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Cellular signalling by primary cilia in development, organ function and disease

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Abstract

Primary cilia project in a single copy from the surface of most vertebrate cell types; they detect and transmit extracellular cues to regulate diverse cellular processes during development and to maintain tissue homeostasis. The sensory capacity of primary cilia relies on the coordinated trafficking and temporal localization of specific receptors and associated signal transduction modules in the cilium. The canonical hedgehog (HH) pathway, for example, is a bona fide ciliary signalling system that regulates cell fate and self-renewal in development and tissue homeostasis. Specific receptors and associated signal transduction proteins can also localize to primary cilia in a cell type-dependent manner; available evidence suggests that the ciliary constellation of these proteins can temporally change to allow the cell to adapt to specific developmental and homeostatic cues. Consistent with important roles for primary cilia in signalling, mutations that lead to their dysfunction underlie a pleiotropic group of diseases and syndromic disorders termed ciliopathies, which affect many different tissues and organs of the body. In this review we highlight central mechanisms by which primary cilia coordinate HH, G-protein-coupled receptor, WNT, receptor tyrosine kinase and TGFβ/BMP signalling, and illustrate how defects in the balanced output of ciliary signalling events are coupled to developmental disorders and disease progression.

Opening section

The primary cilium is a microtubule-based, non-motile organelle that extends as a solitary unit from the basal body (derived from the centrosomal mother centriole of most cell types

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in the human body [1]. The cilium is enclosed by a membrane that is continuous with the plasma membrane but has a unique lipid and receptor composition that enables the cilium to detect changes in the extracellular environment and convey signalling information to the cell to regulate diverse cellular, developmental and physiological processes. Consequently, mutations that lead to dysfunction of primary cilia give rise to a pleiotropic group of diseases and syndromic disorders termed ciliopathies, which can affect many different organs during embryonic development as well as in postnatal life [2]. Primary cilia are dynamic organelles that are assembled and disassembled in coordination with cell cycle and developmental cues. Emerging evidence indicates that the constellation of signalling components within the cilium is also dynamic and closely coupled to the differentiation state and microenvironment of the cell [3]. This versatility of the cilium might explain how specific cell types are able to receive and convert signalling inputs at different time points during development and under physiological conditions. Here we present an overview of the main signalling pathways, including those regulated by Hedgehog (HH), G-protein-coupled receptors (GPCR), WNT, receptor-tyrosine kinases (RTKs) and TGFβ/BMP receptors, that are coordinated by primary cilia to control developmental processes, tissue plasticity and organ function. We discuss the potential mechanisms by which primary cilia regulate signalling pathway interactions and organize spatial-temporal signalling networks during development as well as in the maintenance of tissue homeostasis, and describe how dysfunctional cilliary signalling can lead to a multitude of human diseases.

Intraflagellar transport and ciliopathies

Both motile and non-motile cilia, including primary cilia, comprise a microtubule-based axoneme that extends from a basal body and is covered by a bilayer lipid membrane enriched in specific signalling receptors and ion channels. The axoneme of a primary cilium contains a ring of nine outer microtubule doublets (known as a 9+0 axoneme), whereas the axoneme of a motile cilium has nine outer microtubule doublets around two central microtubule singlets (called a 9+2 axoneme). The basal body is a modified centriole that contains specialized structures at its distal end that regulate critical aspects of ciliary biogenesis and function. For example, transition fibres mediate docking of the basal body to the plasma membrane or vesicles during early stages of ciliogenesis [4, 5] whereas basal feet interact with the actin cytoskeleton **of the cell** to regulate basal body alignment in cells that contain multiple motile cilia, such as epithelial cells that line the mammalian respiratory tract, brain ventricles or oviduct [6]. For cells that form a single primary cilium (Figure 1), the basal body is derived from the mother centriole of the centrosome, and depending on the cell type, axoneme extension can be initiated before or after docking of the basal body at the plasma membrane [4, 5]. The length of cilia is controlled by the actions of various kinases and other proteins [7, 8]; before mitosis the cilium is usually dismantled and centrioles are duplicated for participation in mitotic spindle pole formation [9–14]. Quiescent cells can lose their cilium as a consequence of developmental programming [15–19] or in response to environmental insults such as mechanical stress [20].

Between the basal body and cilium proper is a region known as the ciliary transition zone (TZ), which contains specialized gating structures such as Y-links that along with the basal body transition fibres control the entrance and exit of ciliary proteins, and thereby contribute

to compartmentalization of the organelle (Figure 1). Importantly, a number of genes mutated in ciliopathies such as Joubert syndrome, Meckel-Gruber syndrome (MKS), and nephronophthisis (NPHP), encode protein module components of the TZ or basal body transition fibres, highlighting the physiological importance of these structures [21]. In addition to gating by the TZ and basal body transition fibres, ciliary composition and function are also regulated by active transport mechanisms (Figure 1). These include vesicular transport pathways that target specific receptors or signalling molecules from the Golgi or recycling endosome to the ciliary base where vesicles are exocytosed [22, 23], and the intraflagellar transport (IFT) system that zips up and down axonemal microtubules to mediate the transport of specific ciliary cargo proteins into or out of the organelle [24, 25] (Figure 1). Moreover, ciliary membrane content can be modulated by the ectocytosis of vesicles at the ciliary tip [26–28] (Figure 1). Cilia are unable to synthesize proteins; therefore, cilia-associated transport pathways also function during cilium biogenesis and maintenance by delivering the building blocks required for ciliary axoneme and membrane extension [22–25]. Not surprisingly, mutations in genes that encode proteins involved in cilium-associated transport processes, such as IFT, typically result in absent or defective cilia and are associated with a wide range of human ciliopathies (Figure 1), including polycystic kidney disease (PKD), Bardet Biedl syndrome (BBS) and Short-rib thoracic dysplasia[29]. Indeed, studies of IFT have been instrumental for furthering our understanding of the importance of primary cilia in human health and disease. We therefore provide a brief overview of the process of IFT below. Comprehensive reviews on IFT are available elsewhere [25, 30, 31].

The IFT system

Discovery and characterization of the IFT system

The IFT system was discovered in 1993 following the observation of a continuous movement of particles, sandwiched between the flagellar membrane and axonemal outer doublet microtubules, from the flagellar base to tip (anterograde IFT) and back (retrograde IFT) in *Chlamydomonas reinhardtii* flagella[32]. A few years later, studies using temperature sensitive *C. reinhardtii* mutants with defects in flagellar assembly revealed that anterograde and retrograde IFT movements are powered by heterotrimeric kinesin-2 [33–35] and cytoplasmic dynein 2 motors [36–38], respectively. A large number of studies in different organisms subsequently confirmed a universal requirement for these motors in IFT, but also demonstrated that some organisms additionally use ‘accessory’ kinesins to regulate certain aspects of ciliary assembly or function [23, 39]. For example, metazoans such as *Caenorhabditis elegans* and zebrafish express a homodimeric kinesin-2 motor that functions somewhat redundantly with heterotrimeric kinesin-2 to mediate anterograde IFT and distal singlet axonemal microtubule assembly in certain types of cilia [40–43].

Purification of the polypeptides within the particles moved by IFT [44], demonstrated that they sediment as two larger complexes, termed IFT-A and IFT-B [44, 45]. We now know these polypeptides can be subdivided into IFT-A, IFT-B1 and IFT-B2 sub-complexes that contain 6, 10 and 6 different IFT particle proteins, respectively [25, 30, 31, 46, 47]. The three dimensional structures of many IFT-B polypeptides have been solved, and progress has been made towards understanding the arrangement and interactions of individual IFT polypeptides

within the IFT-A and IFT-B sub-complexes [25]. Likewise, progress has been made towards understanding the interactions between specific IFT particle polypeptides and specific ciliary cargo proteins or motor subunits during anterograde or retrograde IFT, as well as how IFT motor activity is regulated [24, 48–52].

Sequence analysis of IFT polypeptides isolated from *C. reinhardtii* flagella revealed that they are homologous to proteins required for ciliary assembly in sensory neurons of *C. elegans* [44, 53], providing the first evidence that IFT is an evolutionarily conserved process required for ciliary assembly in eukaryotes. Subsequent work in a range of additional organisms, including protists such as *Tetrahymena thermophila* and *Trypanosoma brucei* and metazoans like *C. elegans*, sea urchin, zebrafish, mouse, and human, have substantiated this view, and provided remarkable insight into the molecular workings and physiological roles of IFT [25, 30, 31, 46]. For example, although early work using *C. reinhardtii* and *C. elegans* mutants suggested that IFT-A and IFT-B complexes function in retrograde and anterograde IFT, respectively [30, 46], we now know that these polypeptides participate in ciliary transport in both directions [54–60]. For instance, the IFT-B complex proteins IFT-81 and IFT-74 promote anterograde IFT of tubulin towards the ciliary tip during axoneme assembly [61], whereas the IFT-B complex proteins IFT25 and IFT27 are involved in ciliary export of hedgehog (HH) signalling components via retrograde IFT [59, 60, 62, 63]. Furthermore, the IFT-A complex interacts with the tubby domain proteins TULP3 and TUB to mediate ciliary targeting of certain transmembrane proteins, including G protein-coupled receptors (GPCRs) [56, 58, 64–69]. In addition, a complex of BBS proteins called the BBSome [70], was shown to function as an IFT adapter [71, 72] in transporting ciliary proteins. The BBSome proteins were initially thought to facilitate the delivery of GPCRs to cilia [73–75]; however, it has been proposed that BBSome proteins primarily regulate ciliary export of signalling proteins [72, 76, 77, 78]. For example, flagella in a *Chlamydomonas bbs4* mutant show abnormal accumulation of several signalling proteins, resulting in disrupted phototaxis [72].

Linking IFT to human disease

The above-described studies of IFT in *Chlamydomonas* set the stage for a landmark study which revealed that a hypomorphic mutation in the mouse *Tg737* gene, which encodes an orthologue of the *Chlamydomonas* IFT-B polypeptide IFT88, causes ciliary loss and autosomal recessive (AR) PKD [79, 80]. This study provided the first evidence that defective primary cilia can lead to disease in mammals, and was supported by prior work in *C. elegans* demonstrating that homologs of the human autosomal dominant (AD) *PKD1* and *PKD2* gene products, the transmembrane proteins polycystin (PC) 1 and PC2, localize to neuronal sensory cilia and regulate male mating behaviour [81]. In agreement with these observations, PC1 and PC2 were found to localize to primary cilia in mammalian cells such as those of the renal collecting duct [82–84]. The physiological importance of the polycystins is underscored by the fact that mutations in the corresponding genes lead to ADPKD — a disease characterized by the adult-onset development of kidney and liver cysts [85, 86]. Furthermore, mutations in PKD2 have been linked to left-right laterality defects in vertebrates, including humans (add refs: PMID 12062060 and PMID 21719175). It is generally accepted that proper functioning of PC1 and PC2 relies on their appropriate targeting to the ciliary compartment, but the precise mechanism by which the polycystins function at cilia to

prevent cyst formation and control left-right patterning during development is a matter of intense debate [85]. For many years, the prevailing hypothesis was that PC1 and PC2 comprise a mechanosensitive receptor-Ca^{2+} channel complex, which is activated by fluid flow [87, 88]. This hypothesis was challenged [89, 90], and it was subsequently suggested that PC2, independently of PC1, forms a homotetrameric K^+ and Na^+-conducting ciliary ion channel that is potentiated by intraciliary Ca^{2+} [91–93]. A report of the near-atomic-resolution structure of the human PC1-PC2 complex by single-particle cryo-electron microscopy, however, strongly favours a model in which PC1 and PC2 function together in regulating cation transport [94]. Nevertheless, despite important advances, the molecular mechanisms by which cilia and polycystins cooperate to regulate development and tissue homeostasis remain incompletely understood (discussed elsewhere[85]). Further work in many different organisms, including mouse and human, has substantiated the link between primary cilia and kidney disease, and revealed a requirement for IFT and cilia in a range of other disease-relevant pathways (Figure 1).

