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Review

The cell biology of Smo signalling and its relationships with GPCRs

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Abstract

The Smoothened (Smo) signalling pathway participates in many developmental processes, contributing to the regulation of gene expression by controlling the activity of transcription factors belonging to the Gli family. The key elements of the pathway were identified by means of genetic screens carried out in *Drosophila*, and subsequent analysis in other model organisms revealed a high degree of conservation in both the proteins involved and in their molecular interactions. Recent analysis of the pathway, using a combination of biochemical and cell biological approaches, is uncovering the intricacies of Smo signalling, placing its elements in particular cellular compartments and qualifying the molecular processes involved. These include the synthesis, secretion and diffusion of the ligand, the activation of the receptor and the modifications in the activity of nuclear effectors. In this review we discuss recent advances in understanding biochemical and cellular aspects of Smo signalling, with particular focus in the similarities in the mechanism of signal transduction between Smo and other transmembrane proteins belonging to the *G-P*rotein *c*oupled *r*eceptors superfamily (GPCR).

Keywords: Hh signalling; Smoothened; GPCR; GRKs; β-arrestin

Contents

1. Biological roles of Hh/Smo signalling during development and disease

The components of the Smo signalling pathway were identified by their similar requirements during embryonic segmentation and appendage development in *Drosophila* [1,2]. Since then, the number and variety of developmental

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processes requiring Smo function has increased enormously, both in *Drosophila* and vertebrates [3–7]. Most often Smo functions during embryonic development, in processes such as digit patterning in the chick limb bud and left–right asymmetry of vertebrate embryos. In addition, Smo function is also fundamental for the maintenance of tissue homeostasis in adults, and deregulated Smo signalling is implicated in tumorogenesis. Most of the elements of the Smo pathway have been identified through genetic screenings carried out in *Drosophila* and, more recently, by the use of systematic searches in cell culture experiments [8,9]. Simultaneously, the use of biochemical approaches has unravelled many of the complexities of the pathway, allowing a partial understanding of the molecular interactions that translate the binding of the ligand Hedgehog (Hh) to its receptor Patched (Ptc) into the functional state of the transcription factor Cubitus interruptus (Ci), and hence to the regulation of gene expression in response to Hh [10].

Most of the elements of the pathway are conserved from flies to vertebrates, the main difference being the number of related genes present in different organisms [7]. Thus, there is only one Hh gene in flies and three Hh-related genes in vertebrates, *sonic-hedgehog*, *desert-hedgehog* and *indian-hedghog*, which are expressed in different tissues and stages of development [11]. Similarly, there is only one gene encoding the receptor Ptc in *Drosophila* and two Ptc proteins (PTC1 and PTC2) in vertebrates (Table 1). The main effectors of Smo signalling, the Gli proteins related to *Drosophila* Ci, are also conserved in vertebrates, where at least three Gli proteins are found (GLI1, GLI2 and GLI3). In the mouse, these proteins mediate all Hh-dependent patterning in the neural tube [12,13]. Like Ci, all three vertebrate Gli proteins have five highly conserved zinc finger DNA binding domains and C-terminal activation domains, with Gli2 and Gli3 also having N-terminal repressor domains [12,14]. Truncations, point mutations, and frame shifts changes in human Gli3 lead to a variety of diseases including Greig cephalopolysyndactyly syndrome (GCPS) [15–17], Pallister–Hall syndrome (PHS) [18], and postaxial polydactyly type A (PAP-A) [19].

