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Control of the Hedgehog pathway by compartmentalized PKA in the primary cilium

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Abstract

The Hedgehog (Hh) signaling is one of the essential signaling pathways during embryogenesis and in adults. Hh signal transduction relies on primary cilium, a specialized cell surface organelle viewed as the hub of cell signaling. Protein kinase A (PKA) has been recognized as a potent negative regulator of the Hh pathway, raising the question of how such a ubiquitous kinase specifically regulates one signaling pathway. We reviewed recent genetic, molecular and biochemical studies that have advanced our mechanistic understanding of PKA's role in Hh signaling in vertebrates, focusing on the compartmentalized PKA at the centrosome and in the primary cilium. We outlined the recently developed genetic and optical tools that can be harvested to study PKA activities during the course of Hh signal transduction.

Keywords

Hedgehog signaling; protein kinase A (PKA); primary cilium; centrosome; compartmentalized cell signaling

Introduction

Overview

The Hedgehog (Hh) signaling is an evolutionarily conserved pathway that regulates key events during embryonic development and in adults. During early embryonic development, it plays key instructive roles in pattern formation and cell fate determination in the telencephalon, developing spinal cord, and digits (Fuccillo et al., 2006; Sagner and Briscoe, 2019). Right after birth, Hh signaling controls cell proliferation in the developing cerebellum (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). In adults, it plays critical roles in the establishment and maintenance of stem cells in the nervous system and hair follicles in the skin (Han et al., 2008; Lee et al., 2016). Errors in Hh signaling lead to various birth defects, such as spina bifida, holoprosencephaly, polydactyly, skeletal malformations, heart and lung defects (Ruiz i Altaba et al., 2002; McMahon et al., 2003; Nieuwenhuis and Hui, 2005). Excessive Hh signaling leads to cancers, such as medulloblastoma, a malignant pediatric brain tumor caused by uncontrollable progenitor

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proliferation in the cerebellum (Stone et al., 1996; Pugh et al., 2012; Raleigh and Reiter, 2019). In adults, overactive Hh signaling leads to basal cell carcinoma, one of the most common skin cancers (Johnson et al., 1996; Xie et al., 1998).

The pathway originally got its name from the work of Nüsslein-Volhard and Wieschaus, who isolated *Drosophila* mutants to study body segmentation in the 1970s (Nüsslein-Volhard and Wieschaus, 1980). "Hedgehog" was named because the mutant embryos appeared stubby with denticle hairs pointing in random directions resembling a hedgehog. The mutated gene was later identified to be the eponymous ligand for the Hh pathway. The gene family of this ligand is expanded to three paralogs in amniotes: desert hedgehog (Dhh), Indian hedgehog (Ihh) and sonic hedgehog (Shh). Each ligand is expressed in distinct developing tissues (Bitgood and McMahon, 1995; Pathi et al., 2001). Shh is predominately expressed in tissue such as the central nervous system (CNS), limb, hair, gut, and is the best studied ligand (Goodrich et al., 1996). Shh may function as a mitogen to stimulate cell proliferation, such as the neural progenitor proliferation in the developing cerebellum (Stone et al., 1996; Pugh et al., 2012; Raleigh and Reiter, 2019). It may also function as a morphogen to induce cell fate determination by exposing target cells with different signaling strength and duration, such as the well-studied neuronal cell fate determination in the developing spinal cord (Sagner and Briscoe, 2019).

There are many similarities in the transduction mechanisms of Hh signaling between *Drosophila* and the vertebrate system (Ingham, 2018). In both systems, Hh signaling is transduced via alternative de-repression. When the signaling is off, Ptch inhibits Smo, and SuFu inhibits Gli transcription factors. Upon ligand binding, Ptch is inhibited, leading to the activation of Smo. Active Smo then suppresses SuFu to release SuFu's inhibition on Gli transcription factors (Ci in *Drosophila*). However, details of signal transduction differ significantly in almost every step of the pathway, mainly because of the reliance on the primary cilium in vertebrates. *Drosophila* cells lack cilia, except in mechanical sensory neurons and sperm (Basto et al., 2006). The primary cilium provides a special cellular compartment where receptors and signal transducers are highly concentrated to effectively sense and transduce signaling cues from outside the cell into signaling cascades within the cell (Barr and Sternberg, 1999; Huangfu et al., 2003; Pazour and Witman, 2003). It also provides a privileged space to allow ubiquitous enzymes, such as cAMP-dependent protein kinase A (PKA), to regulate specific cell signaling, such as Hh signaling. A comprehensive review of biochemical events in Hh signaling can be found in the review by Kong et al. (2019). In this review, we will focus on the roles of PKA in Hh signal transduction in the vertebrate system. We will summarize previous discoveries from mouse genetics, cell biology and biochemical studies that implicate PKA as an essential regulator of the Hh pathway, and outline recent techniques that have been used to study PKA regulation of Hh signaling in the primary cilium.

Hh signal transduction relies on the primary cilium

Almost every vertebrate cell has a primary cilium. This specialized microtubule-based, membrane-enclosed organelle projects from the cell surface, and has been historically considered as an evolutionary vestige of the flagellum (Rosenbaum and Witman, 2002;

Satir and Christensen, 2007). However, recent studies have revealed pivotal roles of this organelle in signal transduction. The very first clue that cilia may be involved in transducing cell signaling came from the seminal discovery by Kathryn Anderson. Mutations in genes encoding proteins involved in intraflagellar transport (IFT) cause defects in mammalian Hh signal transduction (Huangfu et al., 2003; Bangs and Anderson, 2017). Since then, almost all Hh signaling components have been found in the cilium, and Hh signal is now known to be transduced through a choreographed trafficking of signaling proteins into and out of the cilium (Mukhopadhyay et al., 2010; Bangs and Anderson, 2017).