Ciliary organization of HH signalling

HH signalling pathways regulate a number of cell fate and self-renewal processes in development and tissue homeostasis [95]. The final transcriptional output of canonical HH signalling is determined by post-translational modifications of GLI transcription factors, which cause intracellular activation or basal repression of pathway targets in the presence or absence of HH morphogens, respectively. Defects in IFT were first shown to disrupt sonic hedgehog (SHH) signalling during mouse embryonic development in a forward genetic screen in 2003 [96]; the primary cilium is now known to be fundamentally important for canonical SHH signalling in vertebrates [97]. Activation of the SHH pathway by formation of the GLI transcriptional activator (GLIA) and basal repression of the SHH pathway by GLI transcriptional repressor (GLIR) are both dependent on the primary cilium [97]. Binding of SHH to its 12-transmembrane receptor Patched-1 (PTCH1) triggers endocytic clearance of PTCH1 from the cilium in a process that relies on ubiquitination of the receptor by the HECT domain E3 ubiquitin ligases SMURF1 and SMURF2, which co-localize with PTCH1 in caveolin-1 (CAV1)-positive lipid rafts and vesicles [98]. CAV1 localizes to the base of cilia in cultured mammalian cells in a manner dependent on the kinesin-3 motor protein KIF13B; both CAV1 and KIF13B have been implicated in the regulation of SHH signalling [99]. CAV1 has also been reported to have ciliary functions in *C. elegans* [100]. Removal of ciliary PTCH1 in response to SHH binding is associated with ciliary enrichment of Smoothened (SMO) [101, 102] –a 7-transmembrane receptor belonging to the class F (frizzled) family of GPCRs – which ultimately leads to the formation of GLIA, predominantly by phosphorylation of GLI2 [103] (Figure 2). In contrast, basal repression in the absence of SHH involves protein kinase A (PKA)-mediated phosphorylation, predominantly of GLI3, and formation of the truncated N-terminal form of GLI, GLIR, in a cilia-dependent manner [104]. Additional ligands of the HH family includes desert (DHH) and Indian (IHH) hedgehog, which also operate via primary cilia but in a tissue-specific manner, such as in cells of the testis and in growth plate chondrocytes [105], respectively.

In addition to canonical pathways, HH signalling can occur though so-called non-canonical pathways, which involve either GLI-independent mechanisms or SMO-independent

regulation of GLI activity [106, 107]. For example, the IFT-B complex protein, IFT80, can repress SHH-mediated noncanonical activation of the GTPase RHOA in differentiating mouse osteoblasts [108], and HH-stimulated chemotaxis is mediated by SMO localized outside of cilia [109]. However, little is still known about the role of primary cilia in regulating non-canonical HH pathways and whether such pathways act in parallel to canonical HH signaling to control cellular processes during development and in tissue homeostasis [106, 108, 110]. Below we summarize current knowledge regarding the organization of canonical HH signalling in primary cilia.

Activation of canonical HH signalling

The mechanism underlying either the repression of SMO by PTCH1 or the activation of SMO upon removal of PTCH1 from cilia is not well understood. However, the solved structures of PTCH1 bound or unbound to SHH [111, 112] and SMO bound or unbound to cholesterol derivatives [113] reveal important insights. First, PTCH1 shows similarity to members of the resistance-nodulation-cell division (RND) family of bacterial efflux transporters and to sterol sensing domains in the cholesterol trafficking protein, Niemann-Pick C1 (NPC1). The transmembrane segments 2–6 in PTCH1 constitute the sterol-sensing domain, and are sufficient to accommodate cholesterol derivatives [111, 112]. The two extracellular domains of PTCH1 interact with SHH, although the two structural studies that investigated SHH binding identified different interacting interfaces with native lipidated SHH and an SHH N-fragment [111, 112]. Interestingly, the extracellular domains also have an interface that accommodates cholesterol derivatives, and binding of PTCH1 to the N-fragment of SHH potentially limits access to or exit of these derivatives from this site. Binding of PTCH1 to the N-fragment of SHH is enhanced by cholesterol derivatives and it is predicted that binding of cholesterol to the extracellular domain interface might arise from cholesterol transport by PTCH1 [111].

Inactive SMO is stabilized by a π-cation lock in the inner transmembrane interface. Binding of cholesterol to the extracellular cysteine rich domain of SMO dramatically changes the conformation of this domain with respect to the transmembrane domains, leading to release of the π-cation lock. Interestingly, a longitudinal tunnel capable of accommodating cholesterol was identified in the transmembrane region of active SMO, suggesting that SMO itself might move cholesterol from the plasma membrane to the extracellular domain [113]. Oxysterols can also bind to the cysteine rich domain and activate SMO downstream and independent of PTCH1 removal from cilia [102, 114]; however, the physiological role of oxysterols in HH pathway activation is not clear.

These findings suggest that SHH-mediated activation and removal of PTCH1 from cilia might increase the availability of endogenous SMO ligands — most likely cholesterol or cholesterol derivatives — in the ciliary membrane, thereby leading to activation of SMO in cilia. Downstream factors that mediate SMO-dependent activation of GLI2 are varied, and include G proteins and other proteins such as the EVC2 complex [104, 115]. Furthermore, the 7-transmembrane receptor GPR175 (also known as TPRA1 or TPRA40) is enriched in cilia in the presence of SHH, and might regulate maximal activation of pathways downstream of SMO by coupling to the inhibitory Gα protein, $G\alpha_i$, leading to decreased cAMP levels and

inhibiting PKA-mediated formation of GLI3R [116]. The intermediate steps between SMO activation, GLIA formation and translocation to the nucleus are not well understood, although GLI2 and GLI3 proteins accumulate in cilia tips upon HH pathway activation, suggesting that GLIA formation is regulated in the vicinity of cilia [117].

Basal Repression of canonical HH signalling

In the absence of SHH, PKA-mediated phosphorylation of GLI3 primes sequential phosphorylation events by casein kinase 1 (CK1) and glycogen synthase kinase-3 beta (GSK3β), which results in binding of GLI3 to the $SCF^{\beta TrCP}$ ubiquitin ligase, and its subsequent proteolysis into GLI3R [118–121]. The cilia-localized class A orphan GPCR, GPR161, was identified as a negative regulator of SHH signalling during early neural tube development in mice [122]. GPR161 regulates formation of GLI3R possibly via constitutive activation of cAMP–PKA signalling. Another negative regulator of SHH signalling, Suppressor of FUSED (SUFU) restrains GLI3 in the cytoplasm and promotes GLI3R processing [123] in a cilia-independent step [124]. Importantly, the absence of GPR161, PKA, and SUFU induces high levels of SHH signalling during mouse neural tube development [122, 125, 126], similar to the effect of *Ptch1* deletion, which induces activation of SMO signalling [127]. Interestingly, mutations in the genes encoding TULP3 or IFT-A subunits phenocopy *Gpr161* mutants by increasing HH signalling in the caudal neural tube [58, 128–131]. The pre-ciliary function of the IFT-A core complex, together with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), in binding and ciliary trafficking of TULP3 — an adapter protein involved in the gating of ciliary GPCRs including GPR161 — explains high SHH signalling observed in IFT-A mutants, despite the presence of abnormal cilia in these models [56, 64, 122]. Thus, IFT-A-regulated trafficking of TULP3 and GPR161 regulates basal suppression of SHH signalling. However, the neural tube ventralization phenotypes of *Tulp3* and *IFT-A* mutants, and *Gpr161* mutants are weaker than those resulting from mutations in downstream negative regulators of HH signalling such as *Sufu* and *PKA* [132], suggesting the existence of additional inputs into PKA activation other than GPR161 [133].

Studies from the past few years suggest that active suppression of HH pathway is as important as activation of the pathway during development and in maintenance of tissue homeostasis. Although SHH secretion from distinct regions, such as the purkinje neurons in the postnatal cerebellum and the zone of polarizing activity in limb buds, patterns these tissues, each of these tissues expresses low or no SHH at distinct stages of development. The importance of HH pathway repression is illustrated by the finding that premature SHH signalling resulting from disruption of GPR161 causes defects in limb and skeletal morphogenesis [134], cerebellar granule cell hyper-proliferation and formation of SHH-subtype medulloblastoma [135]. Thus, active repression of HH pathway by GPR161 regulates tissue architecture, whereas pathway de-repression contributes to disease pathogenesis.

[H2] *Ciliary coordination of HH signalling*

Most components of canonical HH signalling are dynamically associated with cilia. In addition to PTCH1 removal, SMO enrichment and association of GLI2, GLI3 and SUFU with ciliary tips, the microtubule-associated atypical kinesin, KIF7, also becomes enriched in ciliary tips during activation of HH signalling [136, 137], where it. functions as both a

positive and negative regulator of SHH signalling by modifying ciliary architecture [136, 137]. GPR161 is also removed from primary cilia in a SMO-dependent and β-arrestin-dependent manner following activation of HH signalling [28, 138]. β-arrestins are adaptor proteins that are recruited to the proximal C-terminus of GPR161 in a manner dependent on G protein-coupled receptor kinase 2 (GRK2)[138]. GRK2 and GRK3 also transduce high level SHH signals through mechanisms independent of GPR161[133]. Depletion of *Inpp5e* (which encodes a phosphoinositide 5-phosphatase responsible for removing the 5-phosphate from $PI(4,5)P_2$), leads to accumulation of $PI(4,5)P_2$ in cilia and increased steady-state levels of TULP3, IFT-A and TULP3-dependent cargo such as GPR161 and PC2 [139, 140]. In addition, removal of GPR161 from cilia upon activation of the SHH pathway is impaired in *Inpp5e*-knockout cells [139, 140], irrespective of levels of the pathway activator SMO suggesting that a number of factors are responsible for the dynamic regulation of HH signalling components in cilia.

Activation of PKA in different subcellular regions is mediated by local cAMP production and anchoring of PKA to A-kinase anchoring proteins (AKAPs) [141]. The distal C-tail of GPR161 has a conserved amphipathic helix that directly binds to type I PKA regulatory subunits [142] (Figure 2). Since the type I PKA regulatory subunit alpha localizes to cilia [143], direct coupling of this subunit to PKA in this compartment might enhance the ability of GPR161 to activate PKA via constitutive cAMP signalling. nine adenylyl cyclases that regulate downstream cAMP signalling, at least three — ADCY3, ADCY5, and ADCY6 — are localized to cilia [143–145]. In particular, overexpression of ADCYC5 and ADCYC6 partially represses the HH pathway in the developing chicken neural tube [146]. However, factors that regulate trafficking of adenylyl cyclases to cilia are currently unknown, and the role of such factors in SHH signalling has not been established.

Ciliary modulation of GPCR signalling

The cilium is recognized as an important nexus for GPCR signalling. GPCRs comprise the largest signalling receptor superfamily in the human genome, with more than 800 functional GPCRs [147], which can be subdivided into six different classes: class A (the Rhodopsin family), class B1 (the Secretin family), class B2 (the Adhesion family), class C (the Glutamate family), class F (the frizzled–smoothened family) and the Taste 2 family, based on sequence and phylogenetic analysis [147, 148]. The vast majority of GPCRs fall into the class A rhodopsin family, which comprises approximately 700 receptors, including 460 olfactory receptors [147]. GPCRs mediate numerous physiological functions in the human body by responding to a wide range of signals, such as photons, peptides, proteins, hormones, chemicals, lipids and sugars. GPCRs are also the largest group of therapeutic drug targets; about one-third of all US Food and Drug Administration (FDA) approved drugs act on GPCRs [149]. Thus, understanding the molecular mechanism of GPCR signalling has great therapeutic importance.

Canonical signal transduction through GPCRs is mediated by activation of heterotrimeric G proteins composed of three associated subunits, Gα, Gβ, and Gγ. G proteins are classified on the basis of their Gα subunit and at least 20 Gα isotypes exist that are functionally categorized into four major families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$) [150]. Signalling through

these proteins involves a very well characterized sequence of events. When inactive, a heterotrimeric G protein consists of a GDP-bound Gα subunit associated with a Gβγ dimer. Activated GPCRs engage the GDP-bound heterotrimer and facilitate GDP dissociation from Gα, which is rapidly followed by GTP binding to the G protein. Consequently, the Gα and Gβγ subunits dissociate and modulate the activity of downstream effectors (such as adenylyl cyclases by Gα and potassium channels by Gβγ). G protein activity is terminated when the Gα subunit hydrolyses GTP to GDP and re-associates with Gβγ.

