Chapter 3
Smoothened Signaling Through a G-Protein Effector Network

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Introduction

The important role the Hedgehog (HH) signaling pathway plays in cancer was first revealed by patients diagnosed with the familial disorder known as Gorlin Syndrome, who harbor loss-of-function mutations in the HH receptor Patched (PTCH) [1–3]. Besides numerous developmental abnormalities, consistent with disruption of this important developmental signaling pathway, individuals afflicted with this disorder have an inherited predisposition to medulloblastoma, basal cell carcinoma, and rhabdomyosarcoma [2]. Similar mutations found in sporadic cases of these same tumor types implicated *PTCH* as an important tumor suppressor in human cancer [4]. Other components of the HH pathway, such as the gene encoding the seven-transmembrane (7TM) protein Smoothened (SMO), are also found mutated in sporadic forms of these same malignancies [5, 6]. More recently, constitutive activation of the HH pathway has been implicated in other human cancers including those of the breast, prostate, pancreas, and lung, where HH is thought to play a role as a tumor-survival factor [7]. Combined, it has been estimated that approximately 25% of all human tumors harbor a constitutively active HH signaling pathway [8]. As such, considerable effort has gone into identifying novel small-molecule inhibitors of HH signaling. Consistent with the rate-limiting role SMO plays in HH signaling, the vast majority of HH inhibitors isolated from numerous small-molecule screens appear to target SMO [9]. A number of these compounds are currently in clinical trials as anti-cancer agents, targeting tumors that are dependent on HH pathway activity [10]. Thus, a

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clear understanding of the mechanisms by which SMO communicates with downstream pathway components, and how such inhibitors affect these processes, will directly impact human health.

Hedgehog Signal Transduction

Much of what is understood about HH signaling originates from studies of this signaling pathway during the development of the fruit fly *Drosophila melanogaster* [11]. It is now well accepted that the major components, and how they communicate with each other, are highly conserved from Drosophila to man. Although there are significant differences in the importance of some of the signaling components across phyla, it is not yet clear if these variations are due to specific contextual differences or to evolutionary divergence. Thus, in this chapter we generalize about the HH signaling pathway from work derived from numerous animal models, mentioning specific biological contexts only where necessary to illustrate a particular point.

HH is produced and secreted by discrete compartments within a developing field of cells, where it elicits both short- and long-range effects on target cells [12]. The receiving cells interpret the level of HH activation through poorly defined, indirect interactions between the HH receptor PTCH and the signal transducer SMO. PTCH inhibits the activity of SMO in a manner that appears to be catalytic, whereas SMO is constitutively active in the absence of PTCH [13, 14]. One of the mechanisms by which PTCH inhibits SMO activity involves PTCH-dependent trafficking of SMO to lysosomes [15, 16]. In response to HH, PTCH is removed from the cell surface, thereby allowing SMO phosphorylation, stabilization, and accumulation at the plasma membrane [15, 17]. Ultimately all signaling downstream of SMO coalesces to regulate the stability and activity of the GLI/CI family of transcription factors [18]. In the absence of HH, these proteins exist as proteolyzed transcriptional repressors. HH blocks this proteolytic conversion, and stabilizes full-length transcriptional activators. The degree of HH a cell is exposed to ultimately determines the ratio of GLI/CI repressor and activator forms to regulate a spectrum of transcriptional targets that is thought to correlate with the concentration of the initial HH signal.

While the general flow of information through the HH signaling cascade is known, the direct effectors of SMO, and the mechanism(s) by which it communicates with them are still being characterized. The first clue as to how SMO transduces the signal from the plasma membrane to the intracellular effectors came with the observations that Drosophila SMO directly associates with the kinesin-related protein Costal2 (COS2) [19–22], and that mammalian SMO binds the COS2 ortholog KIF7 [23]. The functional consequence of KIF7-SMO binding in mammalian systems is not yet clear. However, in Drosophila, COS2 serves as a scaffold upon which a complex containing CI and the protein kinases Fused (FU), cyclic-AMP (cAMP)-dependent protein kinase A (PKA) and casein kinase 1 (CK1) assembles [24, 25]. As such, a direct association between COS2 and SMO