When the Hh pathway is off, the 12-transmembrane protein Ptch is enriched in cilia to inhibit Smo (Figure 1A). Recent results from cryo-EM suggest that the underlying mechanism may involve the local control of cholesterol composition in the ciliary membrane by Ptch, which structurally resembles a sterol transporter (Huang et al., 2018; Qi et al., 2018a; Qi et al., 2018b; Zhang et al., 2018; Deshpande et al., 2019; Rudolf et al., 2019). Sterol lipids such as cholesterol can activate Smo (Corcoran and Scott, 2006; Huang et al., 2016; Raleigh et al., 2018). The transcription factor Gli3, primed by PKA phosphorylation, is proteolytically processed into a transcription repressor Gli3R that translocates into the nucleus to keep Hh target genes off. PKA also prevents the activation of Gli2, a transcription activator. In the presence of Shh, Ptch binds to Shh and exits from the cilium (Figure 1B). Subsequently, Smo is activated and accumulates in the cilium. Active Smo antagonizes the inhibitory effects of PKA on Gli3 and Gli2. As a result, Gli3R production ceases, and Gli2 accumulates at the tip of the cilium where it is activated. Thus, inhibition from Gli3R is released, and active Gli2 enters the nucleus to turn on the transcription of Hh target genes. Not surprisingly, proteins involved in IFT, a bidirectional trafficking system along ciliary axonemal microtubules, are involved in the regulation of Hh signaling (Huangfu et al., 2003; Keady et al., 2012; Lechtreck, 2015; Eguether et al., 2018). These proteins include motor proteins, such as kinesin (Kif3a, Kif7) and dynein complexes (Dync2h1, Dync2li1, Wdr34), and IFT proteins (IFT172, IFT88 etc.). Comprehensive reviews about IFT in Hh signaling can be found in Lechtreck (2015), Bangs and Anderson (2017) and Eguether et al. (2018). Many steps in this process remain mysterious. For example, why do Hh signaling components need to transit to the primary cilium? How is this complicated protein trafficking coordinated in the cell? How does PKA, a ubiquitous housekeeping kinase, selectively regulate the Hh pathway? We will focus on the last question to summarize the specific regulation of Hh signaling by PKA in the primary cilium and at the centrosome. In ciliated cells, the mother centriole of the centrosome becomes the basal body to organize the cilium microtubules; for simplicity, hereafter we use centrosome to refer to both in this review.

PKA is a strong negative regulator of the Hh pathway

PKA subunits and its activation

The PKA holoenzyme is a heterotetramer consisting of two catalytic (PKA-C) subunits and two regulatory (PKA-R) subunits. When cAMP binds to the R subunits, the PKA holoenzyme dissociates to release active C subunit (Turnham and Scott, 2016). In mice, two genes encode catalytic subunits (Cα and Cβ) and four genes encode regulatory subunits

(RIα, RIβ, RIIα and RIIβ) (Taylor et al., 1990; Torres-Quesada et al., 2017). The expression pattern of these isoforms shows some degree of tissue specificity revealed by *in situ* hybridization in mouse embryos and adults (Cadd and McKnight, 1989; Guthrie et al., 1997). Within the cell, different regulatory subunits may localize to distinct subcellular sites (Ilouz et al., 2017). RI subunits are found in the primary cilium (Mick et al., 2015), whereas RII subunits localize to the centrosome (Barzi et al., 2010).

PKA activity is primarily controlled by the levels of the second messenger cAMP, which directly binds to and activates PKA (Figure 2A). cAMP levels are modulated by signaling cascades initiated by GPCRs. Upon activation by ligands, GPCRs activate heterotrimeric G-proteins by catalyzing the exchange of GDP for GTP on the Gα subunit, resulting in the dissociation of Gα from Gβ-γ subunits. Gαs activates adenylyl cyclases (AC) that synthesize cAMP, whereas Gαi subunits reduce cAMP levels by inhibiting AC. cAMP levels are also controlled by phosphodiesterases (PDEs) that hydrolyze cAMP (Beavo and Brunton, 2002) (Figure 2A). In addition to cAMP, PKA activity is also modulated by phosphorylation in an intra- or inter-molecular manner (Keshwani et al., 2012; Torres-Quesada et al., 2017). C subunits are phosphorylated on T197 and S338. Phosphorylation of T197 in the kinase activation loop is a characteristic of active PKA-C, and is presumably carried out by PDK1 (Taylor et al., 2013). Phosphorylation of the PKA-RII subunits affects the PKA holoenzyme assembly and impacts the dissociation of C subunits from the R subunits upon cAMP binding (Zhang et al., 2015). More details about PKA activity regulation and its implications in pathological processes can be found in these reviews (Gold et al., 2013; Röck et al., 2015; Torres-Quesada et al., 2017).

PKA is involved in myriad cellular processes in development, metabolism, memory formation and proliferation. It is an intriguing question how a specific cell signaling pathway is selectively regulated by such a ubiquitous kinase. An emerging view proposes that the precise spatiotemporal control is achieved via compartmentalization of PKA activity in the cell. A-kinase anchoring proteins (AKAPs), multi-domain scaffold proteins, bind to PKA-R and anchor the holoenzyme to discrete subcellular compartments (Wong and Scott, 2004; Baillie et al., 2005). AKAPs serve as scaffolds to deposit PKA in the vicinity of its substrates together with other enzymes such as phosphatases, phosphodiesterases, or other components of the specific signaling pathways, thereby providing tailored PKA signaling units (aka, signalosomes) (Torres-Quesada et al., 2017). PKA activity at the centrosome and in the cilium is directly involved in the Hh signaling. AKAP9, AKAP450 and Myomegalin target PKA to the centrosome (Verde et al., 2001; Uys et al., 2011; Terrin et al., 2012), and GPR161 is reported to anchor PKA to the primary cilium (Bachmann et al., 2016) (Figure 2B). As detailed in section 3, manipulating local PKA activity at the centrosome and in the cilium, even though the global PKA activity is not impacted, is sufficient to control Hh signaling.

Genetic evidence of PKA as a potent suppressor of the Hh pathway

The involvement of PKA in Hh signaling was first observed in *Drosophila*. Loss of PKA activity leads to activation of Hh target genes in embryonic tissues such as eye, leg and wing imaginal discs (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and

Rubin, 1995; Strutt et al., 1995). Subsequent studies in zebrafish showed that expression of dominant negative or constitutively active forms of PKA in the zebrafish embryo led to transcriptional activation or inhibition of Shh target genes, respectively (Concordet et al., 1996; Hammerschmidt et al., 1996). Pharmacological approaches that increase PKA activity were found to block Shh-mediated induction of dopaminergic neurons (Fan et al., 1995; Hynes et al., 1995).

A transgenic mouse model was generated by Epstein et al. (1996) in which a dominant-negative PKA (dn-PKA) was expressed in the developing CNS. This *dnPKA* encodes a PKA regulatory subunit that is insensitive to cAMP, thereby preventing the activation of PKA-C. The dnPKA transgenic mouse exhibited high levels of Hh signaling activity, including increased Hh-target expression and mis-patterning of the embryonic CNS (Epstein et al., 1996). Huang et al. (2002) generated PKA-deficient mice that retain only one functional PKA-C. Decreased PKA activity led to neural tube patterning deficits that resemble the phenotypes of overactive Hh signaling. Tuson et al. (2011) generated a mouse model that lacks both PKA-Cα and PKA-Cβ, and found that the double mutant caused complete ventralization of the neural tube to the same extent as Ptch1- and SuFu-null embryos (Goodrich et al., 1997; Cooper et al., 2005; Svärd et al., 2006). These results suggest that inhibiting PKA leads to maximal activation of the Hh pathway.