Upon activation, GPCRs are phosphorylated at specific sites within their intracellular domains primarily by GRKs. Phosphorylated receptors are targets for the recruitment and binding of β-arrestins, which are scaffolding proteins that inhibit additional G protein activation and promote internalization of receptors by facilitating clathrin-mediated endocytosis. However, in some cases endocytosis leads to sustained or enhanced G protein signalling from endosomes [151]. Moreover, β-arrestins bind to various signalling proteins, such as c-SRC and ERK1/2, and can promote G protein-independent signalling both at the plasma membrane and within endosomes [151].

Ciliary *GPCR signalling in diverse cell types*

Numerous GPCRs and their downstream effector molecules localize to cilia on a variety of mammalian cell types [152, 153] (Table 1, Figure 3). Intrahepatic bile ducts are lined with ciliated epithelial cells called cholangiocytes, which mediate bile acid transport and bicarbonate secretion [154]. Primary cilia on these cholangiocytes have been proposed to provide mechanosensory, chemosensory and osmosensory functions to regulate cholangiocyte proliferation [155]. Indeed, polycystic liver disease is a ciliopathy, characterized by the formation of fluid-filled hepatic cysts that originate from cholangiocytes [156]. Reports suggest cholangiocyte cilia are enriched for two different GPCRs — purinergic receptor P2Y12 (P2RY12) [155] and G-protein coupled bile acid receptor 1 (GPBAR1, also known as TGR5) [157, 158], as well as the cAMP signalling proteins adenylyl cyclase, PKA, exchange protein directly activated by cAMP isoform 2 (EPAC2), and A-kinase anchoring protein (AKAP150) [155], suggesting that cholangiocyte cilia mediate cAMP signalling in response to biliary factors. In support of this proposal, activation of $G\alpha_i$-coupled P2RY12 by its endogenous ligand, ADP, decreased forskolin-induced cAMP levels in ciliated cholangiocytes but not in non-ciliated cholangiocytes [155]. The presence or absence of cilia might also affect GPBAR1 signalling [158]. Activation of GPBAR1 on non-ciliated cholangiocytes induced co-localization of GPBAR1 with $G\alpha_s$, increased cAMP signalling, inhibited ERK signalling, and increased cellular proliferation [155], whereas activation of GPBAR1 on ciliated cells induced co-localization of GPBAR1 with $G\alpha_i$, decreased cAMP signalling, activated ERK signalling, and decreased cellular proliferation. These findings therefore suggest that GPCR-mediated cAMP signalling in cholangiocyte cilia is distinct from cAMP signalling on the plasma membrane of cholangiocytes.

Ciliary GPCR signalling in nephrons

A clear link also exists between cilia dysfunction and cystic kidney disease [159]. Studies have further implicated ciliary GPCR signalling in renal cilia function and disease. Dopamine receptor type 5 (D5) localizes to cilia on renal epithelial cells, where it may functionally

couple to the CaV1.2 L-type calcium channel [160]. Activation of D5 caused an increase in calcium levels in the cilium along with an actin-mediated increase in cilia length, which might increase sensitivity to fluid flow [161] and inhibit cystogenesis. Type 2 vasopressin receptor (V2R) which regulates Na^+ and water reabsorption in the mammalian nephron has also been reported to localize to cilia on renal epithelial cells [162]. Upon activation, ciliary V2R seems to activate adenylyl cyclase, which increases local cAMP concentrations and activates a cation-selective channel to regulate intraciliary Ca^{+2} signals [162]. Although the precise consequences of V2R ciliary signalling on renal epithelial cell function are unknown, the V2R receptor antagonist tolvaptan slows the decline in glomerular filtration rate in patients with ADPKD[163]. Tolvaptan is now the first FDA-approved treatment for PKD.

Ciliary GPCR signalling in trophoblasts

GPCR signalling in cilia also seems to have an important role in early human pregnancy. During embryo implantation, trophoblasts, which form the outer layer of a blastocyst, invade the uterus in order to secure an adequate supply of oxygen and nutrients for the foetus. Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) is a critical regulator of embryo implantation and placental development. Interestingly, the EG-VEGF receptor, prokineticin receptor 1 (PROKR1) localizes to cilia on human trophoblast cell lines and human first-trimester placental tissue [164]. Treatment of trophoblast cells with EG-VEGF activates ERK1/2 signalling to induce upregulation of matrix metalloproteinases (MMPs) and facilitate cell invasion [164]. Disruption of cilia on trophoblast cells ameliorates EG-VEGF-induced activation of ERK1/2, MMP upregulatoin, and cell invasion [164]. Thus, ciliary-mediated PROKR1 signalling might have an important role in embryo implantation.

Ciliary GPCR signalling on central neurons

Most neurons in the mammalian brain possess primary cilia. The importance of neuronal cilia is highlighted by the fact that ciliopathies are associated with numerous neuropathologies, including anatomical abnormalities and neuropsychiatric disorders [165]. Neuronal cilia are enriched for certain GPCRs and downstream effector proteins [152, 166, 167], suggesting they act as specialized signalling hubs. The importance of GPCR ciliary localization is underscored by the fact that mouse models of the ciliopathy BBS show dysregulation of GPCR ciliary localization. Specifically, deletion of BBS proteins leads to failure of somatostatin receptor subtype 3 (SSTR3), melanin-concentrating hormone receptor 1 and neuropeptide Y (NPY) receptor subtype 2 (NPY2R) to localize to neuronal cilia, whereas, dopamine receptor 1 accumulates in neuronal cilia [73, 74, 168]. In addition, tubby mice, which carry a mutation in the gene encoding the TUB protein and display ciliopathy phenotypes, exhibit defective localization of a number of GPCRs in neuronal cilia [74, 169]. Thus, defects in localization of GPCRs to neuronal cilia likely disrupt ciliary signalling and contribute to ciliopathy phenotypes.

Numerous studies have implicated ciliary GPCR signalling in both neural development and function. Inhibitory interneurons originate in the telencephalon from where they migrate to numerous brain regions and integrate into local neural circuits to regulate the balance of excitatory and inhibitory inputs. Disruption of interneuronal circuits has been linked to

neurodevelopmental disorders such as schizophrenia, autism and intellectual disabilities [170]. Interestingly, conditional disruption of ARL13B, which is a GTPase required for proper ciliary signalling, in post-migratory interneurons perturbs circuit development in the mouse striatum [171]. Specifically, loss of ARL13B in interneurons results in reduced dendritic and axonal complexity, disrupted synaptic connectivity, and functional deficits in synaptic activity [171]. Interneurons lacking ARL13B also show disrupted ciliary calcium dynamics and a dramatic reduction in ciliary localization of SSTR3 [171]. Intriguingly, expression of SSTR3 in ARL13B-deficient interneuronal cilia rescues the morphological and synaptic connectivity defects, whereas expression of a non-ciliary form of ARL13B with normal GTPase activity does not rescue the developmental defects in ARL13B-deficient interneurons [171], suggesting that SSTR3 signalling within interneuronal cilia is critical for proper inhibitory network construction and function.

Ciliopathies are also associated with cognitive deficits. SSTR3 localizes to neuronal cilia in the hippocampus, a region of the brain that is important for learning and memory. Treatment of mouse hippocampal neurons with the SSTR3 ligand, somatostatin, stimulates recruitment of endogenous β-arrestin into SSTR3-positive cilia, leading to a rapid β-arrestin-2-dependent decrease in the ciliary SSTR3 [167], suggesting that ciliary export of activated SSTR3 is mediated by β-arrestin-2. In support of this finding, ciliary export of activated SSTR3 in cultured renal epithelial cells also requires β-arrestin-2 [28, 172]. Interestingly, mice lacking SSTR3, ADCY3, β-arrestin-2 or cilia in the hippocampus all show similar deficits in learning and memory [173–176], implicating SSTR3 ciliary signalling in proper learning and memory.

GPCR signalling in neuronal cilia also contributes to the regulation of metabolic homeostasis. The first hint that cilia affect food intake and energy metabolism arose from the observation that some ciliopathies are associated with obesity. This connection was further supported by studies showing that conditional disruption of cilia in adult mice causes hyperphagia and obesity [177, 178]. More recent studies have provided important mechanistic insights into the metabolic neural circuits that are affected by GPCR ciliary signalling. The $G\alpha_s$-coupled GPCR melanocortin 4 receptor (MC4R) is an important component of the neurocircuitry that regulates food intake and energy expenditure. In fact, mutations in *MC4R* are the most common cause of monogenic obesity in humans, underlying up to 6% of early-onset or severe cases of adult obesity [179]. A 2018 study showed that MC4R localizes to cilia on neurons in the mouse paraventricular nucleus of the hypothalamus — a region of the brain that is important for the regulation of energy homeostasis and metabolism [180]. Interestingly, some *MC4R* mutations associated with obesity in humans impair ciliary localization of MC4R. Moreover, inhibition of ciliary cAMP signalling specifically in cilia on MC4R-expressing neurons of the paraventricular nucleus led to increased food intake and weight gain in mice [180]. Thus, impaired ciliary signalling of MC4R could be a common cause of syndromic and non-syndromic obesity in humans.

NPY also has an important role in the central regulation of food intake and energy expenditure [181]. NPY2R localizes to cilia on hypothalamic neurons in mice [74]. Importantly, BBS mutant mice that lack NPY2R-positive cilia are obese and do not respond to NPY2R ligand [74], suggesting that receptor ciliary localization is required for ligand-dependent

signalling *in vivo*. Moreover, quantification of NPY2R-mediated cAMP signalling in a ciliated cell line revealed that ligand treatment produced a stronger signal in cells with a cilium than in non-ciliated cells [74]. Thus, NPY2R signalling is seemingly enhanced within the cilium. Similar to SSTR3, ligand treatment results in a decrease in NPY2R ciliary localization. NPY2R binds poorly to β-arrestin [182]; however, using live-cell imaging in a ciliated renal epithelial cell line, Nager et al [28] revealed that activated NPY2R accumulates at the ciliary tip and is released in extracellular vesicles called ectosomes [28] (Figure 3), raising the fascinating possibility that ciliary GPCR signals might be transmitted between cells. Interestingly, Nager et al also showed that agonist-dependent ciliary export of heterologously-expressed SSTR3 in inner medullary collecting duct cells lacking either BBSome function or β-arrestin-2, involved active recruitment of SSTR3 to the ciliary tip from where a considerable fraction was released in ectosomes [28]. These findings further support a role for the BBSome and β-arrestin-2 in GPCR ciliary export and led to the suggestion that loss of GPCR localization on neuronal cilia in BBS mutant mice [73] might result from constitutive ectocytosis, rather than a defect in GPCR trafficking to cilia. However, this proposal would suggest that hippocampal neurons that lack β-arrestin-2 would also lack ciliary localization of SSTR3, yet ciliary localization of SSTR3 is not affected in β-arrestin-2-deficient hippocampal neurons[167]. Why SSTR3 would be constitutively ectocytosed from cilia on BBS mutant neurons, whereas D1 accumulates in cilia on BBS mutant neurons even in the presence of agonist, is unclear [168]. Additional studies are therefore required to define the precise roles of the BBSome in GPCR trafficking to and from neuronal cilia.

Ciliary modulation of WNT signalling

WNT signalling comprises an evolutionarily-conserved network of pathways that coordinate a multitude of cellular events over a lifespan [183]. WNT ligands comprise a family of secreted lipoproteins that often activate frizzled receptors of the class F GPCRs in conjunction with a series of co-receptors [184, 185]. Two prominent branches of the WNT signalling network are the so-called canonical WNT–β-catenin and non-canonical WNT–planar cell polarity (PCP) pathways. In the canonical pathway, WNT ligands bind to frizzled to trigger complex formation with the co-receptor LRP5/6 and Dishevelled (DVL), promoting stabilization of cytoplasmic β-catenin, which enters the nucleus to regulate target gene expression with effects on cell proliferation, differentiation and survival [186, 187]. By contrast, WNT–PCP signalling regulates cell morphology, migration and oriented cell division and relies on a multitude of receptor combinations and downstream signalling events [188, 189]. For example, WNT ligands can bind frizzled and members of the RTK family, specifically ROR1/2, RYK or PTK7, which recruit DVL and activate the branches of RAC–JNK and DAAM-1–RHOA–ROCK signalling pathways that control polarity processes while inhibiting canonical WNT–β-catenin signalling [188, 190–192].