Despite the conservation in the components of Smo pathway during evolution, some caveats remain as to the preservation of

Table 1
Elements of the Smo signalling pathway in flies and vertebrates

	Drosophila	Vertebrates	Main features
Ligands	Hedgehog (Hh)	Sonic Hedgehog (SHH)	Secreted signalling protein
		Desert Hedgehog (DHH)	
		Indian Hedgehog (IHH)	
Processing and transport of ligand	Rasp (Rasp)	Skinny Hh (SKN)	Acyl transferase
	Dispatched (Disp)	Dispatched A (DISPA)	Sterol-sensing domain protein
	Dally and Dally-like (Dlp)	?	Glypicans
	Tout-velu (Ttv)	Exotosin (EXT)	HSPGs biosynthesis
	Sister of tout-velu (Sotv)		
	Brother of tout-velu (Botv)		
	Shifted (Shf)		Secreted protein
Receptors	Patched (Ptc)	Patched 1 (PTC1)	12-TM protein
		Patched 2 (PTC2)	
Co-receptors	Interference Hedgehog (Ihog)	CDO	Fibronectin type III and Ig domains
	Brother of Ihog (Boi)	BOC	trans-membrane protein
		Megalin	LDL superfamily protein
Transducter	Smoothened (Smo)	Smoothened (SMO)	7-TM protein
Other Hh-binding factors	Pxb	Hh-interacting Protein (HIP)	1-TM protein
Cytoplasmic regulators	Costal2 (Cos)	KIF7	Kinesin family member
		KIF3a	
		IFT88	Intraflagellar transport protein
		IFT172	
	Fused (Fu)	Fused (FU)	S/T kinase
	Suppressor of Fused (Su(Fu))	Suppressor of Fused (SUFU)	Negative regulator
		Missing in metastasis (MIM)	Positive regulator
		Iguana	Positive regulator
		FKBP8	Negative regulator
		SIL	Negative regulator
		Rab23	Negative regulator
Smo and/or Ci regulators	PKA	PKA	S/T Kinase
	Casein Kinase I (CKI)	CKI	S/T Kinase
	Glycogen synthase kinase 3 (GSK3)	GSK3	S/T Kinase
	?	β-arrestin-2	GPCR binding protein
	Slimb	{beta}TrCP	F-box/WD40
	Gprk2	GRK2	GPCR kinase
Transcription factors	Cubitus interruptus (Ci)	GLI1	Zn-finger transcription factor
		GLI2	
		GLI3	

References are indicated in the text, and can be found in the following reviews: [3–7,10,11,20,22,42–45,90] and for Gprk2 in flies [132].

the key molecular mechanisms operating in different organisms [20]. Particularly perplexing has been the difficulties in relating the mechanisms of Smo signal transduction to that of other transmembrane proteins with a similar molecular structure, the G-protein coupled receptor (GPCR) superfamily of seven transmembrane domains proteins.

In this review we will summarize recent advances in the identification of the molecular mechanisms involved in Smo signal transduction, emphasizing the relationships between Smo and other GPCRs, and the participation in Smo signalling of several components of classic GPCR pathways, such as heterotrimeric G-proteins, β-arrestins and G-protein coupled receptor kinases (GRKs).

2. The elements of the Smo signalling pathway and the mechanisms of Smo signal transduction

The core members of the Smo signalling pathway include the ligand (Hedgehog, Hh), the receptor (Patched, Ptc), several transducers such as Smoothened (Smo) and the cytoplasmic complex formed by the kinesin-like protein Costal-2 (Cos2), the serine/threonine kinase Fused (Fu), the novel protein Suppressor of fused [Su(fu)], and the transcription factor Cubitus interruptus (Ci) [7,21]. In addition, several proteins participate in the correct secretion and movement of active Hh, in the phosphorylation of Ci and Cos2, and in the processing and degradation of Ci [21,22]. In this manner, the Smo pathway can be sub-divided into several "molecular modules" participating in (i) the formation, secretion and movement of active Hh, (ii) the intracellular trafficking, sorting and recycling of Hh/Ptc/Smo complexes, (iii) the relay complex formed by Cos2, Fu, Ci and in some instances Su(fu), and (iv) the enzymatic machinery that by phosphorylation (GSK3, CKI and PKA) or proteolysis (Slimb) modulates the activities of Smo, Cos2, Fu and Ci (Table 1 and Fig. 1).