PKA activity is controlled by cAMP levels. Therefore, disturbing cAMP production in mutants along the axis of GPCR-G protein-AC also impairs Hh signaling in relevant tissues during embryogenesis. Mukhopadhyay et al. (2013) found that loss of GPR161, which is coupled to Gαs, inhibits PKA in the primary cilium and increases Shh signaling in the developing neural tube. Conversely, loss of GPR175, a Gαi-coupled receptor, increases cAMP/PKA activity and suppresses Hh signaling (Singh et al., 2015). Regard et al. (2013) reported that loss of Gαs upregulates Hh signaling via decreased cAMP/PKA activity, leading to ectopic ossification, like what is observed in osseous heteroplasia (POH). In the developing cerebellum and neural tube, overexpressing AC5 and AC6 represses the Hh pathway, whereas their knockdown results in the activation of Hh signaling (Vuolo et al., 2015). Recently, Somatilaka et al. identified that Ankmy2 is responsible for the maturation and trafficking of multiple ACs to the primary cilium, and Ankmy2 knockout mouse exhibits neural tube ventralization, to the similar degree as in *Ptch1*- and *PKA*-null mouse (Goodrich et al., 1997; Tuson et al., 2011; Somatilaka et al., 2020). Furthermore, the action of Ankmy2 is independent of Smo and directly controls Gli2 and Gli3 processing (Somatilaka et al., 2020). In summary, multiple lines of genetic evidence strongly implicate PKA as an essential and potent suppressor of the Hh pathway.

Gli transcription factors are targets of PKA in Hh pathway

The Gli homolog in *Drosophila* is Ci (Cubitus interruptus). In the absence of Hh ligand, Ci is phosphorylated by a protein complex that includes Cos2, PKA, GSK3, and Fused (Fu) (Robbins et al., 1997; Sisson et al., 1997; Zhang et al., 2005). Phosphorylated Ci is proteolytically processed to an inactive form. The presence of Hh ligand stabilizes fly Smo which competes for Cos2 association and prevents Cos2-Kinase complex formation. This prevents Ci from being phosphorylated, allowing it to remain in its full-length active

form (Ruel et al., 2003), resulting in activation of the Hh pathway. In vertebrates, the Ci protein expands into three isoforms, Gli1/2/3, making Gli protein processing more complex (Ruppert et al., 1988). Mouse genetics studies show that Ci's role seems to be unevenly distributed between the three Gli proteins, with Gli1 and Gli2 playing predominantly activating roles and Gli3 playing a repressive role (Ruppert et al., 1988; Ding et al., 1998; Matise et al., 1998; Bai and Joyner, 2001; Bai et al., 2002; Persson et al., 2002; Rallu et al., 2002). Gli1 is a Hh-target gene and acts to amplify Hh signaling (Park et al., 2000), whereas the ON and OFF switch of Hh signaling is determined by the balance between the Gli2 activator and Gli3 repressor.

In the absence of Shh, Gli3 is phosphorylated at six sites by PKA, which primes Gli3 for further phosphorylation by glycogen synthase kinase 3-beta (GSK3b) and casein kinase 1 (CK1) (Wang et al., 2000; Tempe et al., 2006; Wang and Li, 2006). The phosphorylated residues provide a high-affinity binding site for β-TrCP, which then recruits the SCF (Skp1/Cullin1/F-box) ubiquitin ligase complex (Wang and Li, 2006). Ubiquitinated Gli3 gets cleaved by the proteasome, converting the 190-kDa Gli3 full length (Gli3FL) protein into an 83-kD Gli3 repressor form (Gli3R) (Wang and Li, 2006; Pan and Wang, 2007; Wen et al., 2010). Gli3R enters the nucleus and suppresses the transcription of Hh target genes. Activation of Hh signaling represses this processing, via mechanisms including the inhibition of PKA activity, resulting in the existence of Gli3 predominantly in its full-length form. In contrast, Gli2 protein is minimally processed, and the vast majority of Gli2 is present in the full-length form (Pan et al., 2006). However, phosphorylation by PKA facilitates Gli2 degradation, as Gli2 protein lacking PKA phosphorylation sites is more stable than wild type Gli2, and is constitutively active in mouse embryos (Pan et al., 2009; Niewiadomski et al., 2014; Liu et al., 2015). Further, in PKA-null cells, Gli2 accumulates at the cilium tip, suggesting that PKA normally restricts Gli2 transportation into the cilium (Tuson et al., 2011).

The processing and activation of Gli2/3 requires the primary cilium and involves two other proteins, SuFu and Kif7 (Haycraft et al., 2005; Chen et al., 2009; Cheung et al., 2009; Endoh-Yamagami et al., 2009; Kim et al., 2009; Liem et al., 2009). SuFu binds to Gli proteins, preventing their degradation, but also inhibits their activation. Dissociation of Gli/SuFu complexes in cilia is a critical step in the activation of the vertebrate Hh pathway (Humke et al., 2010; Tukachinsky et al., 2010). In the current model, without Shh stimulation, Gli2 and Gli3 complex with SuFu and are primed by PKA at the base of the cilium. The complexes then enter the cilium, where Gli proteins are further phosphorylated by CK1 and GSK3b. After exiting the cilium, Gli3 is converted to Gli3R by the proteasome and Gli2 remains inactive. Upon Shh stimulation, Hh signaling cascade decreases PKA activity at the cilium base, preventing it from phosphorylating Gli proteins. The Gli/SuFu complex enters the cilium, where Gli is not fully phosphorylated by CK1 and GSK3b and the complex dissociates (via unknown mechanisms). Gli3R production ceases, and free Gli2 exits from the cilium and acts as the transcription activator to turn on transcription of Hh target genes in the nucleus.

The subcellular pool of PKA at the centrosome and in the cilium regulates Hh signaling pathway

Accruing evidence from pharmacological, biochemical and genetic studies suggest the inverse relationship between PKA and Hh signaling: increasing PKA activity suppresses Hh transduction, whereas inhibiting PKA activity enhances the strength of Hh signaling (Hammerschmidt et al., 1996; Huang et al., 2002; Barzi et al., 2010; Humke et al., 2010; Tukachinsky et al., 2010; Tuson et al., 2011; Niewiadomski et al., 2013; Vuolo et al., 2015; Williams et al., 2015; Ge et al., 2015; Bachmann et al., 2016; Moore et al., 2016; Kong et al., 2019). It is tempting to speculate that the reverse paradigm is also true: PKA activity must be suppressed in order for the Shh pathway to be activated; in other words, active Hh signaling must suppress PKA activity. However, early attempts at detecting global PKA activity in the entire cell failed to verify such assumptions (Niewiadomski et al., 2013). In fact, under some special circumstances, high total cell PKA levels can occur in parallel with Hh target gene transcription (Niewiadomski et al., 2013). Thus, global PKA levels are not good indicators of Hh signaling levels. Instead, studies in the field support a more precise model that core Hh signaling regulates compartmentalized pools of PKA at discrete subcellular sites, such as the centrosome and the primary cilium.

