP1 with rhodopsin.

ASAP1 is a large (~130 kDa) multifunctional protein that coordinates membrane trafficking and actin remodeling (Nie & Randazzo, 2006; Randazzo & Hirsch, 2004). In ciliary trafficking, ASAP1 is an essential scaffold that promotes communication between the Arf and Rab GTPases (Mazelova et al, 2009a; Wang et al, 2012). The dimeric nature of ASAP1 and its multiple protein and lipid interactions provide an ideal platform that links Arf activation with the assembly of the ciliary targeting complex. ASAP1 is a coincidence detector for activated Arfs, acidic phospholipids and PI(4,5)P₂. In addition to the Arf GAP domain that mediates GTP hydrolysis on Arf GTPases, ASAP1 contains the N-terminal BAR (Bin/amphiphysin/Rvs)(Peter et al, 2004) domain, pleckstrin homology (PH), SH3, and a proline-rich domain. BAR domains recognize acidic phospholipids and are involved in sensing and/or inducing membrane curvature. The BAR domain of ASAP1 mediates membrane tubulation and homodimerization and acts as an autoinhibitor of its GAP activity (Jian et al, 2009; Nie et al, 2006). The PH domain of ASAP1 binds PI(4,5)P₂, which also regulates its GAP activity (Brown et al, 1998; Che et al, 2005). ASAP1 interacts with FIP3, which is a member of the family of Arf- and Rab11-interacting proteins (Hales et al, 2001; Junutula et al, 2004) and a component of the Arf4-based ciliary targeting complex (Mazelova et al, 2009a). Binding of the Arf/Rab11 effector FIP3 to the BAR domain of ASAP1 stimulates its Arf GAP activity (Inoue et al, 2008). Thus, FIP3 may regulate late stages of the assembly of the rhodopsin ciliary targeting complex at the TGN. It may function to terminate Stage I, by partially disassembling or rearranging the complex upon GTP hydrolysis on Arf4, thus allowing the subsequent recruitment of Rab8 and its GEF Rabin8.

4.3. The Rab family of GTPases

The Rab family members comprise another set of small GTPases that are key regulators of membrane trafficking, which are specifically localized to different membrane domains where they regulate the delivery of cargo to their particular intracellular destinations (Stenmark, 2009). Like Arfs, Rab family GTPases are also regulated by GEFs and GAPs that cooperatively control intracellular membrane traffic (Mizuno-Yamasaki et al, 2012). Through the recruitment of specific effectors, Rabs provide the identity to transport membranes and organize functional networks that link together the different stages of a specific transport pathway (Zerial & McBride, 2001).

Functional networks of Rab GTPases are frequently disrupted by disease-causing mutations. A prominent example is the deficiency of Rab Escort Protein-1 (REP1), a subunit of Rab geranylgeranyl transferase (GGTase) that mediates the posttranslational isoprenylation and membrane attachment of Rab GTPases (Fig 5). Defects in REP1 function have been established as a cause of choroideremia, a disease that affects photoreceptors, RPE and the choroid (Pereira-Leal et al, 2001; Seabra et al, 1993; Seabra et al, 1995; Seixas et al, 2013). Notably, different Rab GTPases are responsible for the defects in each cell type that lead to cell specific, yet cooperative retinal degeneration (Tolmachova et al, 2006; Tolmachova et al, 2010). Whereas imperfect prenylation of Rab27 leads to the defects in the RPE and choriocapillaris, the identity of the underprenylated Rab causing photoreceptor degeneration remains to be established (Tolmachova et al, 2006). However, based on the available data some predictions about the identity of the underprenylated photoreceptor Rab are possible. In the neuroretina specific phenotype, the loss of function of REP1 includes a significant shortening of the ROS and the severe photoreceptor degeneration is linked to

underprenylation of one of the larger (~24 kDa) Rabs (Tolmachova et al, 2006). Based on these data, Rab6, or a closely related Rab, is a likely candidate for the photoreceptor-specific REP1-sensitive target. Rab6 is a 24 kDa protein associated with the trans-Golgi that is involved in rhodopsin trafficking and ROS membrane renewal (Deretic & Papermaster, 1993). Furthermore, dissociation of Rab6 from the membranes by the excess of the Rab GDP Dissociation Inhibitor (Rab-GDI), which is functionally comparable to the deficiency in isoprenylation, causes complete inhibition of rhodopsin trafficking in vitro (Deretic et al, 1996). In addition to the defects in Rab protein prenylation, photoreceptor cell death in rd1 retina is also caused by the reduced expression of Golgi-associated Rab-accessory protein, Prenylated Rab Acceptor 1 (PRA1) (also denoted as GDF in Fig 5), which prevents the removal of Rab GTPases from the membrane by Rab-GDI (Dickison et al, 2012). This further underscores the importance of functional Rab proteins for photoreceptor homeostasis.