Controversies in ciliary WNT signalling

Several core WNT pathway components localize to primary cilia (Figure 4) [193–197] and although multiple lines of evidence from gene knockout or knockdown studies have suggested roles for cilia in regulating WNT signalling, the literature in this field is

controversial. The first links between WNT signalling and primary cilia came from functional studies of nephrocystin-2 (NPHP2, also known as inversin, which when overexpressed inhibits DVL1-mediated activation of WNT reporter constructs in mammalian cells and rescues secondary axis formation induced by overexpression of Dsh or CK1μ in *Xenopus laevis* [198]. In addition, morpholino-induced depletion of NPHP2 in *X. laevis* and zebrafish disrupted convergent extension — a process that was rescued by overexpression of mouse NPHP2 or Diversin, respectively [198]. These findings led to the hypothesis that the cilium acts as a switch for diverse WNT pathways by modulating DVL. A subsequent study in *X. laevis* showed that NPHP2 promotes WNT–PCP signalling by facilitating fzd8-mediated recruitment of Dvl to the membrane, and that proximal pronephros extension is disrupted by morpholino-mediated depletion of NPHP2 [199], although normal WNT–β-catenin signalling was unaffected in this study. *In vitro*, NPHP2 controlled fibroblast polarity and directional cell motility through modulation of ciliary WNT signalling pathways that control the activity and localization of polarity proteins to the leading edge of migrating cells [196]. In support of a role for primary cilia in canonical WNT signalling, mouse embryos deficient in the ciliary microtubule-based kinesin-like protein KIF3A, which is a component of the kinesin-2 motor that facilitates anterograde IFT and ciliogenesis, displayed increased levels of canonical WNT reporter activity compared to levels in heterozygous embryos, and loss of the primary cilium in mouse embryonic fibroblasts lacking key ciliogenesis genes (*Kif3A*, *Ift88*- or *Ofd1*)was associated with hypersensitivity to a WNT3A ligand [193]. These findings support the proposal that the primary cilium restrains canonical WNT signalling; however, another study showed that mouse embryos and mouse embryonic fibroblasts with mutations in *Kif3a*, *Ift88*, *Ift72* and a gene that encodes a component of dynein-2 (*Dync2h1*) exhibited normal *Axin2* mRNA expression and canonical WNT reporter activity [200]. A 2016 study contradicted both these reports showing that in lung cancer cell lines, KIF3A restricts WNT canonical signalling, but independently of the primary cilium by restricting β-arrestin interaction with DVL2 and AXIN as well by modulating WNT ligand secretion [201]. Conversely, the *Drosophila* homologue of KIF3A, Klp64D, promotes canonical WNT signalling in the non-ciliated *Drosophila* wing discs, forms a complex with Arm (the *Drosophila* homologue of β-catenin) and Dvl, and probably mediates correct trafficking of Arm during WNT signalling [202]. Finally, a further study showed that a zebrafish maternal-zygotic mutant of *ift88* has normal expression of canonical WNT target genes and normal convergent extension and body axis development, demonstrating that primary cilia are redundant for both branches of WNT signalling [203]. Thus, despite intense investigation, the function of the primary cilium in fine-tuning WNT signalling remains unclear [204].

WNT signalling: the TZ and the basal body

Dvl, β-catenin and several members of the β-catenin destruction complex have been shown to localize to the ciliary base and available evidence suggests that they can be modulated by components of the MKS and NPHP modules, which are protein complexes of the TZ, which forms a prominent part of the ciliary gate that regulate proteins entering and exiting the cilium (PMID: 27770015). For example, in the absence of a primary cilium, the MKS module component Jouberin (JBN, also known as AHI1) promoted WNT and β-catenin signalling by facilitating nuclear localization of β-catenin [205]. The primary cilium induces depletion of nuclear and cytoplasmic JBN and thereby represses the effect of JBN on β-

catenin. Ciliary JBN facilitates the ciliary localization of β-catenin following WNT3A stimulation and thereby negatively affects WNT signalling [206]. JBN therefore has a dual function in regulating WNT–β-catenin signalling, depending on the presence or absence of the primary cilium. Another study showed that the cystic kidney and abnormal brain development phenotypes of mice deficient in JBN are largely caused by dysregulated WNT–β-catenin signalling [207]. The transmembrane protein TMEM67 is another key component of the MKS module that recruits the tyrosine-protein kinase transmembrane receptor ROR2 to the TZ. Together TMEM67 and ROR2 respond to WNT5A to activate RHOA, modulate the actin cytoskeleton and promote epithelial branching morphogenesis; these proteins also partially facilitate WNT5A-mediated repression of the canonical pathway [208]. In addition to MKS module components and NPHP2 (discussed above), evidence suggests that other NPHP module components including NPHP3 [209], NPHP4 [210] and RPGRIP1L (PMID 22927466, [212]) modulate the WNT pathway through DVL. Similarly to NPHP2, NPHP3 and NPHP4 restrain DVL-mediated WNT–β-catenin activation in cell culture, and when depleted, induce WNT–PCP defects *in vivo* (PMID 18371931 and PMID 21498478). In addition, NPHP4 was reported to stabilize the E3 PHD finger domain E3 ubiquitin ligase JADE1 and promote its nuclear translocation [213]. JADE1 is a negative regulator of WNT–β-catenin signalling that ubiquitinates cytoplasmic and nuclear β-catenin irrespective of its phosphorylation status, thereby promoting its proteasomal degradation [214]. In ciliated cells, JADE1 is also localized at the TZ and the basal body, probably through its interaction with NPHP4. Thus, NPHP4, in addition to its negative effect on DVL, also negatively regulates β-catenin levels through JADE1. Similarly, RPGRIP1L regulates WNT–PCP pathways by modulating DVL levels [212, 215]. RPGRIP1L was reported to regulate the proteasome function in a cilia and basal body-dependent manner [211]. Mouse embryonic fibroblasts deficient in RPGRIP1L exhibit impaired proteasomal activity at the basal body — a phenotype that can also be induced by depletion of the 19S proteasome subunits PSMD2, PSMD3 and PSMD4, all of which localize at the TZ and basal body. Indeed a key manner by which cilia might regulate WNT pathway components is through direct proteasomal degradation [212, 216, 217] (PMID: 24691443). Cilia proteomics have identified several proteasomal subunits that bind to BBSome subunits [218], and depletion of BBS4 in cultured cells (which results in both canonical and WNT–PCP defects in zebrafish) reduces proteasomal activity and leads to an accumulation of cytoplasmic and nuclear β-catenin [216].

Thus, available data suggest that various proteins in the TZ and basal body might act as a processing platform to which various WNT signalling components are recruited for post-translational modification and/or elimination through the basal body-associated proteasome pathway. However, it is important to remember that many TZ proteins may have extra-ciliary functions [219] and further work is needed to decipher and distinguish their ciliary and non-ciliary functions and the context in which they regulate the WNT pathways.

Ciliary RTK signalling

Many growth factors and hormones operate through RTKs, which belong the largest family of >50 enzyme-linked receptors. RTKs can be subdivided into different classes based on their structure, domain organization and requirement for co-receptors in signal transduction [220]. In many cases, RTKs are activated by homodimerization or hetero-dimerization, which

leads to activation of their intracellular tyrosine kinase domains followed by autophosphorylation of specific tyrosine residues, which in a sequence-specific context recruit a multitude of adaptor and effector proteins involved in signal transduction primarily through Src homology 2 (SH2) and pTyr-binding (PTB) domains. Prominent RTK downstream signalling components and pathways include ERK1/2, p38 and JNK in the family of mitogen-activated protein (MAP) kinases, PI3K-AKT-mTOR and PLCγ as well as a branch of STAT signalling [220]. In addition, RTKs extensively cross-talk with other receptor systems and may signal through G proteins, GRKs, and β-arrestins to control various cellular responses [221]. Designated subtypes of class II and class III RTKs, including platelet-derived growth factor alpha receptor (PDFGRα), the insulin receptor (IR) and insulin-like growth factor receptor (IGFIR) operate in primary cilia to control specific processes in cells and tissues [222, 223] (Figure 5). A series of other RTKs, including epidermal growth factor receptor (EGFR) [224, 225], fibroblast growth factor receptor 3 (FGFR3) [226], tropomyosin receptor kinase B (TRKB, also known as neurotrophic tyrosine kinase receptor 2 [227] and angiopoetin-1 receptor (TIE2) [228] also localize to primary cilia. Stimulation of cultured retinal pigmented epithelial (RPE-1) cells with brain-derived neurotrophic factor (BDNF) — a secreted neurotrophin that is a ligand for TRKB and required for neuronal development and synaptic plasticity — induced recruitment and activation of TRKB in the primary cilium in a BBS4-dependent manner, thereby linking ciliary TRKB signalling to neuronal phenotypes associated with BBS [227]. FGFR3 regulates ciliary length and IFT20 trafficking in the cilium, and ciliary expression of constitutively active FRFR3 in chondrocytes is linked to skeletal disorders [226, 229]. Finally, RTKs can localize to specified sub-compartments of the primary cilium. For example, ROR, which as described above, might function as a co-receptor for WNT5A-regulated PCP signalling at the ciliary TZ. However, many of these receptors can also localize outside the primary cilium, indicating that cilia could constitute a specialized site for organizing cellular signalling events that might affect cellular processes differently from those organized at extra-ciliary sites.

Ciliary IR and IGF-1R signalling

The family of insulin and insulin-like receptors have critical roles in glucose storage and uptake, protein and lipid synthesis, cell differentiation and mitogenic responses. A series of studies mostly based on cell culture experiments and mutant mouse models indicated links between many of these functions and IR or IGF-1R signalling at the level of the primary cilium. An important contribution to this understanding came with the discovery that although IGF-IR is localized and activated at the plasma membrane, adipocyte differentiation, which is associated with transient ciliogenesis [197, 230], requires activation of IGF-IR in the primary cilium in cultures of 3T3-L1 preadipocytes [230]. Indeed, extra-ciliary receptor populations seemed to be less sensitive to insulin stimulation than those in the cilium, where receptor activation led to activation of insulin receptor substrate 1 (IRS-1) and AKT at the ciliary base, instigating the adipocyte differentiation programme [230]. Likewise, differentiation of human mesenchymal stem cells into adipocytes was demonstrated to involve elongation of primary cilia, associated with recruitment of IGF-1Rβ to the cilia [231]. Ciliary IGF-1R activation also induced ciliary resorption and cell cycle entry through various downstream signalling pathways, including via IGF-1-mediated recruitment of phosphorylated TCTEX-1 to the ciliary TZ via non-canonical G-protein signalling, marking

a mitogenic signalling cascade that accelerates ciliary resorption and G_1/S progression in cultured mouse embryonic fibroblasts and RPE-1 cells [232]. Perturbation of this signalling cascade in cortical neuron progenitors induces premature neuronal differentiation at the expense of proliferation [232] – a scenario that is linked to developmental brain abnormalities [233]. Similarly, insulin-mediated resorption of cilia in mouse 3T3-L1 fibroblasts operates primarily through activation of ciliary IGF-1R to which IRS-1 is recruited and activated, followed by re-localization of IRS-1 to the ciliary neck region, where heat shock protein Hsp90α might function as a hub for activation of AKT [234]. Evidence also suggests that primary cilia in islet β-cells of the pancreas are implicated in insulin production and that dysfunctional cilia in these cells contribute to susceptibility to metabolic diseases such type 2 diabetes mellitus (T2DM) [235], which is present in a subset of ciliopathies such as BBS and Alström syndrome (reviewed elsewhere [236, 237]). Young *Bbs4*-knockout mice demonstrate impaired glucose handling before the onset of obesity, and ciliary dysfunction induced by knockdown of BBS4 or oral-facial-digital syndrome 1 protein (OFD-1) attenuates first-phase insulin secretion in pancreatic islet cells independent of glucose metabolism [235]. These defects in glucose handling are linked to defects in insulin-mediated activation of the PI3K–AKT pathway in β-cells, which relies on ciliary recruitment of activated IR subtype A (IR-A), which controls the production of insulin in these cells [235, 238]. However, the mechanisms by which primary cilia regulate metabolic processes are very complex and probably involve many different signalling systems, which in a spatial-temporal manner regulate cellular processes in multiple tissues, including the hypothalamus as well as adipose and muscle tissues [236237].