connects the membrane signaling components with the cytoplasmic effectors. Subsequent to this finding, a direct association between SMO and FU that drives a feed-forward loop to facilitate high-level signaling was described [26]. We recently demonstrated that the intracellular molar concentration of SMO is significantly lower than that of CI, COS2, or FU, suggesting that direct association between SMO, COS2, and FU is unlikely to facilitate all aspects of HH signaling [27]. It is, therefore, likely that multiple pools of intracellular effectors exist; some that are in direct contact with SMO, and some that are regulated through the use of G-proteins and/or second messengers.

Smoothened as a G-Protein-Coupled Receptor

Much of what we know about G-protein-coupled receptor (GPCR) structure and function has resulted from studies of the prototypical GPCR rhodopsin, the first GPCR to be fully sequenced and to yield high-resolution structural data [28–30]. Sequence analysis of rhodopsin suggested the existence of several distinct functional domains, including seven predicted alpha-helical transmembrane segments, an extracellular amino-terminal domain, three extracellular loops, a carboxyl-terminal domain with multiple phosphorylation sites, and three intracellular loops [29]. Structural analysis of rhodopsin, and more recently of the β_2-adrenergic receptor and A_{2A} adenosine receptor, confirm the existence and conservation of these domains, underscoring their importance in GPCR function [28]. As such, proteins possessing these well-established functional domains in their primary amino acid sequence or predicted tertiary structure are classified as members of the GPCR superfamily, which is estimated to encompass more than 1% of all human genes [30].

SMO was originally identified as a gene necessary for proper organization of the early Drosophila embryo [31]. Subsequent genetic and molecular characterization of SMO revealed it to be a requisite component of the HH signal transduction cascade [32]. Primary sequence comparisons revealed that SMO and the Frizzled (FZ) family of GPCRs are quite similar across distinct functional domains: 37% similarity across the extracellular amino-terminal domains, and 52% similarity across the seven predicted transmembrane domains [32]. As such, SMO has been classified as a member of the FZ family of GPCRs. The specific contributions of conserved GPCR functional domains to SMO-mediated regulation of HH pathway activity are discussed below (Fig. 3.1).

Cysteine-rich domain (CRD). A conserved CRD is situated in the extracellular amino terminus of all FZ family GPCRs [33–35]. Disulfide bonds between amino-terminal cysteine residues and/or cysteine residues in the extracellular loops of FZ drive receptor conformations that are necessary for its ligand binding and ligand-induced dimerization [34, 36]. Like FZ, SMO possesses multiple cysteines in its amino terminus and extracellular loops that are positionally conserved across species (http://www.gpcr.org). In vitro studies in mammalian cell culture suggested that the amino-terminus of SMO, which encompasses the CRD, is not required for GLI activation [37]. However, genetic analyses in both Drosophila and zebrafish

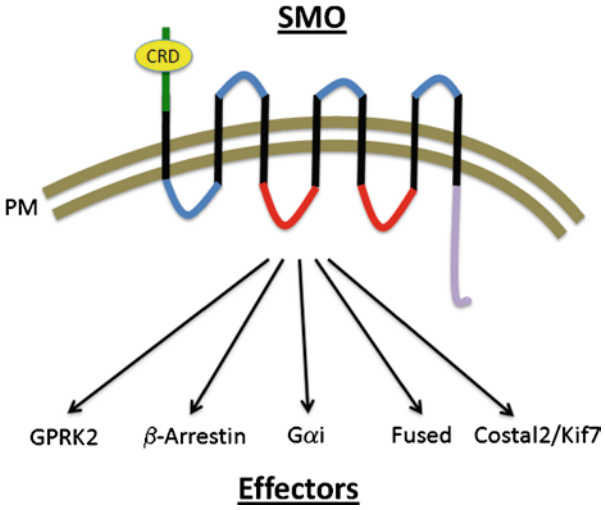

Fig. 3.1 Domains and effectors of smoothened (SMO). A schematic depicting the predicted topology and domains of SMO in the plasma membrane is shown. The seven predicted transmembrane domains of SMO are shown in *black*. The amino-terminal domain is shown in *green* and the cysteine-rich domain (CRD) in *yellow*. Three extracellular and one intracellular loops are shown in *blue*, but intracellular loops 2 and 3 – which are thought to couple to G-proteins – are shown in *red*. The carboxyl-terminal domain is shown in *purple*. The various known direct effectors of SMO are indicated below it