4.4. The Rab11-Rabin8-Rab8 ciliogenesis cascade

The functional networks of Rab GEF and GAP cascades and positive-feedback loops generated by GEF-effector interactions are exceptionally conserved. For instance, the Rab11-Rabin8-Rab8-Sec15 ciliogenesis cascade (Feng et al, 2012; Knodler et al, 2010; Wang et al, 2012; Westlake et al, 2011) completely parallels the Ypt32p-Sec2p-Sec4p-Sec15p cascade that regulates the yeast budding pathway (Medkova et al, 2006; Mizuno-Yamasaki et al, 2012; Ortiz et al, 2002) (Fig 6). Rab8 is the ultimate Rab GTPase within the ciliogenesis cascade and also regulates the final stages of polarized membrane traffic, carrier fusion and lumenogenesis (Bryant et al, 2010; Deretic et al, 1995; Moritz et al, 2001; Murga-Zamalloa et al, 2010; Nachury et al, 2007; Wang et al, 2012; Yoshimura et al, 2007). Rab11a is the penultimate Rab GTPase in these processes, and is essential for the activation of Rab8 (Knodler et al, 2010; Westlake et al, 2011). Rab8 directly interacts with the ciliary cargo, including rhodopsin, Polycystin 1 and fibrocystin (Follit et al, 2010; Wang et al, 2012; Ward et al, 2011). An extraordinary array of retinopathy-associated ciliary proteins is connected to Rab8: 1) RPGR, which is a Rab8 GEF mutated in XLRP (Murga-Zamalloa et al, 2010); 2) the BBSome (Nachury et al, 2007) and 3) CEP-290/NPHP6 that interacts with centriole-distal-end microtubule-binding protein CEP162 and organizes NPHP, MKS and JBTS complexes at the transition zone (Kim et al, 2008; Rachel et al, 2012; Sang et al, 2011; Tsang et al, 2008; Wang et al, 2013). Rab8 also appears to be functionally linked to tubby-like protein 1 (tulp1) and mutations in the TULP1 gene cause autosomal recessive RP and LCA (Hagstrom et al, 1998). In tulp1 knockout mice, rhodopsin and cone opsins are mislocalized, and membranes containing rhodopsin accumulate in the inter-photoreceptor space (Hagstrom et al, 1999). This peculiar phenotype likely arises from the massive accumulation of defective RTCs and also resembles transgenic rods expressing rhodopsin mutant P347S (Li et al, 1996). Notably, Rab8 and Rab11 display abnormal distribution in tulp1 knockouts although the Golgi complex appears unaffected (Grossman et al, 2011). Furthermore, the differential distribution of ROS constituents in these mice suggests a possible role for Tulp1 in the ciliary transport of specific membrane proteins including rhodopsin and GC1, but not peripherin/rds, supporting their different ciliary routes (Grossman et al, 2011; Hagstrom et al, 2012). Tubby, the founding member of the tubby-like family of proteins, appears to function like Tulp1, and mutations in tubby cause retinitis pigmentosa, hearing loss and obesity (Sun et al, 2012). A comparable phenotype is also observed in cc2d2a mutant zebrafish, a model for Joubert syndrome, where cc2d2a localized at the photoreceptor cilium has a role in Rab8-dependent trafficking and fusion (Bachmann-Gagescu et al, 2011).

The guanine nucleotide exchange factor (GEF) Rabin8 is the principal activator of Rab8 (Hattula et al, 2002). Rab11a directly activates Rabin8, which in turn activates Rab8 in a

cascade of interactions similar to the yeast Rab cascade (Knodler et al, 2010; Ortiz et al, 2002; Westlake et al, 2011). In rod photoreceptors, Rabin8 colocalizes with Rab11 and ASAP1 at the TGN (Wang et al, 2012). Rabin8 also associates with the basal body (Nachury et al, 2007), and with Rab11-containing carriers (Wang et al, 2012; Westlake et al, 2011). Rabin8 has to be actively recruited to the membrane to regulate Rab8 activity, by mechanisms that involve Rab11a, specific phospholipids and a serine/threonine kinase NDR2 (also called STK38L) (Chiba et al, 2013). Recently, NDR2 was identified as a canine retinal degeneration gene corresponding to the human ciliopathy Leber congenital amaurosis (LCA) (Berta et al, 2011; Goldstein et al, 2010). Based on its multiple protein and lipid interactions, Rabin8 has the potential to act as a scaffold in ciliary targeting while forming a physical bridge between Rab11a and Rab8. Rabin8 likely acts as a homodimer and interacts directly with the transport protein particle (TRAPP)II complex implicated in intra-Golgi transport (Westlake et al, 2011), and with the Sec15 component of the membrane tethering complex named exocyst (Feng et al, 2012). Rabin8 binds ASAP1 directly and independently of Rab11, and forms a complex with ASAP1 and the inactive (GDP-bound) form of Rab8 (Wang et al, 2012). These particular associations indicate that ASAP1 serves as a platform upon which Rab11-Rabin8-Rab8 ciliogenesis cascade functions, ensuring the spatially restricted activation of Rab8 during ciliary targeting. In support of this concept, rhodopsin FR-AA mutant defective in ASAP1 binding is unable to interact with Rab8 and fails to translocate across the periciliary diffusion barrier (Wang et al, 2012).

4.5. Validation of new regulators of rhodopsin targeting

Validation of protein-protein interactions involved in rhodopsin trafficking to primary cilia was recently facilitated by the advent of the Proximity Ligation Assay (PLA) (Soderberg et al, 2006). The PLA can reveal the distribution of individual endogenous interacting protein pairs within individual cells and even allows detection of very transient interactions. The PLA is based on the dual recognition of a target protein complex by two primary antibodies detected by specific secondary antibodies attached to oligonucleotides, generating a binary output in a form of red fluorescent dots of approximately 0.5 μm in size, which are visualized by microscopy (Fig 7A) (Soderberg et al, 2006). The positive signal is possible if the two secondary antibodies are less than 16 nm away from each other, a distance that is comparable to the distance at which resonance energy transfer occurs between fluorophores (5–10 nm), and is well within the limits of a protein-protein interaction. The maximum distance between the detected protein pairs is approximately 30 nm, including the size of the two antibodies and the oligonucleotides connecting them, which is within the size range of the ciliary targeting complex (see Fig 4). The PLA is particularly suitable for the analysis of the layered structure of vertebrate retinas (Wang et al, 2012), because of the precise alignment of the highly polar rod photoreceptor cells within a single retinal layer that is readily identified by DIC and nuclear staining (Fig 7B and C). The retinas treated with an antibody to rhodopsin and an unrelated protein show no red fluorescent signal in the photoreceptor cell layer (Fig 7B), unlike the rhodopsin-ASAP1 pair that shows protein-protein interactions in the RIS (Fig 7C). Specific organelle markers can be used to aid the subcellular organization of the interaction sites (Fig 7D–F). As illustrated in Fig 7I, the fluorescent signals detected in the RIS suggest that rhodopsin-Arf4 interaction sites are almost completely associated with the Golgi, a conclusion that is further supported by the subsequent staining of PLA-treated retinas with an antibody to Rab6, a trans-Golgi specific marker (Fig 7G and H). By contrast, Rab11-ASAP1 interaction sites are predominantly associated with the nascent buds at the TGN and the RTCs. Rab11-ASAP1 interaction sites nearly outline the path to the cilia (Fig 7J), in agreement with the previous results of Rab11-ASAP1-Rab6 triple labeling (Mazelova et al, 2009a) (Fig 7F). Quantification of the fluorescence signals in the RIS is possible using as a guide the border of the ellipsoid (E) and myoid (M) region, which is readily detectable in the DIC image. The red dots within the