Signaling outcomes that change PKA activity at the centrosome regulate Hh signaling

Early immunocytochemical studies showed that PKA subunits are concentrated at the centrosome and manipulating PKA activity at this local site is sufficient to alter Hh signaling (Barzi et al., 2010; Tuson et al., 2011; Ge et al., 2015). In granule neural precursors (GNPs) from the developing cerebellum, the PKA holoenzyme is anchored to the basal body via an interaction between RII subunit and AKAP proteins. When this interaction is disrupted by a peptide St-HT31, GNP proliferation is inhibited (Barzi et al., 2010). In the developing neural tube, PKA-C immunofluorescence intensity concentrates at the centrosome and PKA-C knockout results in an increased Gli2 accumulation at the cilium tips, leading to the hypothesis that PKA controls Gli2 trafficking at the centrosome (Tuson et al., 2011). Local cAMP level is also controlled by phosphodiesterase 4D (PDE4D), the enzyme that converts cAMP into AMP. When PDE4D is recruited to the cytoplasmic membrane where most cAMP is produced, cAMP is more effectively degraded, leading to enhanced Hh signaling (Ge et al., 2015; Williams et al., 2015). In addition, a signalosome that includes PDE4D3 and the scaffold protein Myomegalin was identified at the centrosome (Verde et al., 2001); Myomegalin loss dislocates PDE4D3 from the centrosome, which subsequently promotes local PKA activity and suppresses Hh signaling (Peng et al., 2021).

PKA in the cilium specifically regulates Hh signaling

Since all Hh pathway components are found in the cilium, it is highly conceivable that PKA resides and signals inside the cilium, although early attempts failed to confirm so (Tuson et al., 2011). However, most Hh pathway transducers in the axis of GPCR-AC-cAMP reside in the primary cilium, such as GPR161 and AC3/5/6 (Mukhopadhyay et al., 2013; Somatilaka et al., 2020). Some G proteins and cAMP specific PDEs are also reported to be present in the cilium (Choi et al., 2011; Ishikawa et al., 2012). Therefore, the cAMP levels inside the

cilium must be tightly controlled, and subtle changes in cAMP levels can only be perceived by local PKA in the cilium. Finally, PKA substrates Gli2 and Gli3 traffic through the cilium, and their transition in the cilium is crucial for their stability and activation (Haycraft et al., 2005; May et al., 2005; Chen et al., 2009; Wen et al., 2010). Hence, the critical phosphorylation events must happen in the cilium. In a recent proximity-based study, the subunits of PKA-RIα, -RIIβ and -Cα were discovered as candidate proteins in the cilium (Mick et al., 2015). When RIα was fused with GFP, the tagged proteins were observed in the axoneme of the primary cilium (Mick et al., 2015). The GFP-tagged PKA-C was also discovered to be present in the cilium (Arveseth et al., 2021; Truong et al., 2021). However, so far direct evidence showing that endogenous PKA is localized to the cilium, such as immunostaining results, is still missing. The endogenous PKA in the cilium is difficult to detect presumably because it rapidly transits through the cilium and the level is under current detection limits, or because of its lability for conventional fixation methods.

The validation of PKA subunits in the cilium indicates that the phosphorylation of Gli2/3 could take place directly in the cilium. Indeed, with an antibody that can recognize PKA's phosphorylated residues in Gli2/3, Li et al. (2017) found that the levels of phosphorylated Gli2 and Gli3 in the cilium are inhibited by Hh signaling. Moreover, in a genome-wide RNAi screen, Jacob et al. (2011) reported that ablation of PKA-RI compromises Hh signaling. Finally, when Mick et al. (2015) targeted a PKA inhibitor, PKI, to the cilium to specifically inhibit local PKA activity, the processing of Gli3 into Gli3R was reduced. Therefore, it is proposed that high basal activity of cAMP/PKA in the cilium contributes to the phosphorylation of Gli3 and promotes its proteolytic processing into Gli3R. Once the Hh pathway is activated, cAMP levels in the cilium decease. Subsequent PKA inhibition reduces Gli3 processing and allows Gli2 to be activated. This model explains the dynamic regulation of Gli3 processing by local PKA in the cilium; however, how Gli2 activation is controlled by cilium localized PKA still remains enigmatic.

The signaling cascade that links Hh signaling with PKA activity

Based on the inverse correlation between PKA activity and Hh signaling, it is believed that the cascade of Hh signaling eventually abrogates PKA activities in the vicinity of the primary cilium, subsequently promoting the activation of Gli. Nevertheless, the specific molecular and biochemical mechanisms that link active Hh signaling to PKA remain controversial. Previous studies have implicated GPCRs, PDEs, and adenylyl cyclase-trafficking proteins in this process. In this section, we will summarize these studies and outline our current understanding of PKA regulation during Hh transduction in the vertebrate system (Figure 3).

From Smo to PKA—Smo belongs to the family of class F (Frizzled) GPCRs. Some evidence in *Drosophila* suggest that Smo may act as a conventional GPCR to activate the trimeric G protein, Gαi (Ogden et al., 2008; Ayers and Thérond, 2010; Qi et al., 2019). As Gαi inhibits AC to reduce cAMP levels, its activation is predicted to reduce PKA activity, thereby promoting Hh signaling (Figure 3). However, contradicting results were revealed in other studies. Li et al. found that active PKA phosphorylates Smo and promotes Smo's membrane localization to activate the Hh pathway (Li et al., 2014; Li et al., 2016). Further,

a constitutively active PKA-C can substitute for endogenous PKA-C to govern the proper patterning during *Drosophila* organogenesis (Jiang and Struhl, 1995; Li et al., 1995). These results suggest a possible dual role of PKA in Hh signaling in *Drosophila*, indicating that the link between Smo and PKA may not be as simple as originally proposed.