The final outcome of Smo signalling consists in modifications to the stability, phosphorylation and subcellular localisation of the transcription factor Ci/Gli, in a process

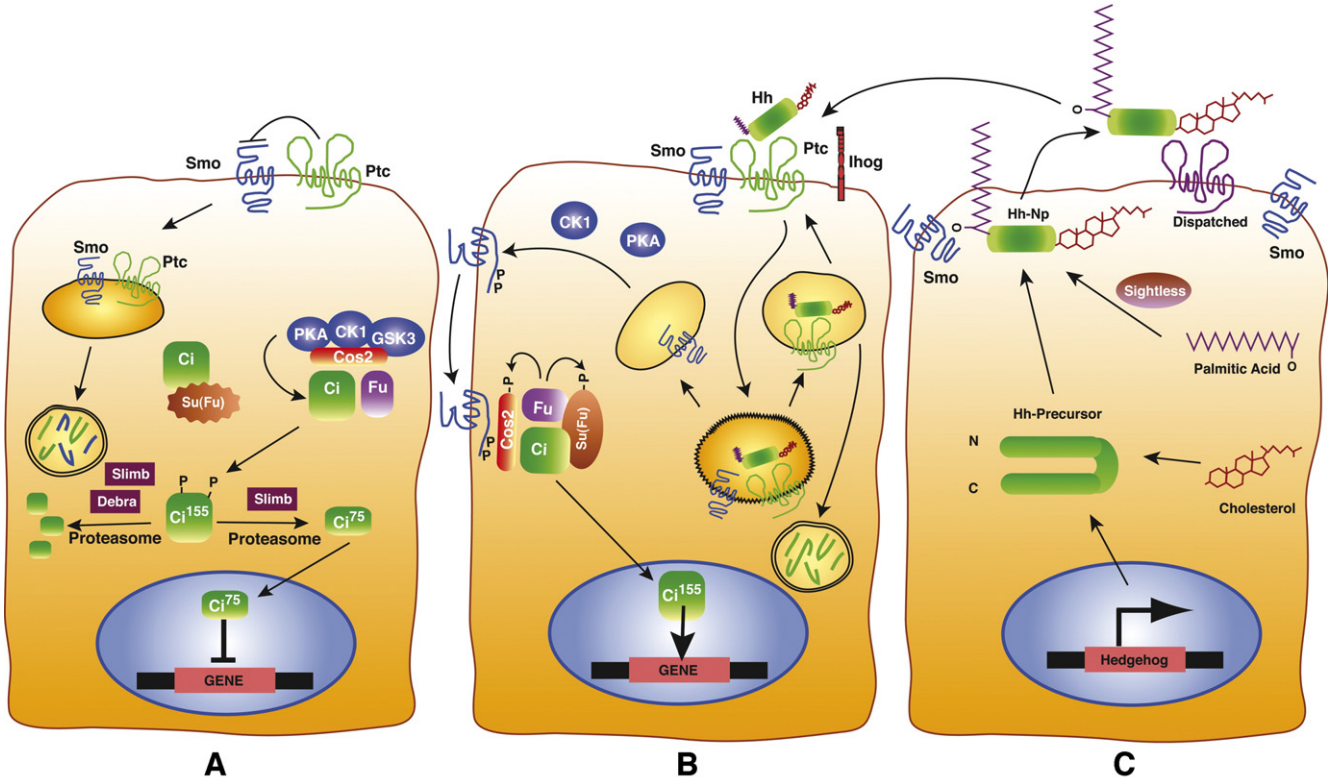

Fig. 1. The Hedgehog signalling network. (i) Right panel, signalling cell (C): Hh auto-processing generates an N-terminal domain with a C-terminal cholesterol motif. Cholesterol-modified Hh can be palmitoylated at its N-terminal, a reaction that is catalysed by the acyl-transferase Sightless. Lipid-modified Hh can be further processed at the plasma membrane, where it multimerizes. Multimeric Hh secretion requires the function of Disp, making Hh available for long-range signalling. (ii) Left panel (A), non-responding cells: In the absence of Hh, Ptc inhibits Smo activity. Ptc acts by directing Smo to endocytic vesicles, where it is targeted for degradation. Ci, the downstream transcription factor of the Hh pathway, is silenced by protein-interactions that take place in two different complexes: most of the Ci protein is part of a microtubule-bound multimeric complex involving Cos2, Fu and different kinases (as PKA, CKI and GSK3β) that phosphorylate Ci, promoting its ubiquitination by Slimb to generate the truncated transcriptional repressor (Ci_{75}), and its degradation by the proteasome in a process dependent on Debra activity. Ci_{75} binds target genes and blocks their transcription. The other Ci pool in the cell appears as complexes between full-length Ci_{155} and Su(fu), resulting in the cytoplasmic retention of Ci. (iii) Central panel (B), Hh-responding cells. When Hh binds to Ptc, releases the repression that Ptc exerts on Smo, allowing the activation of downstream components. The putative co-receptors Megalin, Ihog and Boi are represented to the left of Ptc. Smo is phosphorylated and signals to the Cos2/Fu/Ci complex, causing hyperphosphorylation of Cos2 and Fu and their subsequent release from the microtubules. Fu itself participates in Cos2 phosphorylation. Stabilized Ci_{155} can then travel to the nucleus and function as a transcriptional activator. Smo activation also allows Su(fu) phosphorylation by Fu, promoting the release of full length Ci_{155} from Su(fu) and its nuclear localisation.

regulated by post-transcriptional modifications to Ci. The transition of Ci from the "inactive" state (repressor form), defined by the presence in the nucleus of the processed form Ci-75 and the cytoplasmic accumulation of Ci (Fig. 1A), to the "active" state (activator form), characterised by the presence in the nucleus of full-length Ci-155 and the absence of Ci-75, is triggered by interaction between the receptor Ptc and the ligands of the Hedgehog (Hh) family (Fig. 1B). These ligands are secreted proteins synthesized as precursors containing a signal peptide, and possess auto-proteolytic activity [7] (Fig. 1C). In addition, after its synthesis and