myoid can be considered as the Golgi/TGN interaction sites and those in the ellipsoid as the RTC interaction sites. As the number of rhodopsin interaction sites at the Golgi/TGN falls concomitantly with the increase of RTC interaction sites the further downstream the protein functions in rhodopsin trafficking, this type of analysis can provide valuable information about the order of protein interactions en route to cilia (Fig 7K). For example, interaction between rhodopsin and Arf4 takes place almost exclusively at the Golgi, whereas ASAP1 remains on the RTCs. Other regulatory proteins follow ASAP1, culminating in the rhodopsin interaction with Rab8, which regulates the final stages of ciliogenesis and ciliary targeting.

As the new photoreceptor protein interaction networks are emerging through biochemical, molecular and high content analysis (Kiel et al, 2011; Kwok et al, 2008; Liu et al, 2007), it is essential to validate these associations using methodologies that can reveal precise subcellular localization of stable and transient protein-protein interactions in situ, such as PLA, which was used to demonstrate light-dependent phosphorylation of melanopsin in the mouse retina (Blasic et al, 2012) and to validate interactions within the complexes that regulate ciliary targeting of rhodopsin (Wang et al, 2012). It is anticipated that PLA and similar methodologies will provide further understanding of protein-protein interactions governing membrane trafficking in retinal rod photoreceptors.

Novel insights into the ciliary trafficking pathways made possible spatio-temporal modeling of the complexes that regulate ciliary targeting from the Golgi/TGN. Combining the available structural and functional information a model of the topology of signaling junctions at the Golgi/TGN has been conceptualized (Deretic, 2013). This model, modified to account for the photoreceptor-specific environment, is presented in Fig 8. The model predicts a cascade of events in which the scaffold proteins that interact with Arf and Rab GTPases envelop the membranes in successive layers. The assembly of protein complexes is initiated with ASAP1 that binds rhodopsin and phospholipids directly, and concludes with entrance of the multimeric complex, the exocyst, which likely participates in RTC tethering to the periciliary plasma membrane. This testable model will provide basis for future experimental approaches aimed at the identification and validation of new regulators of rhodopsin trafficking.

5. Intersection of regulators of rhodopsin targeting to the primary cilium

5.1. The Arf4-based ciliary targeting complex and the IFTs

It is plausible that the different functional networks involved in ciliary targeting overlap in their signaling routes. If so, it would be expected that the ciliary targeting complex interacts at some point with Intraflagellar transport (IFT) components. IFTs are involved in the photoreceptor ciliary membrane transport along axonemal microtubules (Pazour et al, 2002). Notably, ROS IFT-cargo complexes contain rhodopsin and GC1 and their interaction is dependant on ATP and chaperones MRJ and HSC70 (Bhowmick et al, 2009). IFTs also interact with the heterotrimeric plus-end directed molecular motor kinesin-2 to mediate microtubule-dependent trafficking into the ROS (Baker et al, 2003; Lopes et al, 2010; Trivedi et al, 2012). One of the best-studied IFT components, IFT20, binds to a protein Elipsa that in turn associates with Rab8, providing a bridging mechanism between the IFT network and regulatory protein complexes in zebrafish photoreceptors (Omori et al, 2008). As the Arf4-based ciliary targeting complex recruits Rab8, it is possible that other components of the complex intersect with the photoreceptor IFTs. IFT20 also plays an essential role in the delivery of ciliary membrane proteins, including rhodopsin, from the Golgi to the cilia (Baker et al, 2003; Follit et al, 2006; Follit et al, 2009; Keady et al, 2011). This suggests an overlap, or coordination between IFTs and the Arf4-based ciliary trafficking complex.

PLA analysis of interactions between IFT20 and the Arf4-based trafficking complex brought some surprising results. Interaction of rhodopsin with Rab8 and Rabin8 was readily detectable, as previously reported (Wang et al, 2012) (Fig 9A and B). Although intersection of IFT20 and the Arf4-based machinery was anticipated, the PLA analysis of photoreceptor IFT20 binding partners revealed no direct interactions with rhodopsin or other components of the Arf4-based trafficking complex analyzed (Fig 9C and E, and data not shown), with the exception of Rabin8, which appears to be an interacting partner for IFT20 in the RIS (Fig 9D). The absence of rhodopsin-IFT20 interactions was unexpected, given the biochemical evidence for the direct interaction of the FLAG-tagged IFT20 coexpressed in IMCD3 cells with the cytoplasmic tail of rhodopsin fused to GFP (Keady et al, 2011). However, because the IFT20 epitope of the antibody that we used for this study can be blocked under certain conditions (Follit et al, 2006), a different IFT20 antibody may be necessary to determine if the interaction of IFT20 with endogenous rhodopsin takes place in photoreceptors in situ.

