Understanding the trafficking mechanisms that underlie the role of ciliary compartmentalizing the role that defects in ciliary compartmentalization might play in disease pathogenesis. We also discuss major unanswered questions regarding the compartment, removal, and retention by diffusion barriers such as the transition zone. Proteins that are concentrated in the ciliary membrane are also localized to other cellular sites. Thus it is critical to determine the particular role for ciliary compartmentalization in sensory reception and signaling pathways. Here we provide a brief overview of our current understanding of compartmentalization of proteins in the ciliary membrane and the dynamics of trafficking into and out of the cilium. We also discuss major unanswered questions regarding the role that defects in ciliary compartmentalization might play in disease pathogenesis. Understanding the trafficking mechanisms that underlie the role of ciliary compartmentalization in signaling might provide unique approaches for intervention in progressive ciliopathies.

Background: The primary cilium has been found to be associated with a number of cellular signaling pathways, such as vertebrate hedgehog signaling, and implicated in the pathogenesis of diseases affecting multiple organs, including the neural tube, kidney, and brain. The primary cilium is the site where a subset of the cell's membrane proteins is enriched. However, pathways that target and concentrate membrane proteins in cilia are not well understood. Processes determining the level of proteins in the ciliary membrane include entry into the compartment, removal, and retention by diffusion barriers such as the transition zone. Proteins that are concentrated in the ciliary membrane are also localized to other cellular sites. Thus it is critical to determine the particular role for ciliary compartmentalization in sensory reception and signaling pathways. Here we provide a brief overview of our current understanding of compartmentalization of proteins in the ciliary membrane and the dynamics of trafficking into and out of the cilium. We also discuss major unanswered questions regarding the role that defects in ciliary compartmentalization might play in disease pathogenesis. Understanding the trafficking mechanisms that underlie the role of ciliary compartmentalization in signaling might provide unique approaches for intervention in progressive ciliopathies.

ABSTRACT The primary cilium has been found to be associated with a number of cellular signaling pathways, such as vertebrate hedgehog signaling, and implicated in the pathogenesis of diseases affecting multiple organs, including the neural tube, kidney, and brain. The primary cilium is the site where a subset of the cell's membrane proteins is enriched. However, pathways that target and concentrate membrane proteins in cilia are not well understood. Processes determining the level of proteins in the ciliary membrane include entry into the compartment, removal, and retention by diffusion barriers such as the transition zone. Proteins that are concentrated in the ciliary membrane are also localized to other cellular sites. Thus it is critical to determine the particular role for ciliary compartmentalization in sensory reception and signaling pathways. Here we provide a brief overview of our current understanding of compartmentalization of proteins in the ciliary membrane and the dynamics of trafficking into and out of the cilium. We also discuss major unanswered questions regarding the role that defects in ciliary compartmentalization might play in disease pathogenesis. Understanding the trafficking mechanisms that underlie the role of ciliary compartmentalization in signaling might provide unique approaches for intervention in progressive ciliopathies.

INTRODUCTION: THE PRIMARY CILIUM AS A COMPARTMENTALIZED ORGANELLE

The primary cilium is a tiny, antenna-like projection from the apical membrane of most vertebrate cells (Rosenbaum and Witman, 2002). Most cilia are a few micrometers in length and are ~200 nm in diameter. Long believed to be vestigial, the primary cilium has now been implicated in multiple cellular pathways, including vertebrate hedgehog signaling (Goetz and Anderson, 2010). Defects in primary cilia result in diseases (ciliopathies) affecting multiple tissues, including the neural tube, brain, and kidney (Hildebrandt et al., 2011).