Studies in vertebrates also revealed mixed results. Early biochemical assays in vertebrate cells overexpressing both Smo and Gαi indicate that Smo may directly activate Gαi (DeCamp et al., 2000; Shen et al., 2013). However, further biochemical and genetic studies failed to fully corroborate this notion. Biochemical studies in mammalian cells revealed that deleting the C-tail in Smo, the sequence required for Gli activation, has no impact on Gαi activation (Riobo et al., 2006). Since the Gαi family in vertebrates are encoded by multiple genes (*Gnai1*, *Gnai2*, *Gnai3*, *Gnao1* and *Gnaz*) that are redundant to each other, many genetic studies employed pertussis toxin (Ptx), an uncoupler of all Gαi proteins (except Gαz) from upstream GPCRs. Ptx locks Gαi proteins in inactive states, resulting in the activation of adenylyl cyclase and increase in cAMP/PKA activity. Expressing Ptx in zebrafish embryos only partially impacted the ventral specification in the nervous system, but not other Hh-dependent patterning (Hammerschmidt and McMahon, 1998). Electroporation of dominant negative Gαi or pertussis toxin into the developing chicken neural tube failed to induce discernible phenotypes that are related to Hh defects (Low et al., 2008). Finally, genetic expression of Ptx in mouse embryos did not impair Hh-dependent limb patterning and skeletal development (Regard et al., 2013). Therefore, the evidence is inconsistent for Smo to act as a Gαi-coupled GPCR to control cAMP/PKA activity.

One recent study hinted at an alternative connection between Smo and PKA (Arveseth et al., 2021). PKA-C can directly bind to the C-terminal tail of active Smo, triggered by GPCR kinase 2 (GRK2)-mediated phosphorylation of the Smo intracellular domain. This sequestration of PKA prevents its phosphorylation of Gli proteins, releasing PKA's inhibition on Gli. However, these results were obtained in non-ciliated 293T cells—whether the same mechanism applies to Smo-PKA interaction in the cilium needs to be confirmed.

GPR161 and PKA activity in the primary cilium—GPR161 is a cilium localized GPCR that negatively regulates Hh signaling (Mukhopadhyay et al., 2013). GPR161 resides in the cilium and activates Gαs to produce basally high cAMP levels within the cilium. Upon Hh activation, GPCR161 is cleared from the cilium within two hours, which dampens local PKA activity to promote Hh signaling (Figure 3). GPR161 knockout mice are lethal by E10.5 with ventralized neural tubes, resembling the phenotypes of higher Shh signaling in Ptch1- and PKA-null mice (Goodrich et al., 1997; Tuson et al., 2011; Mukhopadhyay et al., 2013). The knockout mice also exhibit morphogenesis defects in other tissues that involve Hh signaling, such as the limbs and skeleton (Hwang et al., 2018), as well as medulloblastoma formation in the developing cerebellum (Shimada et al., 2018). Consistent with its role in promoting cAMP/PKA activity, GPR161 regulates the proteolytic processing of Gli proteins. In GPR161 mutants, Gli3R levels are reduced, presumably due to reduced PKA activity, as well as the levels of full-length Gli2 and Gli3 (Mukhopadhyay et al., 2013). However, the link between GPCR and cAMP production needs G proteins, and so far, neither Gαi or Gαs has been reproducibly validated to be present in cilia, raising the question of how GPR161 regulates the local enzymatic activity of ACs and PKA.

Further studies elucidated the trafficking mechanisms of GPR161 into and out of the cilium. The intraflagellar transport A (IFT-A) core complex and the tubby family protein Tulp3 coordinate Gpr161's trafficking to cilia (Badgandi et al., 2017). Indeed, IFT-A or *Tulp3* mutants exhibit high Shh signaling, which is opposite to other mutants that affect the cilia (Goetz and Anderson, 2010). Removal of GPR161 from the cilium requires active GRK2 that recruits β-arrestin to the cilium. Active Smo promotes β-arrestin-GPR161 interaction, and the subsequent clathrin mediated endocytosis clears GPR161 from the cilium (Pal et al., 2016). GPR161 may also function as an AKAP to anchor the subunit of PKA-RI into the cilium with its C-terminal tail (Bachmann et al., 2016). In line with this, a recent study by May et al. (2021) proposed that upon Shh stimulation, PKA-RI reunites with PKA-C, and the holoenzyme piggybacks on GPCR161 to exit the cilium. However, this model assumes that PKA-C localizes to the cilium, which has not yet been verified.

A recent study reported that GPR161 functions as an attenuator for Hh signaling, rather than a negative regulator like Ptch and SuFu. Loss of the negative regulators leads to constitutive activation of the pathway in the absence of ligand; this is not observed in GPR161-null NIH3T3 cells (a ciliated cell line that expresses all components of the Hh pathway) (Pusapati et al., 2018). Instead, Shh and Smo agonists can still activate Hh signaling in GPR161-null cells, although with higher potency and efficacy than wild-type cells. The role of GPR161 as a signaling attenuator may explain why the phenotypes of *Gpr161* mutant mice are much milder than these of PKA-null mutants (Tuson et al., 2011). Thus, GPR161 may function by modulating the cell's sensitivity to Shh, a morphogen that drives cell fate determination and patterning of developing organs, such as the spinal cord, limb bud and paraxial mesoderm (Pusapati et al., 2018).

Control of compartmentalized cAMP/PKA activity by PDE—Within the cell, cAMP levels are controlled by two enzymes: production is regulated by ACs and degradation is regulated by cAMP-specific phosphodiesterases (PDE4,7,8). While most recent studies focus on local cAMP production by AC, PDEs have been long studied to play a pivotal role in shaping the spatial and temporal dimension of cAMP "pulses" in the cell to allow precise control of signal transduction. The concept of compartmentalized signaling is rooted in studies of PDE families in the early 1980s on cardiomyocytes (Hayes et al., 1980; Scotland et al., 1998; Rich et al., 2001; Houslay and Adams, 2003; Houslay, 2010). Visualization of cAMP levels in cardiomyocytes with cAMP reporters revealed strips of cAMP "hotspots" when β-adrenergic receptors (A Gαs-coupled GPCR) were activated. Co-treatment with a PDE inhibitor and the receptor agonist, however, abolished these "hotspots", producing uniform and global increases in cAMP and concomitant activation of all cAMP effectors (Zaccolo and Pozzan, 2002). This led to the concept that cAMP levels are compartmentalized in the cell, and local cAMP levels are precisely sculped by PDEs that anchor to specific subcellular compartments (Wong and Scott, 2004). In other words, PDEs act as local cAMP "sinks", allowing cAMP to accumulate in areas devoid of PDEs to form cAMP "hotspots". In this way, local cAMP gradients can gate the activation of effector proteins, such as PKA, that are sequestered at the same subcellular compartments. Among all PDE proteins, the best characterized is the PDE4D subfamily that contains 11 isoforms derived from alternative splicing. Each isoform of PDE4D has an identical C-terminal

catalytic domain and a unique N-terminal domain. The N-terminus anchors the enzyme to distinct subcellular sites (Scotland et al., 1998; Lefkowitz et al., 2002; Baillie et al., 2005; Lynch et al., 2006), where PDE4D selectively regulates specific signaling pathways (Scott and Pawson, 2009; McCormick and Baillie, 2014).