The IFT20-Rabin8 interaction is particularly interesting because of the relatively complex distribution of Rabin8 in photoreceptor cells. On RTCs, Rabin8 colocalizes with Rab11 and ASAP1 (Fig 9F and I). However, at the Golgi TGN, two populations of carriers can be distinguished: one that contains Rabin8 along with Rab11, and/or ASAP1 (arrows in Fig 9G–K) and the other one that is localized in the close proximity of Rab11 and ASAP1-bearing carriers but excludes both Rab11 and ASAP1 (yellow arrowheads in Fig 9G–K). This localization suggests dual association of Rabin8: i) with the nascent buds at the TGN during incorporation of rhodopsin into RTCs and ii) with a different population of carriers that deliver additional regulatory components to the TGN. This is further supported by the observation that following staining of Rabin8-IFT20 PLA-treated retinas with RTC specific marker Rab8, a population of carriers containing Rabin8 and IFT20 does not overlap with RTCs (Fig 9L). The most plausible interpretation of these findings represented in Fig 9P is that Rabin8, IFT20, and possibly other regulatory components, associate with a population of membrane carriers originating at the base of the photoreceptor cilia that are subsequently delivered to the TGN where they fuse with the rhodopsin-ASAP1-Rab11-containing nascent buds. It remains to be determined if IFT20 is present on RTCs, because it does not interact with Rab11, Rab8, ASAP1, or rhodopsin. Rabin8 is likely a long coiled-coil protein and the possibility remains that IFT20 interacts with the domain of Rabin8 that is sufficiently removed from the rest of the targeting complex on RTCs to be undetectable by PLA. Intriguingly, the known block of IFT20 epitope of the antibody (Follit et al, 2006) does not prevent the Rabin8-IFT20 interaction. The nature and direction of the Rabin8-IFT20 containing carriers, which might deliver other yet to be identified components to the TGN in the process of RTC budding, merits further investigation. Retrograde IFT is essential for ROS extension and IFT protein recycling in vertebrate photoreceptors (Krock et al, 2009), and the retrograde trafficking from the cilia to the TGN may be a part of this process.

6. Tethering of RTCs to the periciliary plasma membrane

In the final stages of polarized membrane trafficking and ciliogenesis, Rab8a-positive carriers are tethered to the periciliary plasma membrane by the conserved octameric complex, the exocyst (or Sec6/8 complex) (Das & Guo, 2011; Guo et al, 1999; Heider & Munson, 2012; Hsu et al, 1998; Hsu et al, 2004; Novick et al, 2006). In a remarkable example of the Rab GEF-effector interaction network conserved from yeast to mammals, the Sec15 component of exocyst, which is a Rab8 effector, interacts with the Rab8 GEF Rabin8 and co-localizes with activated Rab8 in the cilium to regulate ciliogenesis (Das & Guo, 2011; Feng et al, 2012). In neurons, the Sec6/8 (exocyst) complex regulates housekeeping processes such as neurite outgrowth and receptor trafficking to the synapse (Hsu et al, 1996; Murthy et al, 2003; Sans et al, 2003; Vega & Hsu, 2001). In epithelial cells and

photoreceptors, the exocyst complex is localized at the base of the cilium where it interacts with Rab8a and Rab11a (Bryant et al, 2010; Mazelova et al, 2009b; Rogers et al, 2004; Wu et al, 2005; Zhang et al, 2004). The Sec10 component of the exocyst is also involved in the delivery of ciliary cargo. Sec10 interacts directly with IFT20 and the ciliary receptor polycystin 2 and is required for its ciliary localization (Fogelgren et al, 2011).

Recently, the analysis of the mouse rod photoreceptor cilium by cryo-electron tomography revealed tethered vesicles at the base of the cilium (Gilliam et al, 2012) (Fig 10A and B). Although the tethered vesicles were not identified as rhodopsin carriers, they likely correspond to RTCs that colocalize with the exocyst component Sec8 at the base of the frog photoreceptor cilium (Fig 10C). The abundant membrane tethers at the mouse photoreceptor cilium outnumber the tethered vesicles, and those that are attached only to the plasma membrane are shorter than those linking the vesicles to the plasma membrane. The tethers were not identified as the exocyst complexes, but their size and distribution are consistent with the structure and the mode of assembly of the exocyst (Fig 10D), a dynamic complex that goes through the assembly-disassembly cycles in which some exocyst subunits mark the site of exocytosis, whereas others, including Sec15, arrive to the plasma membrane on transport carriers (Munson & Novick, 2006). Interestingly, EM tomography also identified long filaments originating from the RIS that link the vesicles, tethers and microtubule distal appendages, which might hold NPHP-JBTS-MKS ciliopathy complexes at the transition zone. Alternatively, they could be septin filaments, or may embody the protein network that is associated with the basal body and PRC, which is disrupted in human Usher syndrome (USH), the most frequent cause of combined deafness-blindness (Bonnet & El-Amraoui, 2012; Hsu et al, 1998; Hu et al, 2010; Jacobson et al, 2008; Maerker et al, 2008; Williams, 2008).