support that conserved cysteine residues in the CRD are critical for SMO signaling and/or subcellular localization [38–40], suggesting that the CRD is a requisite functional domain. Further studies are needed to more clearly define contributions of the CRD to SMO signaling.

Transmembrane domains and intracellular loops. The topology of the 7TM regions dictates the activation state of a GPCR by directing the conformation of its intracellular loops and cytoplasmic tail [41]. In response to ligand, the TM domains of the receptor shift to allow changes in conformation of the intracellular portions of the protein that facilitate receptor phosphorylation and/or G-protein selectivity, docking, and activation [41]. The importance of SMO TM sequence/structure is underscored by known oncogenic SMO mutations, all of which are localized to predicted TM segments [6, 42]. It is likely that these mutations lock SMO TM and intracellular domains in an activated state, which is insensitive to PTCH-mediated inhibition.

The cytoplasmic tail along with intracellular loop 3 (ic3), and to a lesser extent ic2, constitute the G-protein docking site on the vast majority of GPCRs [43]. While extensive structure/function analysis of the SMO intracellular loops has not been reported, chimeric studies in cultured fibroblasts reveal a critical role for ic3 in activation of the signaling cascade [37]. These findings are supported by a loss-of-function *SMO* mutation in Drosophila of a highly conserved Arg residue localized to the carboxyl-terminal end of loop ic3 [39]. The importance of the intracellular

loops is further supported by a study demonstrating that introduction of peptide analogs of either ic2 or ic3 into cultured cancer cell lines, which have an activated HH signaling pathway, attenuates their proliferation [44]. Further studies are needed to identify binding partners of SMO ic2 and ic3, and to determine whether these domains constitute a binding site for a partner G-protein.

Carboxyl-terminal intracellular tail. Multiple phosphorylation sites, which have been shown to be critical for pathway activation, have been identified in the SMO carboxyl-terminal tail [45–47]. Phosphorylation of such sites in response to ligand is a well-characterized event in canonical GPCR signaling, which generally serves to recruit various adaptor proteins and signaling effectors to the activated receptor [48]. Accordingly, HH-stimulated phosphorylation of SMO by PKA and G-protein regulated kinase 2 (GRK2) triggers both adaptor protein recruitment and SMO multimerization [49–51]. Mutations that prevent phosphorylation of any of these characterized phosphorylation sites compromise the ability of SMO to signal [45–47].

SMO Signaling Through Heterotrimeric G-Proteins

We recently demonstrated that *Gαi* overexpression in Drosophila triggers activation of HH target genes and wing patterning defects consistent with excessive HH signaling [52]. These phenotypes correlated with the activation state of the expressed *Gαi* transgene, as overexpression of a *Gαi* mutant that cannot bind GTP resulted in no observable phenotype. Conversely, overexpression of wild-type Gαi triggered modest gain of function phenotypes, and overexpression of a transgene encoding constitutively active *Gαi* resulted in strong HH gain of function phenotypes.