The effect of compartmentalized PDE4D on Hh signaling was first demonstrated by Ge et al. (2015). This study showed that Semaphorin 3-Neuropilin signaling recruits PDE4D to the juxtamembrane region right underneath the cytoplasmic membrane where cAMP is produced by AC. Due to this close proximity, PDE4D effectively degrades cAMP, resulting in an overall decrease in cAMP levels in the cell. This subsequently inhibits PKA at the centrosome and promotes Hh signaling (Hillman et al., 2011; Ge et al., 2015) (Figure 3). The regulation of Hh signaling by Neuropilin-PDE4D signaling was further corroborated by other studies (Hayden Gephart et al., 2013; Snuderl et al., 2013; Williams et al., 2015; Pinskey et al., 2017). Through a chemical genetic screen in zebrafish, Williams et al. (2015) discovered PDE4D as a positive regulator of the Hh pathway, and identified a small molecule inhibitor that selectively suppresses PDE4D thereby elevating PKA activity. Pinskey et al. (2017) reported that Neuropilin 1 is present in the cilium to promote Hh signaling via a previously uncharacterized cytoplasmic motif. Blocking Neuropilins' activity via shRNA or function-blocking antibodies suppresses the growth of Hh-related tumors (Hayden Gephart et al., 2013; Snuderl et al., 2013). A more recent study showed that one PDE4D isoform, PDE4D3, is anchored to the centrosome by Myomegalin (aka. PDE4DIP), a centrosomal and Golgi scaffolding protein. Myomegalin knockout dislocates PDE4D3 from the centrosome, elevates local cAMP/PKA activity and attenuates Hh signaling (Peng et al., 2021). In renal epithelial cells, a protein complex comprising AC5/6, AKAP150, PKA and PDE4C was found to locate in the primary cilium (Choi et al., 2011). Whether and how this complex regulates Hh signaling remain to be identified. Since extensive arrays of PDE4D inhibitors have been developed for respiratory and inflammation diseases in the pharmaceutical industry (Spina, 2008; Page and Spina, 2012; Zaccolo et al., 2021), the identification of compartmentalized PDE4D activity and its local regulation on Hh signaling may provide opportunities to repurpose these inhibitors for pathway specific therapeutics.

Ankmy2, adenylyl cyclase and PKA activity in the cilium—Ankmy2, an ankytin-repeat and MYND-domain containing protein, binds to multiple ACs (AC3/5/6) and determines their maturation and trafficking to the primary cilium (Somatilaka et al., 2020). The lack of ACs in the cilium minimizes local cAMP levels and PKA activity, thereby promoting Hh signaling (Figure 3). Ankmy2-null mouse exhibits full activation of the Hh signaling in the neural tube, with the ventralization to the similar degree as in Ptch1- or PKA-null mice (Goodrich et al., 1997; Tuson et al., 2011). The levels of Gli3FL/Gli3R and Gli2 accumulation at the cilium tips are also similar to those in PKA-null mice. Intriguingly, genetic studies show that the phenotypes of *Ankmy2*$^{-/-}$, *Smo*$^{-/-}$ mice resemble that of *Ankmy2*$^{-/-}$ mice, suggesting *Ankmy2* is fully epistatic to *Smo*. This result indicates that PKA's effect on Gli activation can be independent of Smo.

Other factors that impact PKA activity in Hh signaling—GPR175 is reported to reside in the cilium in a Hh-dependent manner in NIH3T3 cells. GPR175 moves to the

cilium upon Shh stimulation, where it activates Gαi and subsequently inhibits AC and cAMP production. This in turn dampens PKA activity and Gli3R formation, maximizing Hh signal intensity (Singh et al., 2015). So far there is no genetic evidence of roles of GPR175 in Hh signaling.

ArhGAP36, a Rho GTPase-activating protein, was identified as a positive Hh regulator in a genome-wide screen for Hh agonists (Rack et al., 2014). ArhGAP36 overexpression leads to Smo-independent Hh pathway activation. A later study shows that ARHGAP36 antagonizes PKA activity via two mechanisms: blocking PKA-C activity via its pseudosubstrate motif, like a PKI, and targeting PKA-C for ubiquitin-mediated lysosomal degradation (Eccles et al., 2016; Zhang et al., 2019). Consistent with its role as a PKA inhibitor, overexpressing ArhGAP36 is sufficient to reduce Gli3R formation and promote Gli2 transportation to the cilium tip (Rack et al., 2014).

Ca^{2+} has also been shown to regulate Hh signaling in the cilium via PKA. Studies with Patch-clamp and Ca^{2+} imaging techniques showed that the Ca^{2+} level is seven-fold higher in the cilium than the cytosol (DeCaen et al., 2013; Delling et al., 2013; Su et al., 2013). This high Ca^{2+} concentration is maintained by a heteromeric TRP channel, PKD1L1-PKD2L1 (DeCaen et al., 2013; Delling et al., 2013). In addition, prolonged treatment with Hh agonist increased PKD1L1-PKD2L1 levels in the cilium, leading to further elevation of ciliary Ca^{2+} levels. Moore et al. reported that the high Ca^{2+} level in the cilium inhibits AC5/6 to reduce local cAMP levels in the cilium. The subsequent PKA inhibition promotes Hh signaling (Moore et al., 2016). However, knocking out Ca^{2+} ion channels in the cilium (PKD1L1 & PKD2L1) only produces very mild Hh defects in mice (DeCaen et al., 2013; Delling et al., 2013), and the requirement of prolonged treatment with Hh agonist may not be relevant to the rapid change in PKA activity during Hh activation.

Detection of localized PKA activities at specific subcellular sites

Based on the robust inverse relationship between PKA and Hh signaling, it is believed that activation of Hh signaling suppresses PKA activity in the vicinity of the cilium. Since this subcellular pool of PKA only contributes to a small fraction of global PKA activity, conventional biochemical methods failed to detect this change. Over the years, multiple generations of probes for cAMP have been applied to monitor local cAMP levels in the centrosome and at the cilium (DeCaen et al., 2013; Delling et al., 2013; Moore et al., 2016). Nevertheless, direct monitoring of dynamic PKA activity in the cilium has not been reproducibly achieved. We will summarize recent studies on the detection of 2nd messenger levels in the cilium, as well as currently available tools for PKA activity, to inspire the ultimate direct measurement of PKA activity in the vicinity of the cilium.