7. Fusion of RTCs with the periciliary plasma membrane

In addition to Arf and Rab GTPases and their effectors, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are major components of the intracellular machinery responsible for targeted membrane delivery (Cai et al, 2007). Accordingly, the concluding stages of rhodopsin trafficking to the cilium are regulated by the SNARE protein complexes. In particular, syntaxin 3 and SNAP-25 are the RIS plasma membrane SNAREs involved in RTC fusion (Fig 11A–C) (Mazelova et al, 2009b). SNARE complexes are universal drivers of membrane fusion (Rothman, 2002). Through the formation of helical bundles derived of four amphipatic SNARE domains that bridge the opposing membranes, they bring membranes into proximity sufficient to initiate fusion (Jahn & Scheller, 2006). Fusion with the plasma membrane involves formation of a complex between Qa SNAREs syntaxins and R-SNAREs or VAMPs, each contributing one helix to the four-helix bundle, and the Qbc SNAREs, either neuronal SNAP-25 or non-neuronal SNAP-23 that provide two helices to the core complex (Jahn & Scheller, 2006)(Fig 11D). The polarized distribution of syntaxins contributes additional specificity to membrane targeting by restricting fusion to particular target membranes. As an example, syntaxin 3 is restricted to the RIS plasma membrane in frog photoreceptors (Baker et al, 2008; Mazelova et al, 2009b). However, in mouse rods, syntaxin 3 is also detected in the ROS, suggesting potential species-specific differences in the ciliary membrane targeting and/or ROS morphogenesis (Chuang et al, 2007; Kwok et al, 2008). These potential differences highlight the importance of broadening the network of animal models employed in the research on photoreceptor membrane trafficking, which should help to distinguish between the discrepancies due to method-dependent results and the physiological differences in ROS morphogenesis.

The identity of the VAMP (R-SNARE) that regulates RTC fusion is currently under investigation. The tetanus neurotoxin-insensitive VAMP (TI-VAMP, or VAMP7) is a candidate for the RTC R-SNARE, as it cofractionates with newly synthesized rhodopsin and RTC markers Rab11 and Rab8 (Fig 11H) (Wang et al, 2012). VAMP7 belongs to the family of larger VAMPs, or longins, which are the only R-SNAREs that are common to all eukaryotes (Filippini et al, 2001; Rossi et al, 2004). Unlike short VAMPs, or brevins, that regulate neurotransmitter release, longins regulate neuronal differentiation and neurite outgrowth (Alberts & Galli, 2003; Alberts et al, 2006; Osen-Sand et al, 1996), the same processes that depend on exocyst (Hsu et al, 2004). Thus, the ciliary membrane expansion that generates the ROS might be a process homologous to neurite outgrowth.

SNARE function is also regulated by the local membrane lipid environment (Darios & Davletov, 2006). Therefore, syntaxin 3-SNAP-25 pairing is significantly enhanced by the omega-3 long-chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid [DHA, 22:6(n-3)] (Fig 11E – G)(Mazelova et al, 2009b). Because the concentration of free DHA in the photoreceptors is very low, it is likely that syntaxin 3-SNAP-25 pairing is regulated through localized release of free DHA by phospholipase A2 in the RIS. DHA is a major component of ROS membranes, which is essential for sensory membrane function and for rod cell survival (Bazan, 2006), consistent with its role in promoting rhodopsin trafficking. Despite the acknowledged role of DHA in photoreceptor biology, recent report of the Age-Related Eye Disease Study 2 (AREDS2) Research Team found that oral supplementation with LCPUFAs to the AREDS formulation for the treatment of age-related macular degeneration (AMD) did not further reduce risk of progression to advanced AMD (Chew & Group, 2013). Thus, new approaches are needed to harness the power of DHA into a treatment beneficial for retinal disorders.

8. Membrane trafficking insights may improve diagnosis of retinal dystrophies

Much remains to be learned about the molecular mechanisms involved in regulation of rhodopsin trafficking. However, well-developed model systems for the studies of rhodopsin trafficking, such as the frog retinas, make the mapping of the trafficking routes in the RIS possible, facilitating the correlation of structure and function of rod photoreceptors. This analysis can aid in interpreting optical coherence tomography (OCT) of living frog retinas to improve the understanding of the structures visualized by OCT, as shown in Fig 12 (Lu et al, 2012). OCT analysis of human retinas provides invaluable information to diagnose the degree of retinal damage in retinal dystrophies (Hood et al, 2011; Sallo et al, 2012). The comparison of structures identifiable in living frog retinas may inform diagnostic tools applied to human retinas leading to improved treatments of retinal dystrophies and advance efforts to preserve and restore vision.

9. Concluding Remarks

The molecular assemblies that control rhodopsin transport to the cilia that are described here organize cellular events involved in the cilia formation and function, and account for the largest group among the genes linked to photoreceptor degeneration. Deeper understanding of the cell biology of retinal rod photoreceptors will be instrumental in providing the desired targets for the treatment of blinding diseases. The most exciting discoveries regarding the fundamental questions addressing intricate molecular networks that act in concert to produce the perfection of a healthy rod photoreceptor are yet to come.

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The abbreviations used are

ADRP	Autosomal Dominant Retinitis Pigmentosa
BBS	Bardet-Biedl Syndrome
BBSome	a conserved complex of BBS proteins
CTS	Ciliary Targeting Signal
DHA	Docosahexaenoic Acid
GAP	GTPase Activating Protein
GC1	Guanylyl Cyclase 1
GEF	Guanine Nucleotide Exchange Factor
IFT	Intraflagellar Transport
JBTS	Joubert Syndrome
MKS	Meckel Syndrome
MT	Microtubules
MTOC	Microtubule Organizing Center
NPHP	Nephronophthisis
PLA	Proximity Ligation Assay
OCT	Optical Coherence Tomography
RIS	Rod Inner Segment(s)
ROS	Rod Outer Segment(s)
RPGR	Retinitis Pigmentosa GTPase Regulator
RTC(s)	Rhodopsin Transport Carrier(s)
SNARE	Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor
TGN	Trans-Golgi Network

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Figure 1. ROS is a primary cilium