before their apical secretion to the extracellular space, Hh proteins are modified by the addition of lipid molecules [22]. Thus, Hh is cleaved within the secretory pathway in an autoproteolytic reaction and a cholesterol molecule is incorporated in its C terminus of the N-terminal fragment, giving rise to the Hh-Np active form [22]. A second lipid modification is the addition of palmitic acid to the N terminus of Hh-Np, in a reaction catalysed by the acyl transferase Rasp [23,24]. The analysis of mutated forms of Hh in flies suggests that absence of cholesterol-modification in Hh-Np affects its secretion, multimerization and long-range signalling activity [25,26], as was previously recognized in mammalian systems [27–29]. Similarly, the lack of acylation reduces dramatically Hh signalling, both in *Drosophila* and vertebrates.

Once Hh is modified by lipids, it must be secreted to the extracellular space. It has been shown that the protein encoded by the gene *dispatched* (*disp*) is required to liberate lipid-modified Hh from Hh producing-cells [27,30]. Thus, *disp* mutant cells retain lipid-modified Hh, whereas unmodified Hh-N is secreted independently of Disp function. Disp contains, like Ptc, twelve transmembrane domains and a sterol-sensing domain (SSD), which has been involved in cholesterol homeostasis and cholesterol-linked signalling [22].

Hh interacts with the twelve-pass transmembrane receptor Patched (Ptc) of neighbouring cells, and the range of Hh diffusion and effectiveness varies in different developmental systems [31–35]. Extracellular matrix proteins such as heparan sulfate proteoglycans (HSPGs) participate in the movements of Hh once it has been secreted, and might contribute to the presentation of Hh to its receptor Ptc. Thus, the *Drosophila* EXT family of proteins encoded by the genes *tout velu (ttv), brother of tout velu (botv)* and *sister of tout velu (sotv)* are essential for the synthesis of HSPGs and are required for the diffusion of lipid-modified Hh [36,37]. The proteins Dally and Dally-like (Dlp) are glypicans forming the HSPGs core, and are also required for Hh diffusion [38,39]. Another protein recently implicated in the spreading of Hh is a secreted protein encoded by the gene *shifted (shf)*, that is required for normal accumulation of Hh in the extracellular matrix of Hh-producing cells and for lipid-modified Hh diffusion [40,41]. Shf is the ortholog of the human Wnt inhibitory factor (WIF), a secreted antagonist of the Wingless pathway. However, Shf has not effect on Wingless activity in *Drosophila*. Finally Megalin, a multi-domain transmembrane protein [42] and a novel and evolutionarily conserved family of transmembrane proteins containing Ig domains and two extracellular fibronectin type III domains, Ihog and Boi in flies and CDO and BOC in mammalians, bind to Hh, and might function as co-receptors in Hh reception [43–45].