Most cAMP sensors are based on FRET (Forster resonance energy transfer), which allows for direct monitoring of cAMP levels in live cells. The majority of cAMP sensors are built with the cyclic nucleotide-binding domain (CNBD) of Epac, a family of cAMP-binding effector proteins (Nikolaev et al., 2004; Ponsioen et al., 2004). cAMP binds to these sensors and induces a protein conformation change, resulting in FRET. Moore et al. (2016) targeted one such cAMP probe, cADDis, to the cilium after fusing it to 5-HT6 receptor. They found

that the cilium cAMP level is five times higher than whole-cell levels; Shh reduces ciliary cAMP levels without changing the cAMP concentration in the whole cell. Other studies with a similar Epac-based FRET biosensor also revealed dynamic cAMP changes in the primary cilium in response to activation of Gαs-coupled GPCRs, such as β-adrenergic receptors, and dopamine receptor D1R (Marley et al., 2013; Mukherjee et al., 2016). With Epac-S, a 4th generation Epac-based FRET probe, Stoufflet et al. (2020) detected cAMP hotspots at the centrosome of migrating neurons in the embryonic, postnatal and adult brains. Ablation of the cilium or AC3 abolished the hotspots, leading to neuronal migratory defects. Within the cell, cAMP levels regulate multiple effector proteins, including Epac, cyclic nucleotide-gated (CNG) ion channels, and PKA (Beavo and Brunton, 2002). Therefore, the cAMP levels may not faithfully reflect *bona fide* local PKA activities. Sensitive biosensors that can directly report local PKA activities are needed.

One direct PKA sensor is A-Kinase activity reporter (AKAR) developed by Jin Zhang (Zhang et al., 2001). In this probe, a FRET pair (CFP and YFP) is connected by a linker sequence that contains PKA phosphorylation sites and a phosphoamino acid binding domain (PAABD). PKA phosphorylation induces conformational changes in the linker, bringing the FRET pair in close proximity to efficiently produce FRET. When AKAR3 is targeted to subcellular sites such as the plasma membrane, cytoplasm, nucleus and mitochondria, PKA activity changes in response to related cell signaling were successfully detected (Allen and Zhang, 2006). However, despite the success in these subcellular sites, direct detection of PKA dynamics in the cilium has so far not been achieved, owing to the low sensitivity of the current PKA sensors. The sensitivity is greatly improved in the most recent generation of excitation-ratiometric PKA activity reporter (ExRai-AKAR2) (Mehta et al., 2018; Zhang et al., 2021). Different from its predecessors, ExRai-AKAR is based on single fluorophore cpGFP from GCaMP3 (Tian et al., 2009), in combination with the PKA phosphorylation sequence and PAABD. Phosphorylation by PKA leads to opposite changes in the emission fluorescence intensity at two excitation wavelengths. The resulting ratiometric biosensor greatly increases the dynamic range and the sensitivity to subtle changes in PKA activity within the cell (Zhang et al., 2021). ExRai-AKAR2 may provide hope for the direct detection of PKA dynamics in the primary cilium during the course of Hh signal transduction.

Conclusions

The primary cilium is recognized as a privileged cellular compartment in which many signaling components can interact. 2nd messengers, such as cAMP, Ca^{2+}, and phosphatidylinositol (PIP2, PIP3), have been found to be specially regulated within the cilium, generating a unique signaling environment. The concept of compartmentalized cAMP/PKA signaling in the cell has emerged decades ago. Earlier studies revealed a diverse array of PDEs that can be differentially sequestered to specific subcellular sites to sculpt local cAMP levels. Recent research uncovered the control of cAMP levels by the axis of GPCR-G protein-AC in the vicinity of the primary cilium. Both PDEs and components of GPCR axis are present in the cilium. It remains intriguing to decipher how the two systems cooperate to fine tune the local cAMP levels.

PKA sits at a crucial position in the Hh pathway. Hh signaling is believed to culminate at PKA activity that determines the On/Off switch of the pathway via Gli proteins. Several mechanistic questions remain to be answered regarding PKA's role in this process. What are the molecular and biochemical mechanisms of PKA inhibition in the vicinity of the cilium by Hh signaling? PKA controls the activation and processing of Gli2 and Gli3; why is cilium trafficking required for the formation of Gli activators and suppressors? Where and how does PKA phosphorylate Gli and modulate the SuFu-Gli interaction? Finally, direct monitoring of PKA activity in the cilium has yet to be achieved. Application of the most recent technological advances in super resolution imaging (Milenkovic et al., 2015; Yang et al., 2019), proximity labeling (Mick et al., 2015; May et al., 2021), and nanobody-based optogenetic tools (Hansen et al., 2020) are essential to understanding mechanistic details on how a ubiquitous enzyme such as PKA specifically regulates individual signaling pathways over space and time.

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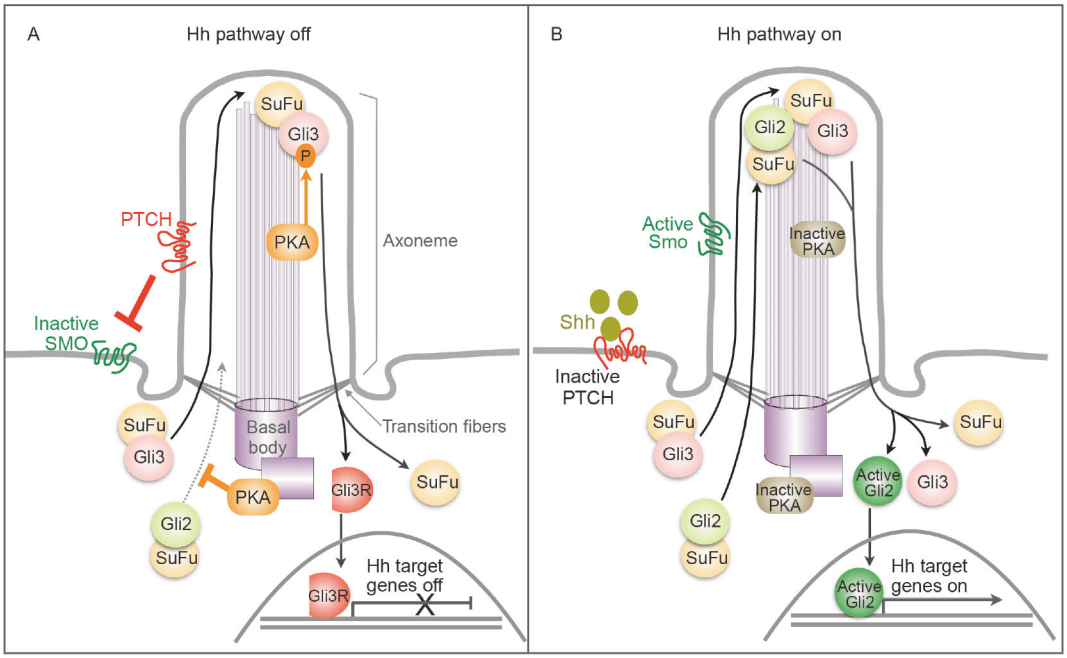

Figure 1.