A. Rhodopsin transport carriers (RTCs) that replenish the ROS sensory membrane are detected by EM immunocytochemistry in the rod inner segment (RIS), at the base of the cilium (C) (also called the connecting cilium) of *Rana Berlandieri* photoreceptors. BB, basal body, m, mitochondria. Modified from Deretic and Papermaster, 1991. Bar = 0.3 μm . **B. and C.** Comparison of *Chlamydomonas* flagellum and rod connecting cilium, respectively. Arrows point to the IFT particles underlying the ciliary membrane. Reproduced from Rosenbaum et al, 1999. **D.** Membranes clustered at the base of the cilium carry newly synthesized rhodopsin, detected by EM autoradiography of *Xenopus* photoreceptors. **E.** Rhodopsin transport vesicles (v) fuse with the plasma membrane at the periciliary ridge complex (PRC) of *Xenopus* rods. **D and E** Adapted from Papermaster et al, 1985. Bar = 0.5 μm . **F. and G.** Schematic representation (F) and the EM (G) of the basal body (centrosome). A, B, and C-tubules in each triplet correspond to the internal, middle and external one, respectively. Mother centriole is surrounded by PCM and contains distal and subdistal appendages on the B and C-tubule, respectively. Bar = 0.2 μm in (G). **H.** Organization of the

axoneme, transition zone and the basal body of *Chlamydomonas* flagellum. An array of doublets arising from the distal end of the centriole fills the axoneme. CW, cartwheel, at the proximal end of the basal body. Bar = 0.25 μm . **F–H** Reproduced with permission from Bettencourt-Dias and Glover, 2007.

Figure 2. Rhodopsin trafficking to the primary cilium: Ciliary targeting signals and regulators
A. Schematic of a rod photoreceptor outlining rhodopsin trafficking pathways. **B.** Schematic of retinal subcellular fractionation that generates fractions enriched in rhodopsin transport membranes. **C.** Immunoprecipitation using the transport membranes as a source of protein complexes reveals regulators of rhodopsin trafficking. Reproduced from Wang et al, 2012. **D.** The rhodopsin cytoplasmic C-terminal VxPx and H-8 FR are its ciliary targeting signals (CTSs), that are recognized by Arf4 and ASAP1, respectively.

Figure 3. Pathways regulated by Arf GTPases

A. Arf proteins have distinct localizations and functions in the endoplasmic reticulum (ER)–Golgi system. Arf1 and Arf4 localize to the early *cis*-Golgi. Arf3 and Arf4 localize to the *trans*-Golgi network (TGN). In addition to the recruitment of coat proteins (coatamer complex I (COPI), GGA (Golgi-localized, γ -ear-containing, Arf-binding protein) and adaptor protein 1 (AP1)) to the Golgi, Arf1 binds to ceramide transfer (CERT) and FAPP2 to mediate the transport of ceramide and glucosylceramide lipids from the *cis*-Golgi to the *trans*-Golgi. At the ER–Golgi intermediate compartment (ERGIC), Arf1 and the GEF GBF1 act with COPII to regulate the formation of lipid droplets and for the replication of several viruses. GBF1 is also localized at the TGN where it activates Arf4 (Lowery et al, 2013). CAPS (Calcium-dependent activator protein for secretion), which is involved in regulated secretion, is recruited to the TGN by Arf4 and Arf5. At the ER, Sar1, activated by SEC12, recruits COPII to allow vesicle transport to the Golgi. **B.** In rod photoreceptors Arf4 binds specifically to rhodopsin in the TGN membrane and, together with FIP3, ASAP1 and Rab11, it facilitates the transport of rhodopsin in transport vesicles from the inner segment to the outer segment, which is a specialized cilium. Arf-like 3 (Arl3) has been localized to the connecting cilium, and retinitis pigmentosa 2 (RP2; also known as XRP2), an Arl3 GAP, localizes to the TGN, the basal body and the membrane adjacent to the connecting cilium. **C.** In primary cilia, Arl6 recruits the BBSome coat complex that facilitates the transport of membrane proteins into the cilium. Arl13 is localized to the cilium and has been implicated in intraflagellar transport. ADRP, adipose differentiation-related protein (also known as adipophilin); ATGL, adipose triglyceride lipase; PtdIns4K, phosphatidylinositol 4-kinase. Figure is reproduced with permission from Donaldson and Jackson, 2011. The figure legend is adapted from Donaldson and Jackson, 2011.

Figure 4. Formation of the rhodopsin ciliary targeting complex at the TGN

The schematic was generated by combining, scaling, merging and outlining the known crystal structures, as described in the text. The width of the complex containing the dimer of the BAR, PH and GAP domains of ASAP1 is approximately 22 nm. Assembly of the complex at the TGN can be divided in two stages: The Arf4-dependent Stage I and the post-Arf4 Stage II. In Stage I, cytosolic Arf4 is activated by the Arf-GEF GBF1 and becomes membrane-associated. Activated Arf4 recognizes the VxPx CTS of rhodopsin. ASAP1 is recruited to the TGN by activated Arf4, acidic phospholipids and PIP₂ (indicated by red head groups). There, it recognizes the FR CTS of rhodopsin. ASAP1 dimerizes through the BAR domain, which likely induces membrane deformation necessary for budding. It selectively binds Rab11a and FIP3, which also acts as a dimer. The CTS proofreading phase is completed through the GTP hydrolysis on Arf4 by ASAP1, assisted by FIP3. In Stage II inactivated Arf4 departs the TGN, but the rest of the complex remains associated to regulate subsequent events. This includes the recruitment of the GEF Rabin8 and the inactive, GDP-bound Rab8 by ASAP1 and Rab11a. This endows the nascent buds with the ciliary targeting information.