Ciliary PDGFRα signalling

Two isoforms of PDGF receptors exist (PDGFRα and PDGFRβ), which function as either homodimers or heterodimers to control diverse cellular and developmental processes. Mutations in the genes expressing PDGFRα or its specific ligand, PDGF-AA, are associated with kidney and liver pathophysiologies, gastrointestinal stromal tumours, glioblastoma and a variety of other cancers [239]. PDGFRα is principally localized to primary cilia [225, 240–243], although non-ciliary localizations for this isoform have been reported for specific cell types [243–245]. PDGFRβ is primarily located and activated outside the cilium [240, 241], but can induce AURKA-mediated disassembly of the primary cilium in cultured fibroblasts and RPE-1 cells through activation of the PLCγ pathway upon stimulation with PDGF-DD [246]. In fibroblasts, ciliary PDGFRα signalling activates the PI3K–AKT and MEK1/2–ERK1/2–RSK pathways to control directional cell migration, which is achieved by targeting the Na^+/H^+ exchanger 1, NHE1, to the leading edge of the migrating cells, which project their cilium towards the leading edge and parallel to the path of motility [240, 247–249]. Consequently, defects in the formation of primary cilia by depletion of IFT-B proteins such as IFT88 and IFT172 block PDGFRα activation [247, 250], rendering the cells unable to respond and move towards a gradient of PDGF-AA [247]. In this context, AKT was shown to be activated at the ciliary base in complex with NPHP2 in a PDGF-AA-dependent manner [251]; these interactions could provide a platform for cross-talk between PCP and PDGFRα signalling pathways in regulating directional cell migration, although further work will be required to understand the potential interaction between these two signalling systems at the level of the cilium. In addition, knockdown of the serine/threonine-protein kinase Ataxia

telangiectasia and RAD3-related protein (ATR), which underlies Seckel syndrome [252], is associated with ciliary shortening and reduced responsiveness to PDGF-AA in cultures of fibroblasts [253], and depletion of the gene that encodes the ciliary TZ NPHP module component, RPGRIP1L, in mouse embryos leads to ventricular septal defects associated with loss of localization, which display cardiac ventricular septal defects [254] is associated with loss of localization of PDGFRα to primary cilia and reduced expression of the PDGFRα target gene, *Hifa*, in cardiac ventricular tissue [243]. The latter observation supports the general concept that that dysfunctional cardiac cilia cause congenital heart defects [255].

Further studies have revealed diverse mechanisms that regulate ciliary targeting of PDGFRα and balance the output of PDGF-AA-mediated signalling. IFT20 appears to have a central role in this context by stabilizing the RING-finger domain family of CBL E3 ubiquitin ligases, c-CBL and CBL-b, which are tumour suppressor proteins that target active PDGFRs for internalization and degradation [256]; dysfunctional CBL E3 ubiquitin ligases are associated with cancer development [257]. Upon PDGF-AA stimulation CBL proteins are recruited to the primary cilium to mediate the ubiquitination and internalization of PDGFRα for feedback inhibition of signalling, whereas in IFT20-depleted cells, which lack the cilium, PDGFRα mislocalizes to the general cell surface, where PDGF-AA-mediated signalling is greatly overactivated due to autoubiquitination and proteasomal degradation of the CBL proteins [258]. Similarly, co-depletion of c-CBL and CBL-b leads to reduced levels of IFT20 in combination with mislocalization and overactivation of PDGFRα [258]. These results suggest the existence of a biochemical and functional relationship between IFT20 and CBL proteins in ciliary receptor sorting and modulation of PDGFRα signalling, which when defective underlies tumorigenic signalling in various tissues [239]. Indeed, expression of the oncogenic mutant PDGFRα Asp842Val, which localizes to the Golgi [239] and is the most common PDGFRα mutation in gastrointestinal stromal tumours [259], promotes ciliary disassembly and cell proliferation by phenocoyping PDGF-DD-mediated signalling [246]. Furthermore, rapamycin-mediated inhibition of mTOR signalling, which is upregulated in cells deficient in both c-CBL and CBL-b [260], rescues PDGF-AA–mediated signalling in IFT88 and IFT172 mutant fibroblasts that are devoid of primary cilia [250], although further studies are required to understand the relationship between aberrant mTOR signalling and mislocalization of PDGFRα in the context of ciliary signalling and tumorigenesis. Finally, INPP5E, was suggested to control ciliary PDGFRα signaling by inhibiting PDGF-mediated AKT activation[261, 262, 263]. These findings could indicate that INPP5E contributes to regulation of the balanced output of PDGF-AA-mediated AKT signalling at the cilium and that ciliopathies associated with mutations in INPP5E, such as Joubert syndrome, are partly linked to defects in this pathway.

TGFβ/BMP signalling in primary cilia

The transforming growth factor-β (TGFβ) superfamily comprises a highly pleiotropic group of ligands that signal via hetero-tetrameric receptor complexes of type I (RI) and type II (RII) serine/threonine kinases and a series of co-receptors to control a multitude of cellular processes during development and in the maintenance of tissue homeostasis in the adult [264, 265]. In canonical signalling, transcription factors of the R-SMAD family, typically SMAD2/3 and SMAD1/5/8 in the TGFβ–activin–nodal or the bone morphogenetic protein

(BMP) –müllerian inhibiting substance (MIS) branches of ligand signalling, respectively, are phosphorylated and activated by the receptors to form a complex with SMAD4, which translocates to the nucleus for targeted gene expression [265]. By contrast, ligands of the growth and differentiation factor (GDF) subfamily can operate either through SMAD2/3 or SMAD1/5/8, whereas other ligand subtypes may antagonize R-SMAD signalling [265].

The TGFβ superfamily also operates through so-called non-canonical pathways, including NF-κB signalling, Rho-like GTPases and PI3K-AKT pathways as well as MAP kinases (for example, ERK1/2, p38 and JNK), which may cross-talk with R-SMAD signalling and/or become integrated into larger signalling networks that contribute to the diverse mechanisms by which ligand stimulation controls diverse complex cellular responses [265, 266]. Finally, several studies have indicated a major role of receptor internalization in modulating the balanced output of TGFβ/BMP signalling, through clathrin-mediated and caveolin-mediated endocytosis. Clathrin-mediated endocytosis compartmentalizes the ligand–receptor complex in early endosomes, where SMAD anchor for receptor activation (SARA) binds to the PI(3)P-enriched membrane of the endosomes via its FYVE zinc finger domain to facilitate the association between RI and R-SMADs for robust SMAD2/3 signalling [265]. However, the function of SARA in the context of R-SMAD activation, nuclear translocation and SMAD-dependent gene expression might not be equivalent in all cell types [267]. Caveolin-mediated endocytosis has an inhibitory role in canonical TGFβ–BMP signalling through the proteasomal degradation of internalized receptors [265].

Consequences of TGFβ/BMP signalling

Increasing body of evidence indicates a prominent role for the primary cilium in co-organizing the balanced output of TGFβ/BMP signalling. The first report of active TGFβ signalling in the primary cilium was based on studies in cultured mouse and human fibroblasts in which TGFβ-RI and TGFβ-RII were shown to localize along and at the tip of the cilium [268]. Upon ligand stimulation, the receptors accumulated at the ciliary base region, which is enriched in SMAD4, after which the receptors were internalized by clathrin-mediated endocytosis at the ciliary pocket to activate SMAD2/3 [268] (Figure 6). Similarly, TGFβ signalling was associated with activation of ERK1/2 at the ciliary base, but in contrast to SMAD2/3, TGFβ1-induced phosphorylation of this MAP kinase seemed to occur independently of clathrin-mediated endocytosis [268]. These findings indicate that the primary cilium uses diverse mechanisms to fine-tune the output of canonical and non-canonical TGFβ signalling to control varied cellular responses, which could be relevant for processes such as those related to heart development, where TGFβ-dependent differentiation of mouse cancer stem cells and human embryonic stem cells into cardiomyocytes is associated with the temporal accumulation of TGFβ receptors and activation of SMAD2/3 at the ciliary base [268]. TGFβ and BMP receptors also localize to primary cilia in other cell types [241, 269–273] to regulate mouse heart development in vivo [273], migration of human bone mesenchymal stem cells in bone remodelling assays [270] and osteogenic differentiation *in vitro* and bone formation *in vivo* [269, 271, 274]. Primary cilia were also identified as major regulators of TGFβ-mediated activation of R-SMADs in the differentiation of human adipose progenitors into myofibroblasts [275]. Further, endothelial primary cilia, which function as flow sensors in the vasculature and contribute to blood–brain barrier integrity [276–279], counteract

endothelial-to-mesenchymal transition in a process that is associated with reduced canonical TGFβ signalling in the endocardial cushion area [280], whereas endothelial primary cilia cooperate with BMP signalling to stabilize vessel connections in the developing mouse retina [281]. More directly, shortening of primary cilia by TGFβ signalling [282–284] is associated with epithelial-to-mesenchymal transition in mouse kidney epithelial cells [284] and impairment of mechanosensation and maturation in human osteoblasts [283].

The importance of the cilium in coordinating TGFβ/BMP signalling is underscored by the fact that additional sets of proteins, which regulate receptor trafficking and mechanisms in feedback inhibition, are enriched at the cilia-centrosome axis (Figure 6). RAB11 [285], which is responsible for endosomal recycling of TGFβ receptors [286], is recruited to the cilium in a process that is mediated by the subdistal appendage protein, CEP128 [287]. Consequently, loss of CEP128 leads to impaired phosphorylation of R-SMADs, which in zebrafish is associated with defective organ development [287]. Conversely, feedback inhibitors of TGFβ/BMP signalling, including SMAD7 and SMURF1 [265, 288], localize to the ciliary base [268, 273] and restrain excessive signalling from the primary cilium. SMAD7 has been proposed to interact with TGFβ receptors to limit ARL6-mediated ciliary localization of the receptors and suppress tumour cell migration and invasion by restricting cross-talk between TGFβ receptors and SMO in HH signalling [272]. In this context, hamartin (also known as tuberous sclerosis complex protein 1, TSC1, which originally was found to form a heterodimeric complex with TSC2 that negatively regulates mTOR signaling, is required for TGFβ-induced phosphorylation of SMAD2/3 and subsequent expression of GLI2 and WNT5A in an mTOR-independent manner [289], signifying an additional layer of cross-talk between multiple signaling pathways at the primary cilium. Finally, the E3 ubiquitin ligase SMURF1 negatively regulates phosphorylation of SMAD1/5/8 at primary cilia to control cell-type specification during *in vivo* heart development, which in *Smurf1*$^{-/-}$ mouse embryos is associated with delayed outflow tract septation [273].

Conclusions and future directions

A growing body of research over the past 15 years has uncovered important cellular signalling pathways that function via primary cilia. Cilia are present on most cell types, but not all cell types in a given tissue or organ will be ciliated. In addition, the ciliary composition of receptors and regulatory proteins seems to be cell and tissue specific. In the individual cell the constellation of receptors and regulatory proteins might change over time, enabling the cell to carry out specific functions during development and in the maintenance of tissue homeostasis in the adult. The importance of cilia in signalling is demonstrated by the fact that mutations in >200 genes that lead to dysfunction of primary cilia underlie a pleiotropic group of >30 ciliopathies, which affect many different tissues and organs during embryonic and postnatal development as well as in adulthood [29].

Current evidence suggest that primary cilia coordinate a variety of signalling pathways, including those regulated by HH, GPCR, WNT, RTK and TGFβ/BMP, to control developmental processes, tissue plasticity and organ function. In this regard it is important to recognize that while some pathways are considered to be *bona fide* ciliary pathways, such as canonical HH signalling, other pathways might be only partially associated with cilia and

probably act in cell type-specific and developmentally dedicated contexts. A growing body of evidence points suggests that temporally regulated trafficking as well as activation or deactivation of receptors and/or regulatory signalling modules at the cilium–centrosome axis are critical for orchestrating the balanced output of these pathways. Some of these events are regulated within different sub-compartments of the cilium–centrosome axis, such as through E3 ubiquitin ligases, which in conjunction with endocytic pathways at the ciliary pocket provide feedback loops to control the balanced output of HH, PDGFRα and TGFβ/BMP signalling pathways. The role of IFT proteins, the BBSome, kinesin motor proteins and endocytic events in regulating ciliary signalling has been discussed elsewhere [23, 77, 78, 290].