Activation of heterotrimeric G-proteins of the Gαi family frequently serves to decrease intracellular pools of cAMP through Gαi-mediated inhibition of adenylate cyclase (AC) [53]. Accordingly, in our study, we observed a SMO- and Gαi-dependent reduction in total intracellular cAMP within 5–10 min of HH stimulation [52]. Modulation of cAMP appears to be critical for in vivo HH signaling, as a mutant allele of the cAMP phosphodiesterase *DUNCE* [54] enhanced the HH loss-of-function phenotype induced by expression of a dominant negative SMO mutant in the Drosophila wing [52]. The ability of cAMP to modulate HH pathway-dependent patterning events is further supported by studies demonstrating that overexpression of an anthrax virulence factor, which functions as a potent bacterial AC, triggers wing phenotypes similar to HH loss-of-function mutations [55]. Further, modulation of cAMP by Sonic HH (SHH) has also been demonstrated in vertebrate systems: retinal ganglion cell axons exposed to recombinant SHH reduce their intracellular pools of cytoplasmic cAMP [56], while frog melanophores exposed to SHH aggregate their melanosomes, a process favored by low concentrations of intracellular cAMP [57]. Taken together, these studies support that one mechanism by which SMO initiates HH signal transduction is to regulate cAMP production through the activation of Gαi family heterotrimeric G-proteins.

Gαi as a context-specific modulator of HH signaling. In vitro studies on vertebrate SMO support the ability of SMO to activate a subset of heterotrimeric G-proteins, with strongest effects on those of the Gαi family, for which SHH-induced GTP binding has been demonstrated [57–60]. Activation of Gαi in these systems fulfills the requirements of canonical HH pathway induction, as it can be inhibited by PTCH and/or small-molecule SMO inhibitors, and can be activated by SHH stimulation [57, 60]. SHH target gene induction in cultured fibroblasts is sensitive to pertussis toxin (PTX), a potent Gαi inhibitor, further supporting that Gαi is engaged by vertebrate SMO in response to ligand stimulation [60].

The above studies provide support for involvement of Gαi in HH signal transduction. However, studies performed in differing developmental or cellular contexts failed to identify a role for Gαi in the HH pathway [37, 61–63]. RNAi screens in cultured Drosophila cells did not implicate Gαi as a component of the HH signaling pathway [62, 63], while studies performed in cultured 10T1/2 cells failed to detect changes in intracellular cAMP following SHH stimulation [37]. The latter might be explained by findings that the bulk of HH signaling in vertebrate cells appears to occur in the primary cilium, a small sensory organelle that is present on most vertebrate cell types [64]. Because the volume of the primary cilium is negligible when compared to the body of the cell, localized changes in ciliary cAMP may be undetectable in whole cell lysates.

Conflicting results have also been obtained from in vivo studies examining the role of Gαi in HH signaling. Uncoupling of SMO from Gαi by expression of the PTX catalytic subunit in chick neural tube did not demonstrate compromised SHH-dependent neural cell type specification, suggesting that Gαi is not required for SHH patterning events in this developmental context [61].

Taken together, these seemingly conflicting results raise the possibility that Gαi is required only in certain cellular or tissue contexts during development. This suggestion is supported by the observation that while chick retinal ganglion axon explants are sensitive to SHH-mediated cAMP modulation and growth suppression, chick neural tube explants are not [56]. Signaling redundancy in specific tissues and/or at distinct developmental time points may also account for the apparent lack of Gαi involvement in HH signaling in some in vivo systems. This possibility is supported by work in both Drosophila and cultured vertebrate cells, which show multiple activating signals and feed-forward loops originating from SMO following ligand stimulation [26, 65, 66]. Further studies are required to ascertain if these additional SMO signals are dominant, or can compensate when Gαi is compromised.

A SMO-Dependent G-Protein Signaling Network

Based on a series of elegant biochemical reconstitution experiments, heterotrimeric G-proteins are proposed to function as ligand-gated switches [53]. Ligand stimulation triggers the GPCR to serve as a guanine nucleotide exchange factor (GEF) for its partner heterotrimeric G-protein, allowing the GDP-bound Gα subunit to bind

GTP and become activated. Upon activation, Gα was originally thought to dissociate from its Gβγ subunits and interact with its effector(s) through random collision along the plasma membrane. Initially the only known effector of G-proteins was adenylate cyclase (AC), the enzyme that converts ATP into cAMP. As such, the Gα subunit responsible for *s*timulating AC, and driving cAMP production was named Gαs, while the Gα subunit that inhibited AC activity to lower cAMP production was named Gαi [53]. Gα subunits were originally believed to be attenuated by their own intrinsic GTPase activity to return the Gα to its inactive GDP-bound form, thereby allowing it to reassociate with its partner Gβγ subunits.