Figure 5. Rab lipid modification and the GTPase cycle

Newly synthesized Rabs bind to Rab-escort protein (REP) encoded by the choroideremia gene, which presents them to Rab geranylgeranyl transferase (RGGTase) that prenylates Rabs (1) and delivers them to membranes of donor compartments (2). Rabs are activated by Rab GEFs and recruit effectors, which regulate budding (3), movement (4) and tethering/docking (5) of the carrier vesicle. As membrane carriers fuse with the acceptor compartment membrane, Rabs are inactivated by Rab GAPs. Rab guanine-nucleotide dissociation inhibitor (Rab GDI) extracts GDP-bound Rabs into the cytosol (6) and delivers them to donor compartment membranes with the assistance of a GDI-displacement factor (GDF), also known as Prenylated Rab Acceptor 1 (PRA1). Adapted with permission from Seixas et al., 2013. Note that only Qa SNARE (syntaxin) and R-SNARE (VAMP) are presented in this schematic, whereas the Qbc SNAREs that provide two helices to the SNARE complex are omitted. See text for the details on SNARE complex assembly.

Figure 6. Localization of Rab11-Rabin8-Rab8 GTPase cascade in a ciliated cell

Rab GTPases involved in ciliary trafficking are conserved from yeast to mammals. In yeast, Ypt31p/Ypt32p interacts with Sec2p, a GEF for the next Rab in cascade, which is Sec4p. Sec2p activates Sec4p on the secretory vesicle, which leads to vesicle tethering and fusion with plasma membrane. Mammalian homologues Rab11, Rabin8 (a GEF for Rab8) and Rab8 participate in the ciliogenesis cascade, which regulates targeting of ciliary transport carriers, including RTCs. Both Rab8 and Rabin8 are required for primary cilium formation. At the plasma membrane, Arf6 activates the small GTPase Rac1. Rab5 and Rab7 regulate early and late endocytic events, respectively. Modified with permission from Mizuno-Yamazaki and Novick, 2012.

Figure 7. Interactions that regulate rhodopsin transiting from the Golgi/TGN into ciliary-targeted RTCs

A. Proximity Ligation Assay (PLA) involves dual target recognition by antibodies covalently linked to DNA primers. Circularization and ligation takes place only between the connector oligonucleotides located at <16 nm. DNA amplification is followed by hybridization with fluorescent oligos (excitation: 598 nm). **B.** When PLA is performed on $100\ \mu\text{m}$ thick sections of Rana Berlandieri retinas using the modified method adapted for brain slices (Trifilieff et al, 2011) with antibodies to rhodopsin and an unrelated Golgi-associated protein no red fluorescence is detected, signifying an absence of protein-protein interactions. **C.** By contrast, rhodopsin-ASAP1 interaction sites (red dots) are detected by PLA. **D.** Frog retina labeled with mAb 11D5 to the VxPx motif of rhodopsin. Rhodopsin is concentrated in the ROS and the photoreceptor Golgi (G). **E.** The Golgi complex is localized in the photoreceptor myoid (M). The *cis*-Golgi is visualized by GM 130 (red) and the *trans*-Golgi by Rab6 (green). E, Ellipsoid. **F.** ASAP1 (blue) and Rab11 (red) co-localize on the nascent buds at the tips of the *trans*-Golgi (Rab6, green). **G. and H.** ASAP1-Arf4 interaction sites (red dots) are detected by PLA and retinal sections are subsequently stained with Rab6-Alexa Fluor 488 conjugate (green). Arrows indicate ASAP1-Arf4 interaction sites juxtaposed to the *trans*-Golgi. **I. and J.** Rhodopsin-Arf4, or ASAP1-Rab11 interaction sites are detected by PLA, respectively. Bar = $5\ \mu\text{m}$ in D, E, I and J; $1\ \mu\text{m}$ in B, C, G and H; $0.5\ \mu\text{m}$ in F. **(K)** Red fluorescent signals are quantified, the data are expressed as a percent of total interaction sites within the RIS and presented as the means $\pm$ SEM (***, $p < 2.27 \times 10^{-6}$; *, $p = 0.01$). Modified from Wang et al, 2012.

Figure 8. Molecular interactions and the sequence of events taking place during rhodopsin trafficking to the cilia

At the TGN, rhodopsin forms a complex with activated Arf4 and ASAP1, which recognize its VxPx and FR CTSs, respectively. This leads to the formation of the Arf4-based ciliary targeting complex, which, in addition to Arf4, contains ASAP1, Rab11 and FIP3. Following GTP hydrolysis on Arf4, the remaining components recruit Rabin8 and Rab8, which is a regulator of fusion with the plasma membrane. On RTCs, ASAP1 serves as a scaffold, or an affinity adaptor for spatially restricted activation of Rab8. Activated Rab8 permits RTCs fusion and cargo delivery across the diffusion barrier surrounding the cilium, based in part on its ability to interact with the Sec15 subunit of the exocyst, a membrane tethering complex involved in ciliary targeting. The approximate size of the targeting complex is as in Figure 4.