The interaction between Hh and Ptc releases the repression that Ptc exerts on Smo, allowing the activation of downstream components. The mechanism of Smo activation includes the internalisation of Hh/Ptc/Smo complexes from the cell membrane, the sorting of Hh/Ptc from Smo in endocytic vesicles, the phosphorylation of Smo by several Ser/Thr kinases including CKI and PKA and the accumulation of phosphorylated Smo in basolateral cell membranes [10,21] (Fig. 1B). The intracellular trafficking of Ptc and Smo through the late endosome–lysosome system is thought to be critical for the regulation of Smo activity by Ptc [46], although the analysis of a *Drosophila* mutant Ptc protein defective in Hh internalisation suggests that Hh internalisation and Smo signalling can be uncoupled [47]. It has been suggested that Ptc, in the absence of Hh and acting in a similar manner to other proteins containing Sterol-sensing domains, directs Smo to a cellular compartment where it is targeted for degradation [48–50]. The exposure of cells expressing Ptc and Smo to Shh leads to the co-internalisation of Ptc, Smo and Shh, and to the degradation of Ptc and Shh in lysosomes [51]. Thus, after entering late endosomes together, Smo is segregated from the Ptc–Shh complex, and returns to the cell surface, where it is now ready to signal [51]. Interestingly, constitutively active mutant Smo proteins that are not inhibited by Ptc (isolated from sporadic basal cell carcinomas), fail to co-localise and co-internalise with Ptc, allowing the activation of the pathway independently of Hh [50].

Once activated, the interactions between Smo and the cytoplasmic complex formed by Cos2, Fu, Su(fu) and Ci changes in a way that the processing and degradation of Ci stops, allowing the accumulation of full-length Ci and its entrance into the nucleus, where it can bind DNA and regulate the expression of its target genes [7,21]. Hh does not alter Smo–Cos2 affinity, but it does increase the total amount of Smo–Cos2 complex and alter its location. Thus, in the absence of Hh, or when Ptc is present in excess, Smo is localised in cytoplasmic vesicles, and Cos2 scaffolds multiple kinases, increasing the accessibility of Ci to these kinases and facilitating extensive phosphorylation of Ci by PKA, CKI and GSK3, targeting Ci for proteolytic processing mediated by the ubiquitin ligase Slimb ([52]; Fig. 1A). Dbr enhances poly-ubiquitination of Ci, promoting its degradation [53] (Fig. 1A). In this manner Ci is either degraded by the proteosome, or converted into a 75 Kd form (Ci-75) that enters the nucleus and represses the transcription of target genes (Fig. 1A). In the presence of Hh, or when Ptc is absent, Cos2 complexes are recruited to the cell surface via Smo, and its components Cos2, Fu and Su(fu) become phosphorylated by the Fu kinase, among others [54] (Fig. 1B). This leads to the disassembly of Cos2-Ci-kinase complexes. As a consequence, Ci phosphorylation is compromised and Ci processing does not take place, allowing the accumulation of the full-length form of Ci (Ci-155) in the cytoplasm and its entrance into the nucleus [55–60]. A significant fraction of Su(fu) associates with Ci, whereas a smaller fraction may also associate with Cos2 and Fu, in at least two distinct complexes, one comprising Ci/Su(fu) and