The identification of additional G-proteins, the advent of molecular biology, and the subsequent investigation of G-protein function in vivo culminated to show that more regulators of the G-protein GTPase cycle were required than initially predicted by the in vitro model [67, 68]. These regulators consist of non-receptor GEFs that promote GDP release, novel inhibitors of GDP release (GDI), *r*egulators of *G*-protein *s*ignaling (RGS) that significantly increase the GTPase activity of the Gα subunit, and GRKs that function to desensitize the activated GPCR and/or propagate receptor signaling [69]. Numerous Gα and Gγ subunits have now been described and demonstrated to be capable of signaling themselves, regulating their own spectrum of specific effectors [69].

The discovery that SMO can signal as a *bona fide* GPCR has the potential to quickly expand the number of signaling proteins regulated by SMO, to include those that act as part of a SMO regulated G-protein signaling network (SGN). Thus, we anticipate that like other GPCRs SMO might regulate a large network of signaling proteins, including other G-proteins, and modifiers and effectors of these G-proteins. We discuss below evidence for such a network of regulators, and what some of the novel components of this SGN might be.

G-protein modulators. Although overexpression of activated Gαi in vivo resulted in strong HH gain of function phenotypes, we found that attenuation of Gαi function triggered only mild HH loss of function phenotypes [52]. These weak phenotypes might indicate that another *Gα* gene product, of which there are five in Drosophila, functions in a redundant manner with *Gαi* during Drosophila development. A likely candidate gene for this redundant function is *Gαo*, a member of the *Gαi* family that can function redundantly with Gαi in other systems [53]. Further, the mammalian homologue of the Drosophila Concertina α subunit, Gα12/13, has been implicated in SHH-mediated regulation of the small GTP-binding protein Rho [58]. Although our survey of three *Gα* gene products did not implicate Gαs in HH pathway regulation, a genome-wide screen in cultured Drosophila cells showed that knocking down *Gαs* could enhance HH signaling activity [63]. Further work is needed to determine if Gαs might represent a feedback mechanism that resets the basal level of cAMP after HH induces a decrease in cellular cAMP concentration via Gαi.

Another group of G-proteins we anticipate will serve as novel SMO effectors are the Gβγ subunits of its partner heterotrimeric G-protein(s). At a minimum, these proteins could function as negative regulators of Gαi by acting as GDIs [69]. The Gβγ subunits might also have the capacity to regulate their own novel set of effectors in the HH signaling cascade and/or modulate effectors that they share with

their Gα subunits. One such effector, AC, is stimulated by Gβγ proteins [69]. Therefore, like Gαs, Gβγ subunits activated in response to HH might be utilized to reverse Gαi-induced decreases in intracellular cAMP.

GPCR kinases and arrestins. As discussed above, phosphorylation of GPCRs on the cytoplasmic carboxyl-terminal tail is a common event following ligand stimulation. Phosphorylation regulates wide-ranging events including receptor subcellular localization, association with downstream pathway effectors, and commonly serves to recruit β-arrestin type adaptor proteins, which can propagate receptor signaling and/or drive receptor internalization and desensitization [70, 71]. Phosphorylation of activated receptors is driven primarily by the GRK family of kinases. GRK2 regulation of SMO follows a well-established GPCR paradigm: GRK2 phosphorylation triggers β-arrestin recruitment, which drives SMO to clathrin-coated pits where it undergoes activation-dependent internalization [72]. Interestingly, rather than desensitizing SMO to attenuate transduction of the HH signal, GRK-mediated phosphorylation and subsequent β-arrestin recruitment appear to regulate positive steps in HH signaling. Co-expression of GRK2 with SMO in cultured C3H10T1/2 cells enhances SMO-dependent activation of GLI, while GRK knockdown in cultured HEK293 cells attenuates SMO signaling in response to the SMO agonist SAG [50, 73]. An in vivo requirement for GRK2 in HH signaling was confirmed through studies analyzing zebrafish and mice lacking GRK2 function. In both cases, these animals demonstrated developmental phenotypes consistent with HH loss of function [73].