The membrane of the primary cilium envelops the microtubular axoneme that templates from the basal body and is continuous with the rest of the plasma membrane. However, the ciliary membrane is believed to be partitioned from the rest of the plasma membrane by the transition zone (Reiter et al., 2012; Figure 1). At least 25 rhodopsin-family G protein–coupled receptors (GPCRs) have been reported to localize to cilia, particularly in neurons in the brain and in other cell types (Hilgendorf et al., 2016). Proteins linked to polycystic kidney disease, such as the TRP-channel family proteins polycystin-1 and 2 (PC1/2; Pazour et al., 2002; Yoder et al., 2002), and the single-pass transmembrane protein fibrocystin (Ward et al., 2003), also localize to cilia. In addition, sonic hedgehog (Shh) pathway components such as the Shh receptor Patched (Ptc1), the pathway activator Smoothened (Smo), and the orphan GPCR, Gpr161, a negative regulator of the pathway localize to ciliary membrane in a dynamic manner (Corbit et al., 2005; Rohatgi et al., 2007; Mukhopadhyay et al., 2013). Other cilia and ciliary pocket-coordinated signaling pathways involve transforming growth factor β, receptor tyrosine kinase, Wnt, and Notch signaling (Ezratty et al., 2011; Wallingford and Mitchell, 2011; Pedersen et al., 2016). Signaling mediated by cilia is an ancient phenomenon; for example, interactions between receptors (agglutinins) on plus and minus gamete cilia during fertilization in the green alga Chlamydomonas stimulate a signaling pathway involved in gamete activation that ultimately leads to cell–cell fusion (Wang et al., 2006). Thus the ciliary membrane serves as a compartment for subcellular localization of...
The lipid composition of the ciliary membrane is different from the rest of the plasma membrane (Lechtreck et al., 2013). In particular, phosphoinositide 5-phosphatases Inpp5e and Ocr1 localize to cilia (Bielas et al., 2009; Jacoby et al., 2009; Luo et al., 2012), and the ciliary compartment lacks phosphoinositide 4,5-bisphosphate (PI(4,5)P2), similar to endosomes (Chavez et al., 2015). The ciliary pocket flanking the primary cilium is rich in coated vesicles and actin microfilaments (Rohatgi and Snell, 2010; Benmerah, 2013; Pedersen et al., 2016). In addition, there are distinct lipid barriers between cilia and rest of the plasma membrane (Vieira et al., 2006), and proteins such as septins that localize to the cilia and transition zone restrict ciliary membrane components from diffusing into the rest of the plasma membrane (Hu et al., 2010; Chih et al., 2012; Ghossoub et al., 2013). Certain membrane proteins are prevented from trafficking to cilia by being immobilized by the apical actin network outside cilia (Francis et al., 2011). Thus factors that affect ciliary pools include trafficking into cilia, removal from cilia, retention inside cilia, restriction outside cilia, and recycling of membrane components in the endosomal compartment (Figure 1; Bloodgood, 2012). Finally, loss of proteins in extracellular vesicles might also regulate ciliary content (Wang et al., 2014; Cao et al., 2015; Wood and Rosenbaum, 2015).

Localization of endogenous proteins in the ciliary membrane of vertebrate cells has been mostly determined by immunolabeling techniques. However, it is important to realize that these proteins are not exclusive to cilia; rather, they are enriched in cilia. The ciliary membrane is ~1/1000–1/5000 of the total cellular surface, and the ciliary volume (~0.5 fl) is about ~1/30,000 of the total cellular volume (Delling et al., 2013). The small size of the cilium enables enrichment of proteins with respect to the rest of the plasma membrane and establishing an effective signaling compartment by local concentration of second messengers and effectors. However, the absolute amounts of cilia-localized proteins are likely to be minute in comparison to total cellular levels. Thus, to understand the role of compartmentalization in ciliary signaling, it is imperative to determine mechanisms underlying ciliary trafficking, and identify functional consequences upon disruption of ciliary localization. Unfortunately, we are lacking in understanding of signaling inside cilia, mostly due to the difficulty of working with such a tiny compartment. We are also extremely limited in the availability of tools that allow us to address the role of ciliary compartmentalization while maintaining the architecture of cilia and/or retaining the functionality of the studied proteins.

**FIGURE 1:** Regulation of ciliary pools of membrane-targeted proteins. Factors that determine the levels of a protein in the ciliary membrane include trafficking into cilia, removal from cilia, retention inside cilia by membrane barriers and transition zone, exclusion of certain proteins from cilia by the cortical cytoskeleton, and recycling of membrane components in the endosomal compartment. Loss of proteins in extracellular vesicles might also regulate ciliary content. BB, basal body; CP, ciliary pocket; PM, plasma membrane; TZ, transition zone.

**ITINERARY FOR MEMBRANE PROTEIN TRAFFICKING TO CILIA**

Membrane biogenesis has to be closely coordinated with axonemal growth during ciliogenesis. Key players in this process have been identified and include a Rab cascade consisting of Rab11 and Rab8 (Moritz et al., 2001; Nachury et al., 2007; Westlake et al., 2011; Lu et al., 2015). Because disruption of these factors affects ciliogenesis per se, it is important to distinguish between factors that affect biogenesis of the ciliary membrane and those that affect trafficking. An increasing number of pathways linked to the secretory pathway have been implicated in trafficking of membrane proteins to cilia. These include the small G protein ARF4 for rhodopsin and fibrocytin trafficking (Mazelova et al., 2009; Follit et al., 2014) and the GGA1 adapters for PC1/2 trafficking (Kim et al., 2014a). The BBSome proteins regulate membrane composition (Lechtreck et al., 2009, 2013) in addition to regulating ciliary GPCR pools and removal of GPCRs, polycystins, and membrane-associated proteins from cilia (Berbari et al., 2008b; Lechtreck et al., 2009, 2013; Jin et al., 2013; Domire et al., 2011; Loktev and Jackson, 2013; Eguether et al., 2014; Liew et al., 2014; Xu et al., 2015). Thus the BBSome proteins have multiple effects on ciliary trafficking and in maintaining membrane composition.