Figure 9. Interactions between the Arf4-based ciliary targeting complex and IFT20

A. – E. Rhodopsin-Rab8, -Rabin8, -IFT20, and Rabin8-IFT20, or Rab8-IFT20 interaction sites were detected by PLA on *Rana Berlandieri* retinas, respectively. The antibody to IFT20 was a kind gift of Dr. Greg Pazour. **F.** By confocal microscopy Rabin8 (green) and Rab11 (red) colocalize (yellow) on RTCs (arrow). **G.** Rabin8 (green) and Rab11 (red) are present on nascent buds associated with the trans-Golgi (G), revealed by Rab6 (blue). N, nucleus. **H.** Magnified Golgi area from G. Arrows indicate carriers containing Rabin8 (green) and Rab11 (red, yellow in the merged image). Rabin8-positive carriers (green, yellow arrowheads in G. and H.) are juxtaposed to Rab11-positive carriers (red, white arrowheads). **I.** Rabin8 (green) and ASAP1 (red) colocalize (yellow) on RTCs (arrow). Yellow arrowhead indicates Rabin8-positive carrier (green) juxtaposed to ASAP1-positive carrier (red, white arrowheads). **J. and K.** Microtubule depolymerization with nocodazole, or treatment with propranolol (Ppl), respectively, does not interfere with the interaction of Rabin8 with ASAP1. An arrow and arrowheads in K point to the same structures as in I. that are enlarged upon treatment with propranolol, as reported (Mazelova et al, 2009a). **L.** Rabin8 and IFT20 were detected by PLA (red dots) and retinal sections were then stained with Rab8 Alexa Fluor 488 conjugate (green). Arrowheads indicate separate structures containing Rabin8 and IFT20, or Rab8. **M.–O.** Rabin8 and IFT20-containing carriers (red, arrows) are found in the proximity of the trans-Golgi labeled with Rab6 Alexa Fluor 488 conjugate (green), in control, nocodazole or Ppl-treated retinas, respectively. Bar = 5 μ m in A–E and M–O; 2 μ m in F–L. **P.** A model summarizing interaction sites of IFT20 with Rabin8, as suggested by our study.

Figure 10. Tethering complexes at the periciliary plasma membrane

A. Three consecutive tomographic slices of the mouse photoreceptor cilium. Filaments originating from the IS (red) interact with vesicles (yellow), C-microtubule distal appendages (green), and ciliary membrane filaments (blue). **B.** Segmentation from A. of vesicles (yellow), IS filaments (red), distal appendages (green), and membrane tethers (blue) in the pericentriolar region. Bar = 0.1 μm . Reproduced with permission from Gilliam et al, 2012. **C.** Rab8-positive RTCs (red, arrows) are in close proximity and partially overlap (yellow) with Sec8-labeled structures (green) in Rana photoreceptors. Bar = 1 μm . Reproduced from Mazelova et al., 2009b. **D.** A structure and a model for the assembly of the exocyst, modified with permission from Munson and Novick, 2006. A quick-freeze/deep-etch EM of purified mammalian brain exocyst complexes, either unfixed or fixed with glutaraldehyde (from Hsu et al., 1998). Bar = 0.1 μm . The schematic representation of the yeast exocyst complex as an elongated helical-bundle structure packed together to form a structure similar to the fixed complex. The exocyst is linked to the carrier membrane via the interaction of Rab8 with the Sec15 subunit. Note that the size of the exocyst closely matches the size of the membrane tethers (blue) in A. and B.

Figure 11. SNAREs regulate fusion of RTCs with the periciliary plasma membrane
A. In *Rana* photoreceptors SNAP-25 (green) and Syntaxin 3 (STX3, red) co-localize in the RIS at the RTC fusion sites (arrows, yellow). **B.** Syntaxin 3 (red) is highly concentrated in the RIS PM (arrows), but is absent from the ROS and from the RPE. AJ, adherens junctions that form the outer limiting membrane (OLM, dotted line); retinal layers: ONL-outer nuclear, OPL-outer plexiform, INL-inner nuclear. Syntaxin 3 is also abundant in the OPL. Asterisks indicate the protruding RIS of green rods that account for ~5% of total rods. Nuclei are stained with TO-PRO-3 (blue). **C.** Anti-SNAP-25 (green) outlines the photoreceptor PM. The CPs, which are in continuum with the RIS PM, also contain SNAP-25. The ROS, which are visible by DIC, are completely devoid of this SNARE. SNAP-25 co-localizes with synaptophysin (SYP, red) in the OPL. **D.** Space-filling model of the SNARE helical bundle. **E. and F.** DHA treated retinas display increase in Syntaxin 3 (red) content of the PM surrounding the cilium. Upon DHA treatment Syntaxin 3 (red) and SNAP-25 (green) increasingly colocalize (yellow, merge). **G.** DHA significantly increases Syntaxin 3-SNAP-25 pixel co-localization (**, $p=0.003$). Bar = 10 μm in B and C; 5 μm in A, and in upper panels in E and F; 0.5 μm in lower panels in E and F. **H.** PNS isolated from one frog retina fractionated by velocity sedimentation followed by 20–39% sucrose density

gradients. Membrane and cytosolic proteins were separated by SDS-PAGE. Radiolabeled rhodopsin was detected by autoradiography. VAMP7, Rab8 and Arf4 were detected by immunoblotting on a single blot, whereas Rab11 was probed on a duplicate blot. Arf4 is also detected in the cytosol (arrow on the right). A.-C. and E.-G. Reproduced from Mazelova et al., 2009b. H. Modified from Wang et al., 2012.

Figure 12. Comparison of the OCT and DIC image of frog retinas

A. Cross-section optical coherence tomography (OCT) image of living frog retinas averaged over 120 μm along the y-axis. **B.** Confocal optical section of the fixed frog retina imaged by differential interference contrast (DIC). Nuclei are stained with TO-PRO-3. Retinal layers: ONL-outer nuclear, OPL-outer plexiform, INL-inner nuclear, OPL-outer plexiform, GCL-ganglion cell layer. Note that the hyper-reflective band visualized by OCT and commonly attributed to the rod photoreceptor inner/outer segment (RIS/ROS) junction originates from the ellipsoid region of the RIS, which is detectable in the DIC image by the change in optical properties associated with the tight mitochondrial packing. Bar = 10 μm . Modified from Lu et al., 2012.