Drosophila GRK2 (dGRK2) has been demonstrated to be both a positive regulator of SMO signaling as well as a HH target gene, suggesting that it functions in a ligand-induced feed-forward loop [74]. As is the case in vertebrate systems, phosphorylation of SMO by dGRK2 results in both β-arrestin recruitment and SMO internalization in HH receiving cells [49]. dGRK2 appears to function only on activated SMO that has transduced a signal in response to ligand, as dGRK2 overexpression in wing discs triggers the removal of SMO from the plasma membrane without attenuating HH target gene induction. dGRK2-mediated internalization of SMO is independent of PTCH-driven removal of SMO from the plasma membrane in cells not receiving the HH signal, further suggesting that dGRK2 functions solely to regulate activated SMO [49]. This supports that SMO plasma membrane localization is regulated in a manner similar to numerous other GPCRs: in the absence of ligand stimulation, SMO undergoes a tonic endocytosis that is regulated by PTCH [75], while ligand stimulated SMO is internalized by the combined activity of dGRK and β-arrestin [49, 72]. As is the case with vertebrate SMO, assembly of the dGRK/β-arrestin complex on dSMO appears to be a positive regulatory event, despite it resulting in the eventual removal of SMO from the plasma membrane [49, 72]. Taken together, these studies highlight the importance of an evolutionarily conserved regulatory complex that assembles in response to ligand-induced GRK phosphorylation of SMO, of which β-arrestin appears to be paramount.

Protein kinase A. PKA was originally identified as a cAMP stimulated protein kinase, consisting of two regulatory subunits and two catalytic subunits [76].

The regulatory subunit inhibits the activity of the catalytic subunit, and this repression is released when the regulatory subunit binds to cAMP. PKA phosphorylates a broad spectrum of substrates, resulting in many diverse biological outputs. The various functions of PKA are thought to be spatially distinct, with PKA binding to its substrates and regulators on scaffolding proteins called A kinase anchoring proteins (AKAPs) [77]. AKAPs cluster relevant GPCRs, G-proteins, kinases, and other downstream effectors to discrete localizations within a cell [69, 77]. COS2 has been demonstrated to associate with SMO, downstream HH effectors, as well as with PKA and CK1 [20, 21, 24, 25]. As such, we hypothesized that COS2 might act as a nexus for HH signaling in a manner akin to that of AKAP proteins. We tested this hypothesis and noted that Gαi and COS2 do associate, and that this association was enhanced by HH [52]. It is, therefore, likely that COS2 acts as a scaffolding protein to recruit SMO, Gαi, and PKA, and likely, analogous to how AKAPs function, might also act to locally modulate the levels of cAMP.

PKA was initially shown to function as a negative regulator of HH signaling, phosphorylating CI in order to convert it to its repressor form [78, 79]. It was later identified as a positive regulator of HH signaling, through its ability to phosphorylate and stabilize SMO to result in SMO enriching at the plasma membrane in a highly active form [25, 46, 80]. Thus, PKA plays two seemingly opposite roles in HH signaling – in the absence of HH it acts to keep the HH pathway in its off-state and in the presence of HH functions to convert SMO into its active form. Consistent with the important role PKA plays in HH signaling, *Costal1 (COS1)* mutations, which enhance the phenotype of *COS2* mutations, were recently shown to encode mutations in both the regulatory and catalytic subunits of PKA [81].