The mechanisms that control the balanced output of ciliary signalling described in this Review might also apply to other pathways such as Hippo and Notch signalling pathways or those regulated by receptors of extracellular matrix molecules, which also have been linked to primary cilia in various cell types and tissues [23, 291, 292]. Indeed, the growing list of signalling pathways that are connected to primary cilia is indicative of the importance of these organelles in orchestrating the integration and cross-talk between these pathways in a spatiotemporal manner. However, we still know very little as to how the temporal and spatial dynamics of major signalling networks are encoded by the primary cilium and how the integration of information in such networks is able to generate diversity in signalling outputs. In this context, it is important to recognize that primary cilia are highly dynamic organelles whose configuration is tightly coupled to the differentiation state and microenvironment of the cell, and that this flexibility of the cilium might explain how cells are able to receive and convert signalling inputs in different cellular, developmental and homeostatic settings. Despite enormous progress in this field, several key questions remain to be addressed including the mechanisms by which primary cilia orchestrate individual signalling pathways in a temporal manner, the mechanisms by which multiple ciliary pathways integrate into higher order signalling networks, the mechanisms by which cilia dictate cellular activity and fate in the context of stem cell microenvironments, cell differentiation states and organ development, and how ciliary protein composition differs at different stages of the cilia life cycle. Addressing these questions is crucial to improve our understanding of fundamental cilia biology and disease etiology as well as for developing new treatment approaches for ciliopathies.

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Key points

- Primary cilia emanate in a single copy from the centrosomal mother centriole (basal body) at the surface of most vertebrate cell types.
- Primary cilia possess a unique lipid and receptor composition and detect and convey extracellular cues to control cellular processes during development and in tissue homeostasis.
- Current evidence suggest primary cilia coordinate a variety of signalling pathways, including those regulated by HH, GPCR, WNT, RTK and TGFβ/BMP, to control developmental processes, tissue plasticity and organ function.
- The ability of primary cilia to balance the output of cellular signalling is dynamic and relies on the differentiation state and microenvironment of the cell.
- Dysfunction of primary cilia underlies a pleiotropic group of diseases and syndromic disorders termed ciliopathies, affecting many different organs in the body.
- Mechanistic insight into ciliary coordination of spatiotemporal signalling networks is critical for understanding the etiology of ciliopathies and for the discovery of novel ciliopathy disease genes and drug targets.

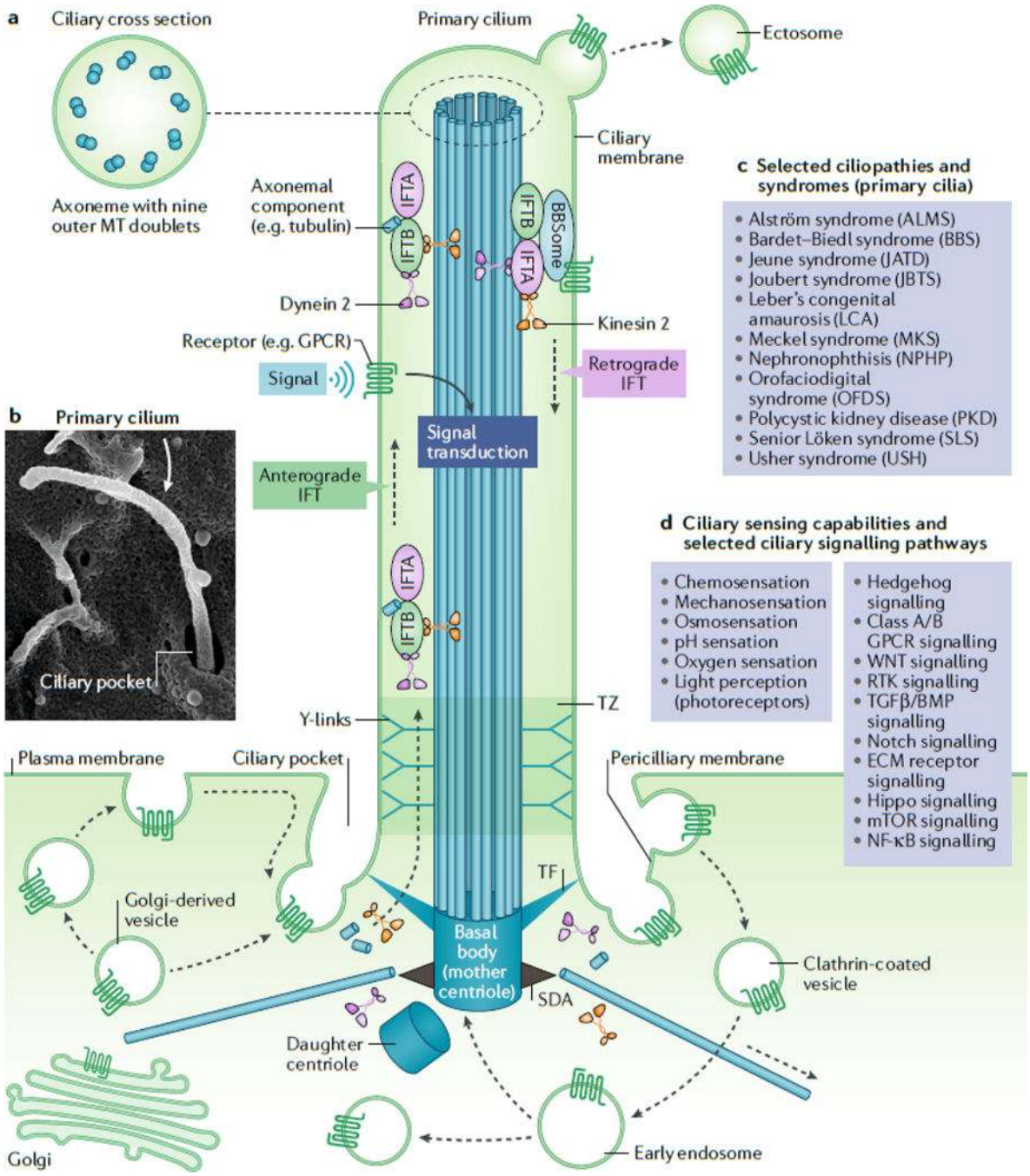

Figure 1. Overview of primary cilia, cellular signalling and ciliopathies.

a| The primary cilium is a non-motile organelle that extends as a solitary unit from the centrosomal mother centriole (basal body). a | The cilium comprises a microtubule (MT)-based axoneme containing a ring of nine outer microtubule doublets. Between the basal body and cilium is the ciliary transition zone (TZ), which contains specialized gating structures such as Y-links that along with the basal body transition fibres control the entrance and exit of ciliary proteins, thereby contributing to compartmentalization of the organelle. The intraflagellar transport (IFT) system zips up (anterograde) and down (retrograde)

axonemal microtubules to mediate the transport of specific ciliary cargo, such as receptors, into or out of the organelle, whereupon they are degraded or recycled. Cilia can also release ectosomes by shedding off membrane-enclosed material from the surface of the organelle. The function of these extracellular vesicles has been linked to maintenance of ciliary integrity, balancing of intraciliary signalling events and/or in transmission of signals across cells [28, 293]. **b|** Image of a primary cilium in a mouse embryonic fibroblast analysed by scanning electron microscopy. **c|** Selected ciliopathies are caused by dysfunctional primary cilia. **d|** Overview of diverse sensory capabilities of primary cilia and their associated signalling pathways. BBSome: protein complex of eight Bardet–Biedl syndrome proteins; SDA, sub-distal appendages, TF, transition fibres.

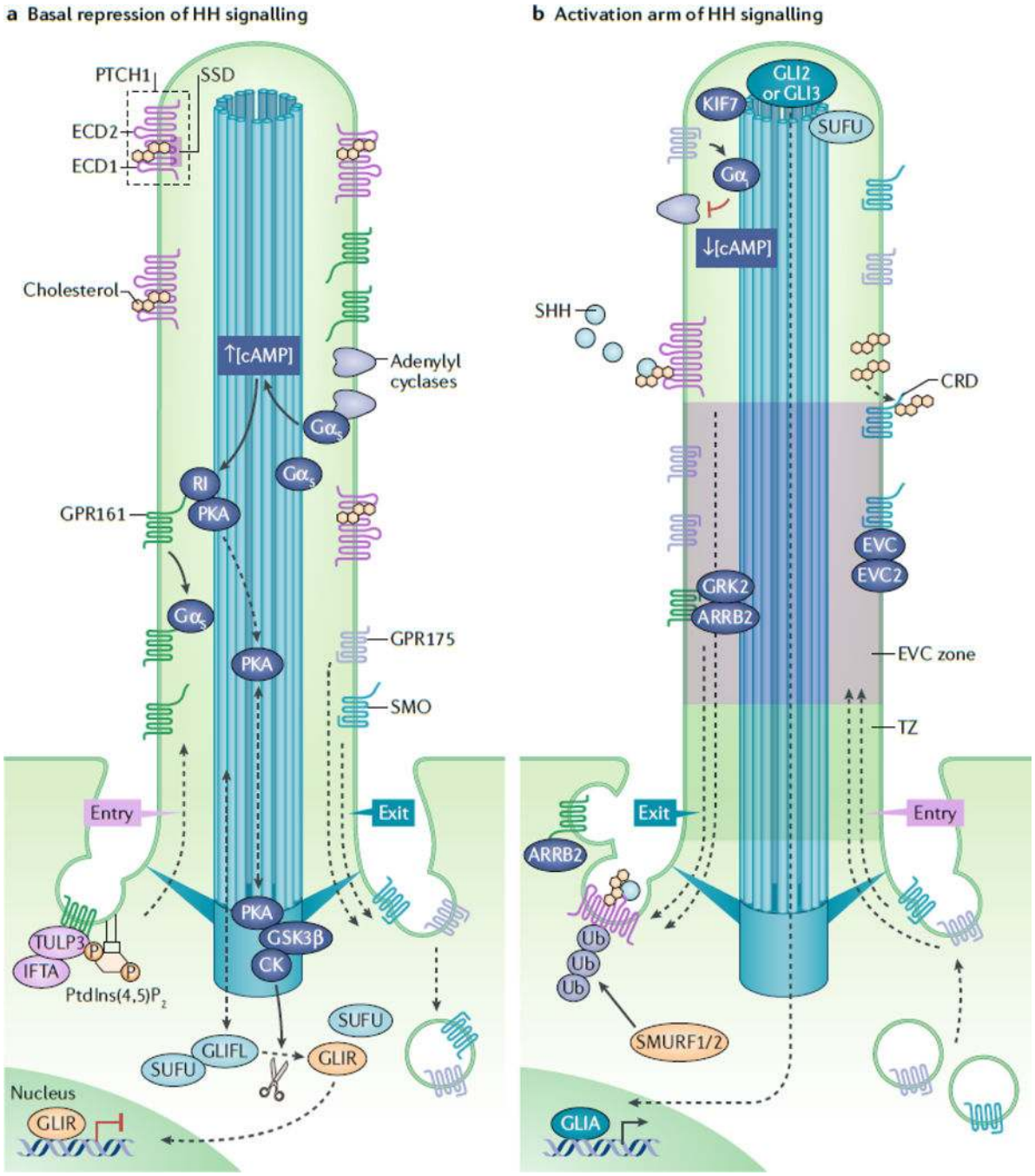

Figure 2. Overview of canonical hedgehog signalling in primary cilia.

a| In the absence of sonic hedgehog (SHH; that is, under conditions of basal suppression) the receptor patched-1 (PTCH1) is enriched in the ciliary membrane, preventing ciliary accumulation of smoothened (SMO). A transmembrane sterol sensing domain (SSD) in PTCH1 can accommodate cholesterol or cholesterol derivatives. The class A GPCR, GPR161, is targeted to the cilium by tubby-like protein 3 (TULP3) and intraflagellar transport complex A (IFT-A) to activate adenylyl cyclases via G-proteins (Gαs), leading to increased ciliary levels of cAMP. Increased cAMP levels release protein kinase A (PKA)

from regulatory subunits (RI), which in conjunction with glycogen synthase kinase 3 beta (GSK3-β) and casein kinases (CK) promote the limited proteolytic cleavage of full-length versions of GLI transcription factors (GLI-FL) into their repressor form (GLI-R) in a cilia-dependent manner. Suppressor of FUSED (SUFU) restrains GLI3 in the cytoplasm and promotes GLI3 processing. **b|** Binding of SHH to PTCH1 extracellular domains (ECDs) is regulated by cholesterol derivatives. In addition, SMURF1/2-mediated ubiquitination regulates PTCH1 exit from cilia and internalization at the ciliary base. Removal of PTCH1 causes concomitant enrichment and activation of SMO in cilia by cholesterol or derivatives. A longitudinal tunnel in the transmembrane region of SMO (dotted arrow) could move cholesterol from the ciliary membrane to its binding domain in the cysteine rich region (CRD). SHH stimulation results in dissociation of SUFU from GLI transcription factors, formation of full-length activator forms of GLIs (GLI-A), and accumulation of these proteins along with the microtubule-associated atypical kinesin KIF7 at ciliary tips. Downstream effectors for SMO include the EVC–EVC2 complex, which localizes in cilia distal to the transition zone. SHH stimulation further triggers the ciliary exit of GPR161 and ciliary entry of GPR175, which inhibits the production of cAMP. Both GLI-R and GLI-A translocate from the cilium into the nucleus to repress and induce transcriptional activation of HH target genes, respectively. Abbreviations: $PI(4,5)P_2$: phosphatidylinositol 4,5-bisphosphate, Ub: ubiquitination.