Irrespective of the role of factors in the secretory pathway and in ciliary membrane biogenesis, the final critical step in ciliary trafficking is the targeting of GPCRs into cilia from the plasma membrane or juxtapacillary vesicles. A ciliary targeting sequence needs to be carefully considered because lack of ciliary localization in mutants might result from defective transit or recycling through the secretory pathway, both of which are steps distinct from direct trafficking into the compartment. Multiple sequences that target proteins to ciliary membrane have been determined (Deretic et al., 1998; Jenkins et al., 2006; Berbari et al., 2008a,b; Follit et al., 2010; Loktev and Jackson, 2013; Mukhopadhyay et al., 2013). The lack of a consensus sequence that could exclusively predict ciliary localization (Loktev and Jackson, 2013) and the multiplicity of pathways implicated in trafficking argue for multiple ways for finally targeting proteins to cilia (Pazour and Bloodgood, 2008). Alternatively, binding of these motifs with a few adapters that is dictated by structural elements in these varied sequences could determine trafficking into cilia.
The tubby-family proteins Tulp3 and tubby (Tub) have been implicated as adapters in trafficking of multiple GPCRs into the ciliary membrane (Mukhopadhyay et al., 2010, 2013; Sun et al., 2012; Loktev and Jackson, 2013). These tubby-family proteins have an N-terminal intraflagellar complex A (IFT-A) core-binding conserved helix and a C-terminal tubby domain that binds to PI(4,5)P₂ (Santagata et al., 2001; Mukhopadhyay et al., 2010). Disrupting either of these domains prevents trafficking of these GPCRs to cilia, suggesting that Tulp3 “bridges” the GPCRs with IFT-A core in targeting them into cilia (Mukhopadhyay et al., 2010). The generality of this model in targeting all cilia-localized rhodopsin-family GPCRs, the parallels between Tulp3 and Tub in ciliary trafficking, and the role of Tulp3/Tub as adapters in ciliary trafficking of other integral membrane proteins are important future directions to pursue.

In contrast to transmembrane protein trafficking to cilia, lipidated membrane-associated protein trafficking to cilia is mediated by a set of proteins that serve as carriers for the lipid modifications (Unc-119 and Pde6β for myristolylated and prenylated proteins, respectively; Wright et al., 2011; Humbert et al., 2012). The lipid-binding carriers release the lipidated cargo into cilia in an Arl3-GTP-dependent cycle in which Arl13b functions as a guanine nucleotide exchange factor for Arl3 (Goethardt et al., 2015). Disruption of trafficking of lipidated cargo to cilia causes profound defects in ciliary function, including disrupted Shh signaling, photodegeneration, and ciliopathies (Caspany et al., 2007; Cantagrel et al., 2008; Hanke-Gogokhia et al., 2016). An important future direction here is to determine factors that regulate trafficking of the Arl proteins, such as Arl13b, to cilia.

**THE VERTEBRATE SHH PATHWAY, PRIMARY CILIUM, AND GPCR SIGNALING**

The vertebrate Shh pathway is one of the best examples in which the primary ciliary axis has been implicated in cellular signaling (Goetz and Anderson, 2010). The final output of the Shh pathway is the formation of Gli transcriptional repressors or activators, both of which occur in a cilia-dependent manner (Goetz and Anderson, 2010). Although the Gli3 repressor is critical in basal suppression of the pathway, the Gli2 activator is the major activator for signaling (Goetz and Anderson, 2010). The Gli2 transcriptional activator is formed by a Smo-dependent process, which is initiated upon binding of Shh to Ptc1, removal of Ptc1 from cilia (Rohtagi et al., 2007), and ciliary retention of Smo (Corbit et al., 2005). The Gli3 repressor is formed in a protein kinase A (PKA)-dependent manner by limited proteolysis, with the N-terminal acting as a transcriptional repressor (Chen et al., 2009; Jia et al., 2009; Humke et al., 2010; Wang et al., 2010; Wen et al., 2010). Phenotypes resulting from loss of cilia depend on the predominance of the role of repressor or activator in development and patterning of the particular tissue. For example, disruption of cilia in the neural tube results in decreased Shh signaling, predominantly in a Gli2-activator-dependent manner (Goetz and Anderson, 2010).