Overview of Hh signal transduction. Vertebrate Hh signaling relies on protein trafficking at the primary cilium, a centrosome organized, microtubule-based cell surface organelle. The membrane of the cilium is continuous with the cytoplasmic membrane, but the ciliary cytosol is separated from the cytoplasm via transition fibers that function as filter for material exchange between ciliary axoneme and the cytoplasm. A, When the Hh pathway is off, Ptch resides in the cilium and inhibits Smo. Active PKA phosphorylates Gli3, resulting in Gli3's proteolytical processing into Gli3R, a transcription repressor. Active PKA also phosphorylates Gli2 and inhibits its trafficking to the cilium tip, preventing its activation. Note that both Gli proteins complex with SuFu, a negative regulator of the Hh pathway. B, Upon binding to the ligand Shh, Ptch exits from the cilium, and Smo is activated and accumulated in the cilium. PKA is inactivated and stops phosphorylating Gli proteins. As a result, both Gli proteins dissociate from SuFu. Gli3 remains in the full-length form and Gli2 is activated. Active Gli2 enters the nucleus to turn on the transcription of Hh target genes.

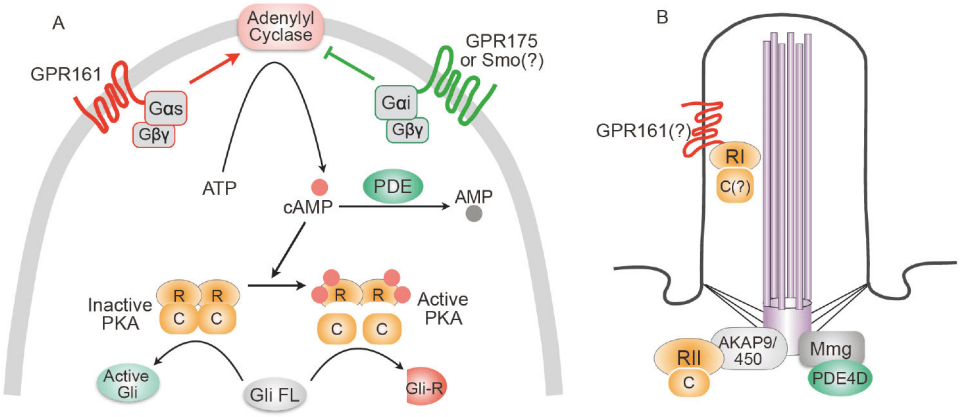

Figure 2.

Regulation of PKA activity and its compartmentalized localization in the cell. A, AC is activated by Gαs-coupled GPCR (such as GPR161), and inhibited by Gαi-coupled GPCR (GPR175 or potentially Smo; the mechanism of how Smo activates Gαi remains to be determined). AC converts ATP into the 2nd messenger cAMP, which binds to PKA-R and dissociates it from PKA-C. Active PKA-C suppresses Hh signaling by promoting the formation of Gli repressor. Inactive PKA allows the formation of active Gli, resulting in activation of Hh signaling. PDEs hydrolyze cAMP to inhibit PKA. B, PKA is sequestered to discrete subcellular compartments by binding to A kinase anchoring proteins (AKAPs). PKA-RII subunit is anchored to the centrosome by AKAP9 and AKAP450 (for simplicity, only one subunit is shown). PKA-RI subunit is found to be present in the cilium, potentially via binding to the C-terminus of GPR161. Some isoforms of PDE4D are anchored to the centrosome via myomegalin (Mmg), and degrade local cAMP to attenuate PKA activity.

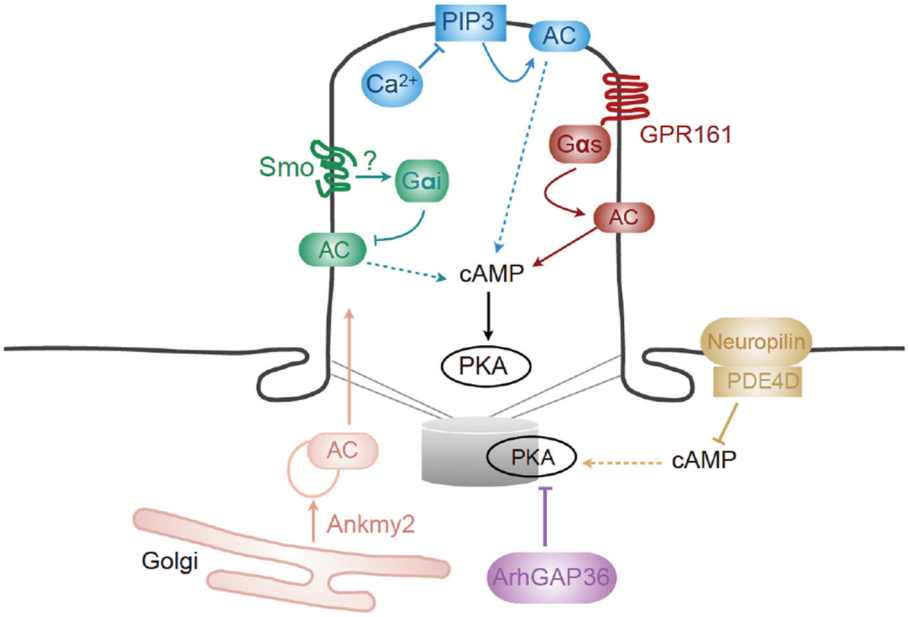

Figure 3.

Current understanding of how PKA activity is regulated in the vicinity of the cilium. Each pathway is colored coded. Green path: Smo may activate Gαi via hitherto unknown mechanisms, which inhibits adenylyl (AC) and reduces cAMP levels to inhibit PKA activity. Red path: GPR161 is present at the cilium before Hh activation, where it activates Gαs to increase PKA activity. Blue path: High Ca^{2+} levels in the cilium inhibits PIP3-mediated AC activation, and contributes to the reduction of cAMP/PKA activity. This path is observed only after prolonged (>24 h) Hh agonist stimulation. Yellow path: Neuropilin recruits PDE4D to the cytoplasmic membrane to effectively degrade cAMP, and subsequently reduces PKA activity at the centrosome. Pink path: Ankmy2 is responsible for the maturation and trafficking of multiple ACs to the primary cilium; loss of Ankmy2 leads to marked elevation of Hh signaling *in vitro* and *in vivo*. Purple path: The Rho GTPase-activating protein ArhGAP36 blocks PKA activity by acting as a PKA pseudo-substrate or inducing PKA degradation. Dashed lines indicate that activation of this pathway will suppress cAMP production and PKA activity.