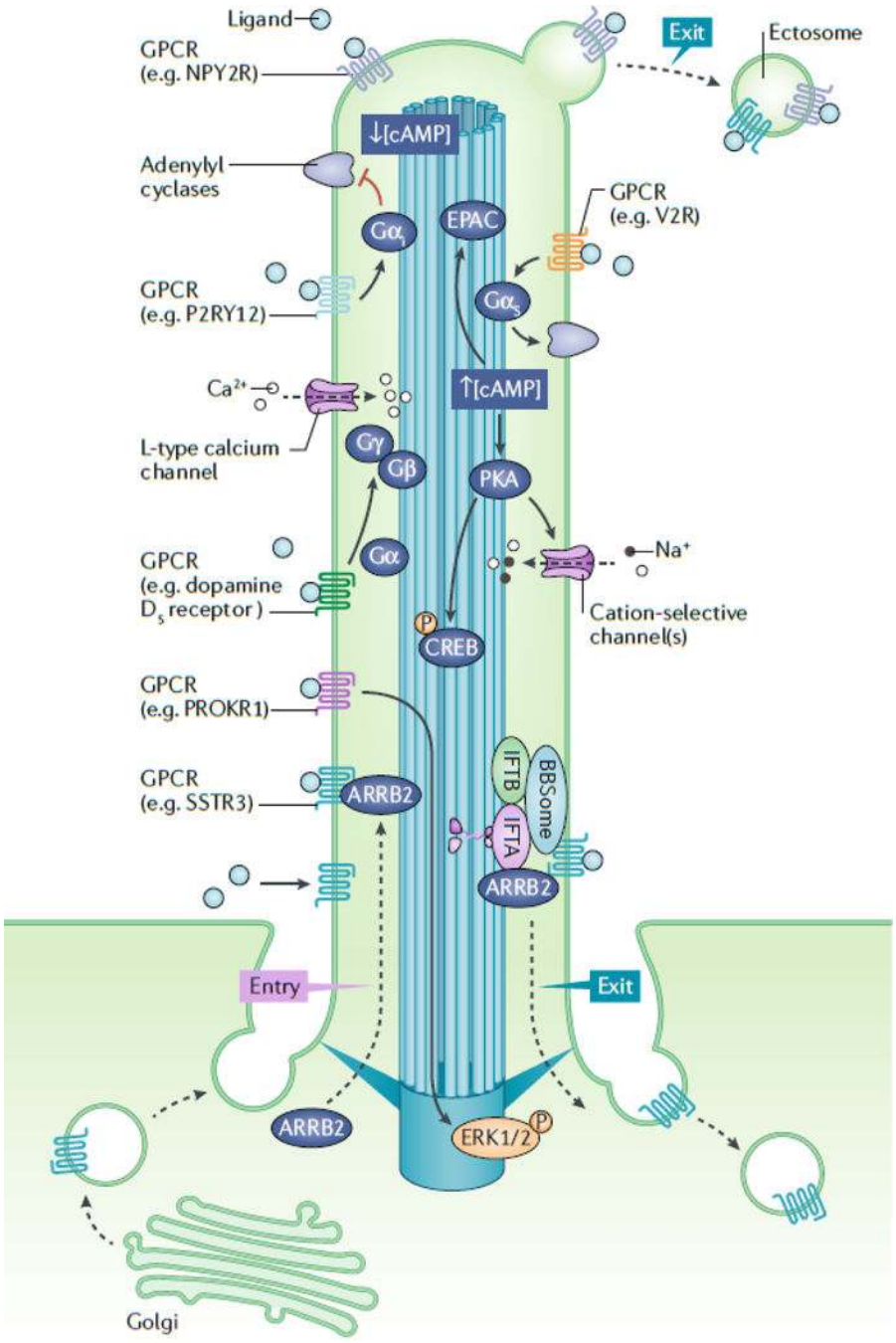

Figure 3. Overview of ciliary GPCR signalling.

Binding of agonists to various G-protein-coupled receptors (GPCRs) expressed on cilia modulates a variety of signalling pathways on different cell types. For example, binding of endocrine gland-derived vascular endothelial growth factor to prokineticin receptor 1 (PROKR1) on trophoblast cell cilia activates mitogen-activated protein kinase kinase 1/2 (ERK1/2) at the ciliary base, presumably through a $G\alpha_q$-mediated increase in calcium levels. Dopamine binding to dopamine receptor 5 (D5) on renal epithelial cell cilia facilitates $G\beta\gamma$ subunit dissociation and activation of an L-type calcium channel, resulting in an

increase in ciliary calcium levels. ADP binding to the purinergic receptor P2Y12 (P2RY12) on cholangiocyte cilia stimulates $G\alpha_i$, which inhibits adenylyl cyclase activity and subsequently reduces cAMP levels. Vasopressin binding to the type 2 vasopressin receptor (V2R) on renal epithelial cell cilia results in activation of adenylyl cyclase, increased cAMP levels, activation of PKA, and stimulation of a cation selective channel on the ciliary membrane. Increased cAMP levels and activated PKA can also activate exchange protein directly activated by cAMP (EPAC) and cAMP response element binding protein (CREB) in the cilium, respectively. Somatostatin binding to somatostatin receptor subtype 3 (SSTR3) on neuronal cilia stimulates β-arrestin (ARRB2) recruitment into the cilium where it mediates SSTR3 ciliary export in cooperation with the BBSome and intraflagellar transport (IFT) complex. Neuropeptide Y (NPY) binding to neuropeptide Y receptor subtype 2 (NPY2R) on neuronal cilia causes accumulation of the receptor at the ciliary tip and release through ectosomes.

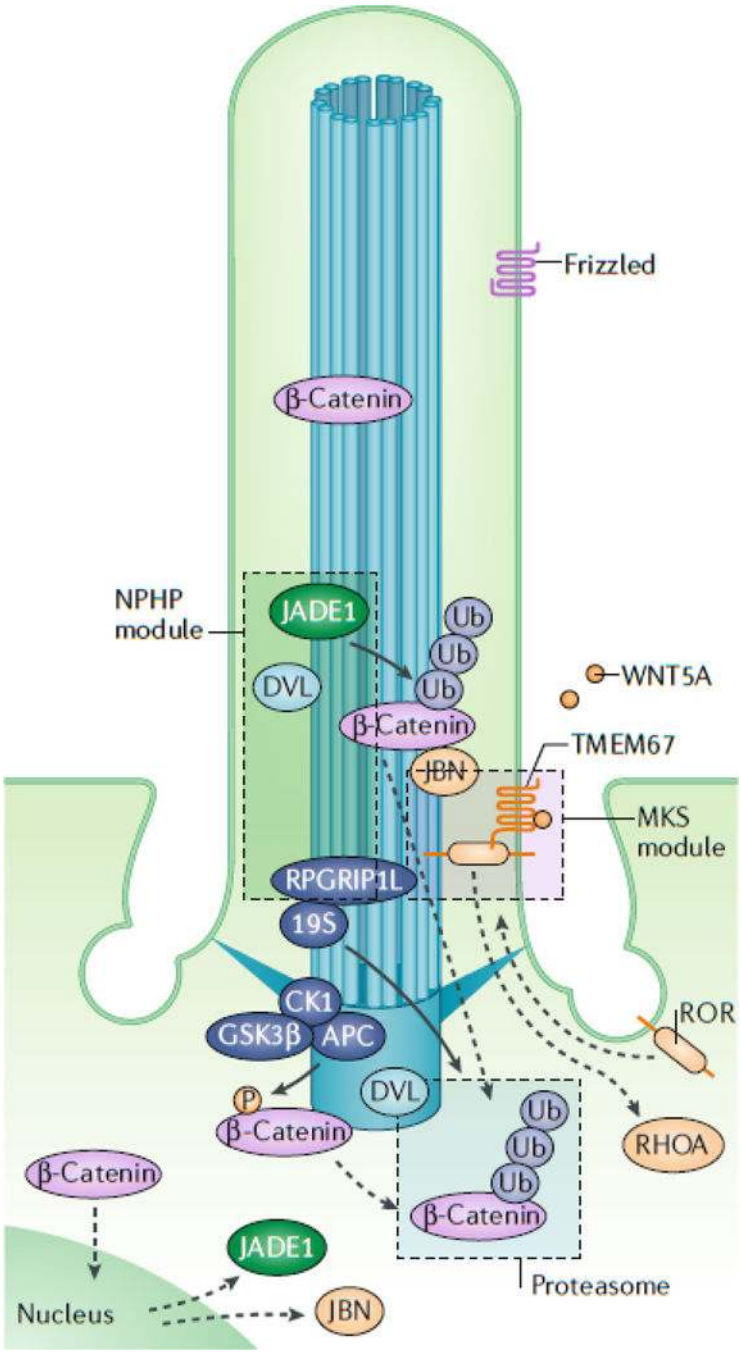

Figure 4.

Overview of ciliary transition zone and basal body modulation of WNT signalling. The ciliary transition zone (TZ) is composed of several interacting protein modules or complexes, including the nephronophtisis (NPHP) and Meckel-Gruber Syndrome (MKS) modules that establish ciliary gating and contribute to the regulating of WNT signalling. The NPHP module protein retinitis pigmentosa GTPase regulator-interacting protein 1-like (RPGRIP1L) inhibits canonical WNT signalling through its interacting with a component of the 19S proteasome subunit, PSMD2, thereby promoting ubiquitin (Ub)-mediated

proteasomal degradation of β-catenin and DVL. Further, β-catenin is ubiquitinated by the E3 ubiquitin ligase JADE1, which is present in the TZ as well as at the basal body. In the MKS module, transmemembrane protein 67 (TMEM67) recruits receptor tyrosine kinase-like orphan receptor 2 (ROR2) to the TZ to form a receptor complex that binds WNT5A, which is a ligand for non-canonical WNT signalling. In the presence of a cilium, the MKS module component Jouberin (JBN) recruits cytoplasmic β-catenin, which accumulates in response to WNT activation. In the absence of a cilium, JBN facilitates β-catenin nuclear entry. Several key components of the β-catenin destruction complex localize to the basal body, including the scaffold protein adenomatous polyposis coli (APC), GSK3-β glycogen synthase kinase 3 beta (GSK3-β) and casein kinases CK1-α, CK1-μ and CK1-δ [193–195, 294]. The main product of the destruction complex, phosphorylated β-catenin, is also concentrated at the basal body, where it undergoes degradation by the proteasome. Whether β-catenin is actively phosphorylated and ubiquitinated within the cilium, or whether the modified protein is recruited to the basal body, is unknown. Frizzled receptors can accumulate in the primary cilia but the functional relevance of this localization is unknown. RHOA: Ras homolog gene family member A.