Loss of Tulp3 and IFT-A complex results in increased Shh signaling in the neural tube as opposed to decreased signaling with loss of cilia (Norman et al., 2009; Obcina et al., 2011; Qin et al., 2011). This suggests that there are Tulp3/IFT-A-regulated negative regulators of Shh signaling. The orphan GPCR Gpr161 localizes to cilia in a Tulp3/IFT-A-regulated manner and negatively regulates Shh signaling via cAMP signaling (Mukhopadhyay et al., 2013). A null murine allele of Gpr161 phenocopies Tulp3/IFT-A mutants in causing increased Shh signaling with concomitant lack of Gli3 repressor. Constitutive CAM signaling by Gpr161 suggests that it regulates PKA-mediated Gli repressor formation, possibly by increasing cAMP levels in cilia (Mukhopadhyay et al., 2013). Because cAMP production by GPCRs is mediated by Gα₅i coupling and activation of adenyl cyclases, it is interesting to note that Nnas (Gα₅i)-knockout mice exhibit increased Shh signaling (Regard et al., 2013). Finally, cAMP binds to PKA regulatory subunits, which are spatially restricted by A-kinase anchoring proteins (AKAPs), releasing protein kinase A catalytic subunits in close vicinity. PKA catalytic subunit mutants demonstrate increased Shh signaling (Tuason et al., 2011). Of interest, PKA regulatory subunits that localize to cilia (Mick et al., 2015) directly bind to an amphiphatic helix at the Gpr161-distal C-terminal tail (Bachmann et al., 2016), suggesting that Gpr161-PKA coupling occurs in cilia, with Gpr161 functioning as an AKAP. Key future directions here are to determine whether lack of Gpr161 localization to cilia results in a phenotype similar to lack of Gpr161. In addition, phenotypic characterization of Gpr161 conditional mutants should provide important clues regarding the role of this GPCR in the basal suppression of the hedgehog pathway in normal development and in pathogenesis of Shh-dependent tumors (Wong et al., 2009; Han and Alvarez-Buylla, 2010).

Whereas the characterization of Tulp3/IFT-A-regulated Gpr161 and downstream factors such as Gα₅i and PKA provide important clues to the role of maintaining Gli repressors in basal suppression of Shh pathway activity, the role of cAMP-generating adenyl cyclases in cilia is not clear. At least three of the adenyl cyclases (ACIII, ACV, ACVI) localize to cilia in cultured cells and in brain (Berbari et al., 2007; Choi et al., 2011; Vuolo et al., 2015); however, their role in cilia is difficult to ascertain because of redundancy (Vuolo et al., 2015). Levels of cAMP in cilia have been measured using cilia-localized sensors; however, the results are controversial. Whereas high levels of cAMP in cilia with respect to rest of the cytoplasm were detected using an intensimetric sensor (Moore et al., 2016), no differences were found using a fluorescence resonance energy transfer–based sensor (Marley et al., 2013). Phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃) levels in cilia, instead of Gα₅i, have been recently implicated in tonic regulation of ciliary cAMP levels by adenyl cyclases (Moore et al., 2016). However, the presence of 5’-inositol phosphatases Inpp5e and Ocrl in cilia would be counterproductive to PI(3,4,5)P₃ generation inside this compartment (Bielas et al., 2009; Jacoby et al., 2009; Luo et al., 2012; Chavez et al., 2015; Garcia-Gonzalo et al., 2015). In addition, factors that target adenyl cyclases to cilia are unknown, and ciliary localization signals are not well defined. If cAMP signaling in cilia is critical, loss of trafficking of adenyl cyclases to cilia should result in increased Shh signaling, phenocopying other factors in the basal suppression machinery. Identification of factors important in trafficking of adenyl cyclases to cilia and their role in development and disease are important directions to pursue in the future.

**MEMBRANE PROTEIN FLUX IN CILIA**

One of the best examples of transmembrane protein flux in the ciliary membrane in vertebrates is provided by Smo (Corbit et al., 2005). Smo is a seven-transmembrane receptor that has an external cysteine-rich domain similar to Frizzled (Byrne et al., 2016; Huang et al., 2016). A potential route for Smo trafficking into the ciliary membrane is by lateral diffusion from the plasma membrane (Milenkovic et al., 2009). Unlike certain class A GPCRs, trafficking of Smo to cilia is Tulp3 independent (Mukhopadhyay et al., 2010; Qin et al., 2011). The Smo ciliary targeting sequence has been mapped to the C-terminal tail of the protein (Kim et al., 2015). However, upon activation of Shh signaling, Smo is retained in cilia. Recent exciting results suggest that cholesterol binding to Smo at its...
Dynamic redistribution of proteins also occurs in the flagella of *Chlamydomonas* plus gametes in a signaling-dependent manner during fertilization. Signaling in plus gametes induces rapid redistribution of the plus agglutinin (SAG1) from the plasma membrane to the periciliary region and the ciliary membrane (Belzile et al., 2013). Of interest, the entire complement of cellular SAG1 is shed during signaling in the form of ciliary exosomes (Cao et al., 2015).