It has been suggested that the role PKA plays in HH signaling is cAMP independent [78, 79]. This hypothesis was presented to explain the observation that a mutant mouse PKA catalytic subunit was able to rescue a *PKA* null mutation in Drosophila. These experiments assumed that the mutant PKA catalytic subunit would be unable to associate with the regulatory subunit of Drosophila PKA. However, recent demonstrations of SMO coupling to Gαi and regulating cAMP levels suggests that a cAMP independent role of PKA in HH signaling may not be correct [52, 60, 65]. Moreover, the identification of a *COS1* mutation encoding a PKA regulatory subunit, which modulates HH signaling, is consistent with a cAMP-dependent activation of PKA [81].

Small-Molecule Modulators of SMO

Mice engineered to lack SHH die shortly after birth and exhibit a wide range of developmental defects, including cyclopia [82]. A similar phenotype was observed in offspring of livestock that ingested the corn lily *Veratrum californica*. Two groups recognized the similarities between these phenotypes and tested the hypothesis that a chemical derived from this plant, cyclopamine, functioned as an inhibitor of HH signaling [83, 84]. Cyclopamine turned out to be a potent inhibitor

of HH signaling, in vitro and in vivo, and was subsequently shown to bind directly to the heptahelical bundle of SMO to functionally antagonize its signaling capability [85]. Consistent with SMO facilitating a rate-limiting step in HH pathway activation, numerous small-molecule screens for novel HH inhibitors have identified distinct SMO antagonists [9]. Many of these SMO inhibitors act in a competitive manner with cyclopamine for binding to SMO, supporting that they bind the 7TM segments. However, some of these SMO modulators bind to SMO in a non-competitive manner and/or activate SMO, suggesting that SMO may have a number of different small-molecule binding sites, as is the case with numerous GPCRs [43].

One basic tenet of pharmacology is that drugs themselves do not possess intrinsic biological properties, but rather can only act to modify existing biological processes [86]. Thus, the identification of small-molecule modulators of SMO implied the existence of endogenous SMO modulators. Furthermore, it has been known for a number of years that the HH receptor PTCH has significant homology with a family of physiological pumps in bacteria, leading to the speculation that PTCH functions to regulate the concentration of such an endogenous SMO modulator [13, 87]. Consistent with this homology, a recent study using a mixed-cell culturing system provided evidence for a lipophilic molecule being pumped into the culture medium in a PTCH-dependent manner [88]. This molecule was identified as the oxysterol, pro-vitamin D3, which was demonstrated to bind SMO in manner similar to that of cyclopamine. Purified pro-vitamin D3 inhibited HH activity, both in vitro and in vivo, with a potency similar to that of cyclopamine [88]. This was one of the first identifications of an endogenous SMO modulator, in this case an antagonist. Two other groups subsequently identified oxysterol molecules that functioned as HH activators [89, 90], suggesting that, like numerous GPCRs, SMO activity is controlled by endogenous small-molecule ligands.

Future Directions

As the critical role(s) that HH signaling plays in tumor growth and progression continues to emerge, and the clinical use of SMO antagonists increases, the impact of on-target adverse effects is likely to become evident. For example, one SMO antagonist was recently demonstrated to have significant efficacy against medulloblastoma [91], but to induce growth defects when administered to young mice [92]. This study was performed to reveal potential problems that may be encountered by inhibiting a developmentally relevant signaling pathway in a pediatric population, the most common class of patients presenting with medulloblastoma [93]. Long bones of the limbs of animals exposed to this compound during early development were found to be significantly shortened, an effect likely due to specific inhibition of Indian HH-regulated bone growth [92]. This observation underscores the importance of delineating all the signaling events immediately downstream of SMO, as one could anticipate that various classes of small-molecule SMO inhibitors

might affect distinct signaling arms. Moreover, classes of SMO antagonists that only inhibit a distinct subset of SMO effectors, such as the SGN effectors, might be used clinically for specific classes of cancer patients. Ideally, such compounds would inhibit the effectors relevant to tumor growth while having minimal impact on effectors more relevant to the role HH plays in tissue homeostasis. Such drugs would be particularly useful to medulloblastoma patients, whose ability to take at least a subset of the SMO antagonist currently in clinical trials would be severely compromised by on-target developmental defects.

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