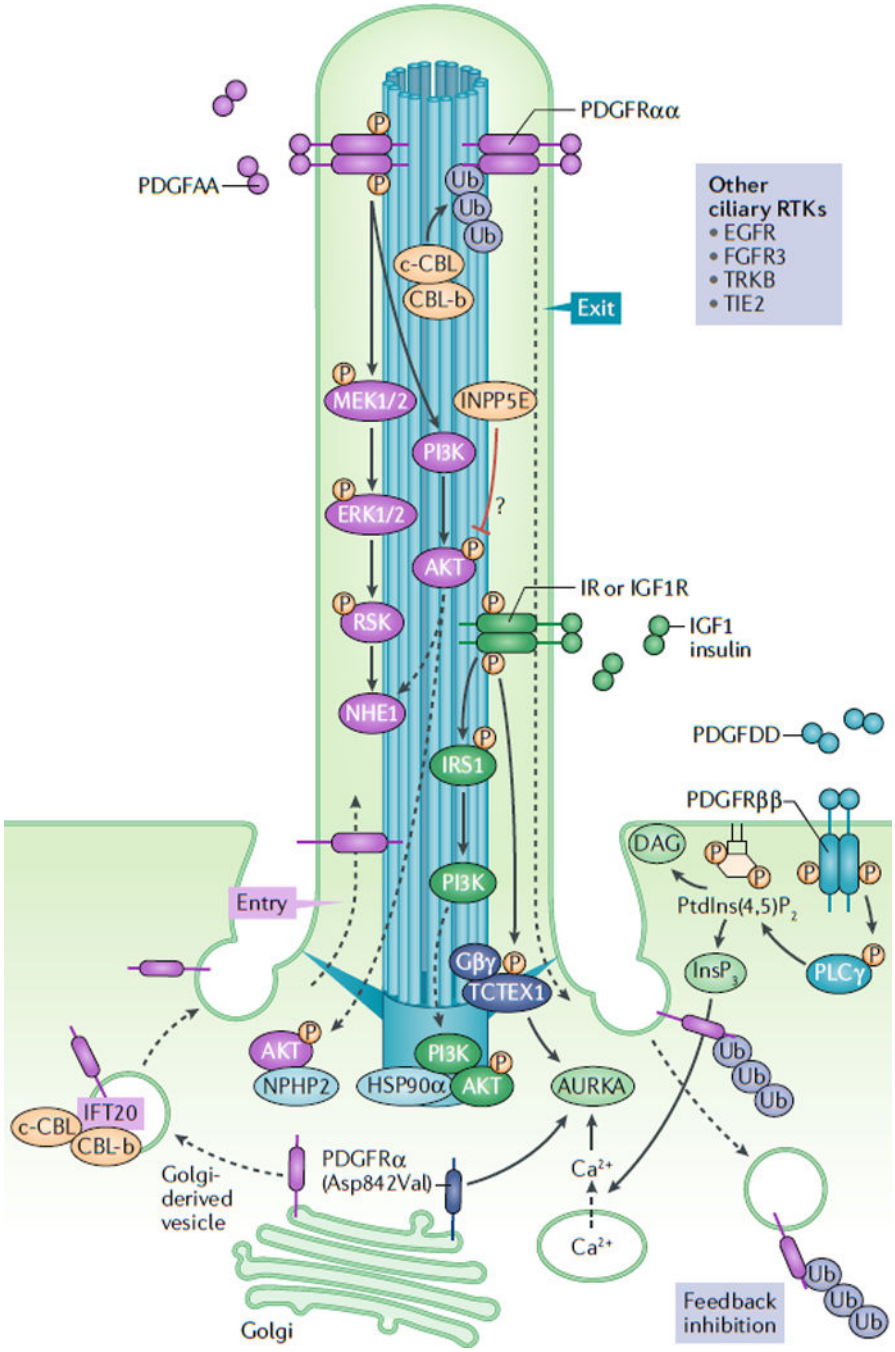

Figure 5. Overview of ciliary PDGFRα, insulin and IGF-1 signalling.

Targeting of platelet-derived growth factor receptor alpha (PDGFRα) to the cilium relies on intraflagellar transport protein 20 (IFT20) in complex with the E3 ubiquitin ligases c-CBL and CBL-b; activation of the receptor in the cilium activates the MEK1/2–ERK1/2–RSK and PI3K–AKT pathways, which in turn control activation and translocation of the Na^+/H^+ exchanger 1 (NHE1) to leading edge of the cell for directional migration. AKT might also become activated at the ciliary base in complex with NPHP2 (also known as inversin). Feedback inhibition of PDGFRα signalling might be controlled by CBL-mediated

ubiquitination (Ub) of the receptor in the cilium as well as by inositol 1,4,5-trisphosphate 5-phosphatase (INPP5E), which inhibits AKT signalling. PDGFRβ localizes at the plasma membrane to induce aurora kinase A (AURKA)-mediated disassembly of the primary cilium, which promotes cell cycle re-entry. Similarly, expression of the oncogenic mutant PDGFRα Asp842Val, which localizes to the Golgi, promotes ciliary disassembly and cell proliferation via activation of AURKA. Insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) also localize to primary cilia to balance the regulation of various cellular processes, including adipogenesis, neuronal differentiation, mitogenic signalling and insulin production in islet β-cells of the pancreas. Ciliary IGF-1R activation also induces ciliary resorption and cell cycle entry possibly via IGF-1-mediated recruitment of phosphorylated TCTEX-1 to the ciliary TZ via non-canonical G-protein signalling, PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; IRS-1: insulin receptor substrate 1; PLCγ, phospholipase C gamma; PIP_2, Phosphatidylinositol 4,5-bisphosphate; IP_3, Inositol trisphosphate; HSP90α, heat shock protein 90 alpha.

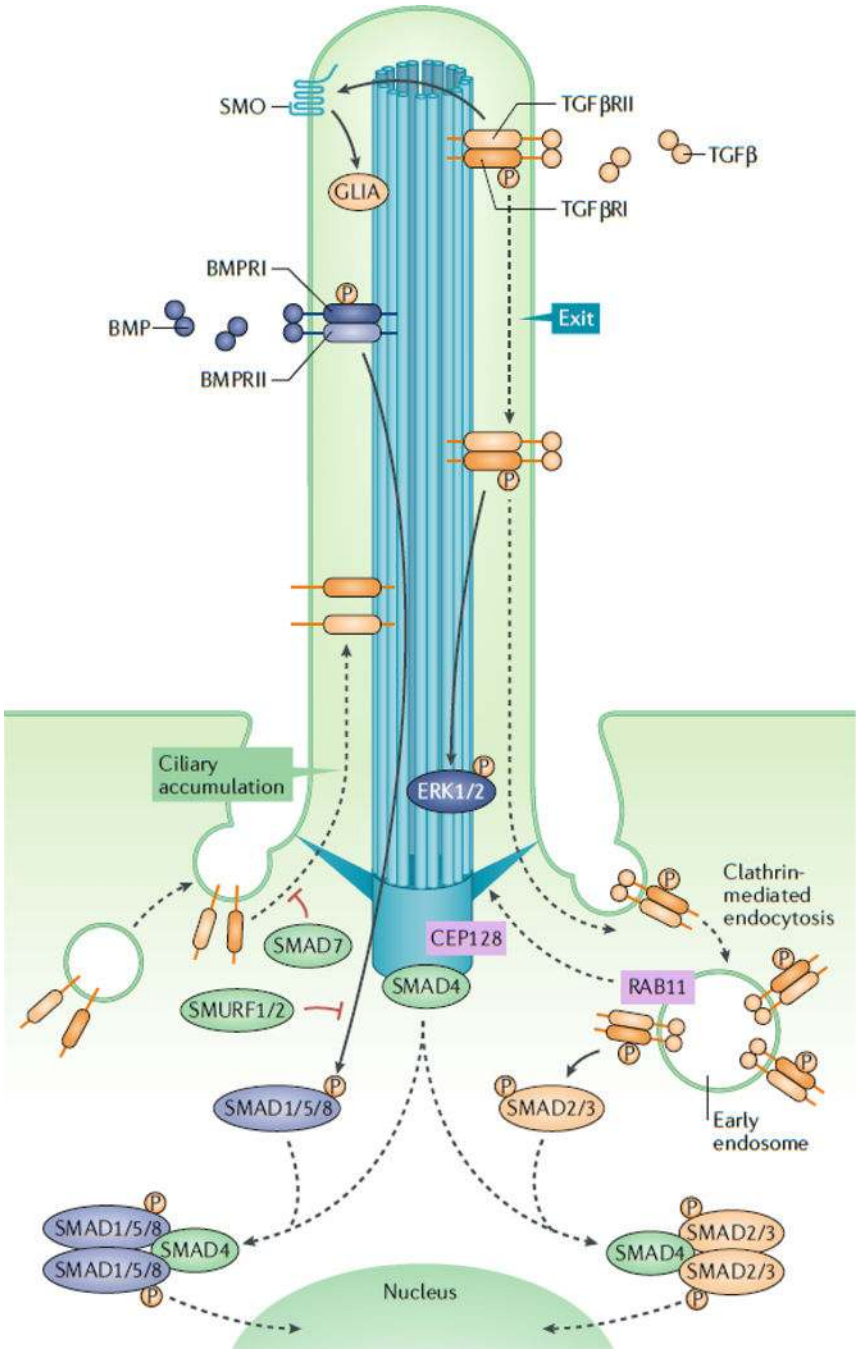

Figure 6. Overview of ciliary TGFβ/BMP signalling.

Receptors of the transforming growth factor beta (TGFβ) and bone morphogenic protein (BMP) family are recruited to the primary cilium to activate receptor (R)–SMAD transcription factors (SMAD2/3 and SMAD1/5/8) partly via internalization of active receptors by clathrin-mediated endocytosis (CME) at the ciliary pocket. Activated R-SMADs form a trimeric complex with the co-SMAD, SMAD4, which translocate to the nucleus for targeted gene expression. TGFβ may activate ERK1/2 in the cilium independently of CME. RAB11-mediated recycling of TGFβ receptors to the primary cilium

may be regulated by the subdistal appendage protein, CEP128. Feedback inhibition of ciliary TGFβ/BMP signalling is under the control of the inhibitor SMAD, SMAD7, as well as the E3 ubiquitin ligase, SMURF1. Ciliary TGFβ receptors may further stimulate the hedgehog pathway component, smoothened (SMO), leading to activation of GLI transcription factors. Abbreviations: TGFβ-RI/II, TGFβ receptors I and II; BMP-RI/II, BMP receptors I and II; GLI-A: activator form of GLI transcription factors; ERK1/2: extracellular signal–regulated kinase 1/2; EE: early endosome.

Table 1:

Non-olfactory and non-visual class A and B G-protein-coupled receptors that localize to primary cilia

GPCR	Cell type	Reference(s)
β2-adrenergic receptor (β2AR)	Neurons	274
Bile acid receptor 1 (GPBAR1 or TGR5)	Cholangiocytes	158, 295
Dopamine receptor 1 (D1)	Neurons	168
Dopamine receptor 5 (D5)	Vascular endothelial cells, renal epithelial cells	160, 296
Galanin receptor 3 (GALR3)	Neurons	74
GPR19	Neurons, Glial cells	64
GPR83	Neurons	74
GPR161	Neurons, mouse embryonic fibroblasts	122
GPR175	Mouse fibroblasts	297
Kisspeptin receptor 1 (KISS1R)	Neurons	298
Melanin-concentrating hormone receptor 1 (MCHR1) Melanocortin 4 receptor (MC4R)	Neurons Neurons	299 180, 299
Muscarinic acetylcholine receptor 3 (M3R)	Olfactory sensory neurons	300
Neuropeptide Y receptor 2 (NPY2R)	Neurons	74
Neuropeptide Y receptor 5 (NPY5R)	Neurons	74
Parathyroid hormone receptor 1 (PTH1R)	Nucleus pulposus cells	301
Prokineticin receptor 1 (PROKR1)	Human trophoblast cells, human placental tissue	164
Prolactin-releasing hormone receptor (PRLHR)	Glial cells	302
Prostaglandin E receptor 4 (EP4)	Human retinal pigment epithelial cells	303
Purinergic receptor P2Y12 (P2RY12)	Cholangiocytes	155
Pyroglutamylated RFamide peptide receptor (QRFPR)	Neurons	74
Serotonin receptor 6 (HTR6)	Neurons	304
Somatostatin receptor 3 (SSTR3)	Neurons	305
Trace amine-associated receptor 1 (TAAR1)	Thyroid epithelial cells	306
Vasopressin receptor 2 (V2R)	Renal epithelial cells	162