**CONSEQUENCES OF LACK OF TRAFFICKING TO CILIA**

To understand the role of cilia in cellular pathways, it is critical to determine whether localization of proteins in the cilium membrane is important for signaling. GPCRs that localize to cilia also localize to the plasma membrane and in the recycling endosomal compartment (Marley and von Zastrow, 2010; Leaf and Von Zastrow, 2015). Similarly, the polycystin PC2 is present mostly in the endoplasmic reticulum (ER), and PC1 promotes its exit from ER and trafficking to cilia (Cai et al., 2014; Kim et al., 2014a; Gainullin et al., 2015). Apart from localizing to cilia, the polycystins PC1/2 and fibrocystin are also present in urinary exosomes (Pazour et al., 2002; Yoder et al., 2002; Ward et al., 2003; Hu et al., 2007; Hogan et al., 2009; Chapin and Caplan, 2010). Localization of GPCRs or polycystins in cilia does not necessarily imply that they function in this compartment in the context of a particular pathway.

To understand the role of trafficking of a protein to the ciliary membrane in relation to a cellular pathway, it is important to maintain intact cilia and prevent ciliary localization without affecting other functions of the trafficked protein. Affecting the IFT machinery or transition zone complex proteins results in gross ciliary defects. In addition, in many ciliary membrane proteins, such as Ptch1, the function of the protein is still speculative (Bazan and de Sauvage, 2009). Thus it is difficult to ascertain whether a Ptch1 mutant that does not traffic to cilia retains its native function. In such cases, ciliary-trafficking mutants can be fused with heterologous ciliary-targeting sequences and tested for rescue in knockout cells. If the mutant chimeric construct rescues the knockout phenotype, the mutant nonciliary form possibly retains native function and can be assessed for its role in the pathway. Similar experiments suggest that a Ptch1 mutant lacking in trafficking to cilia is ineffective in Shh signaling (Kim et al., 2015). Certain mutants in the polycystin PC2 suggest that trafficking to cilia is important. A mutation in the highly conserved extracellular polycystin domain in PC2 (PC2W414G) in polycystic kidney disease patients prevents trafficking to cilia but has wild-type channel properties (Cai et al., 2014). A mutant allele in mouse (PC2m4; E442G within the conserved ion channel region of PC2) that is not trafficked to cilia retains channel activity but causes left–right asymmetry similar to a germline PC2 knockout (Ermakov et al., 2009; Yoshida et al., 2012). In the case of GPCRs, a similar approach would be to test whether mutants that are defective in ciliary trafficking but are otherwise functional result in phenotypes similar to the germline knockouts.

Phenotypes resulting from a lack of cilia-localized proteins are further modified in the background of cilia mutants. Whereas lack of PC1/2 causes severe polycystic kidney disease, the cysts are suppressed in the absence of cilia (Ma et al., 2013). Thus phenotypes arising from lack of PC1/2 require cilia, and a cilia-dependent cystogenesis pathway is suppressed by PC1/2 ciliary localization. Unlike PC1/2, the related polycystins PKD1L1/PKD2L1 function as calcium-selective ion channels in cilia, as detected by patch clamping of cilia (DeCaen et al., 2013; Delling et al., 2013). However, PC1/2 might be functioning in cilia as “regulated” cation channels, with yet-unidentified ligands. Determining factors that target polycystins to cilia and identifying the cilia-regulated cystogenic pathway are important future directions in studying polycystic kidney disease pathogenesis.
CONCLUSIONS
Although the cilium is a tiny cellular compartment, it has profound implications in signaling pathways. Because ciliary membrane–enriched proteins are not exclusive to this compartment, it is critical to identify factors important in trafficking them to the ciliary membrane. It is also important to determine the role of ciliary localization in the respective pathways both in GPCR-regulated signaling and in polycystic kidney disease. Furthermore, measurement of ciliary levels of second messengers and lipids are exciting newer directions in this rapidly evolving field. Finally, identifying factors in trafficking of ciliary membrane proteins and determining their role in the pathology of ciliopathies might provide unique approaches to targeting these debilitating diseases.

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