another Cos2/Fu/Ci/Su(fu) [61]. In this scenario, Cos2 plays a key role in the transition from Ci-75 to Ci-155 through interactions with Smo. Early studies of Cos2 identified a negative role for Cos2 in pathway regulation, because *cos2* mutations caused inappropriate activation of signalling [62,63]. This antagonism is due to the requirement of Cos2 for both cytoplasmic retention of Ci and its proteolytic processing to produce Ci-75 [64–66]. More recently, additional requirements for Cos2 have been identified, including the stabilization of Fu and the accumulation of activated Smo. The transition of Cos2 from a pathway suppressor to activator requires adequate levels of activated Smo, and it has been proposed that Cos2 acts as a scaffold and sensor that, by transducing pathway activation from Smo to Ci, actively participates in the transition of Ci from the inactive to the active state [60].

Although some details of the molecular interactions that participate in Ci modifications are still lacking, it is clear that Hh binding to Ptc triggers modifications in Smo that alter its stability and subcellular localisation, shifting the Cos2/Fu/Su (fu) complex from a state that promotes the formation of Ci-75 to another state in which the accumulation of Ci-155 is favoured, leading to changes in the expression of genes containing Ci-binding sequences (Fig. 1B). In what follows, we will consider the similarities between Smo and other proteins of the GPCR superfamily, and discuss the possibility of shared mechanisms regulating Smo and other GPCRs.

3. Smoothened as a member of the GPCR family

The super-family of G-protein coupled receptors (GPCRs) is one of the largest families of proteins in vertebrates, with circa 1000 genes encoding for such receptors identified in the human genome [67–69]. GPCRs participate in a variety of important physiological functions and are targets for many drugs. The ligands that activate GPCRs are molecularly diverse, and include ions, organic odorants, amines, peptides, proteins, lipids, and nucleotides. In addition many GPCRs have also been denominated ‘orphan receptors’, because their natural ligands have escaped identification so far [13]. The main structural characteristic of the family is the seven membrane-spanning α-helices (TMHs), which span the membrane in an anti-clockwise manner and are formed by 25–35 consecutive amino acid residues with some degree of hydrophobicity. These helices are connected by extracellular and intracellular hydrophilic loops, with an extracellular N-terminus and a cytoplasmic C-terminus. Most GPCRs, as its name indicates, mediate their intracellular actions through pathways involving interaction and activation of heterotrimeric G-proteins, although G-protein-independent signalling mechanisms have also been reported for some GPCRs. In addition several non-GPCR receptors use hetero-trimeric G-proteins and other cytoplasmic proteins related to GPCR activity as transducers in their signalling. For these reasons, some authors used alternative names for this protein superfamily such as “7-transmembrane receptors”, “serpentine-like receptors” or “heptahelical receptors” [70–72].

The Hh/Smo signalling pathway differs in several aspects from the canonical mechanisms of 7-transmembre receptors activation. Smo lacks the ability to directly interact with the secreted ligand, and uses Ptc as the receptor for Hh. In this case, receptor activation involves the release of Smo from Ptc inhibition, triggered by Hh/Ptc interactions, and the intracellular sorting of activated Smo from Hh/Ptc complexes [51]. However, several features of the Smo pathway are related to those of the GPCR superfamily and are discussed below, including structure and membrane localisation, the possible implication of dimerization in its mode of activation, and the existence of post-transcriptional modifications and internalisation mechanisms from the cell membrane in the presence of ligand.

3.1. Smoothened receptor structure and sequence similarity

All published classifications of GPCRs superfamily members based on sequence or structural features include the Frizzled and Smo receptors as related to the GPCR secretin family [73–76]. Both Frizzled and Smo receptors display low but significant sequence similarity to other GPRCs of the secretin family particularly in their transmembrane domains [77,78]. The alignment of representative members of the Fz, secretin and Smo receptors is presented in Fig. 2. The Smo protein has a long extra-cellular N-terminal domain about 250 amino acids long, and presents a conserved cystein-rich domain (CRD). The cysteines in this domain are predicted to be essential for acquiring the correct tertiary structure. In the Smo-related Fz receptor, the CRD binds its ligand, the Wnt protein [79]. Although the CDR of Smo binds neither Wnt nor Hh protein [80], its evolutionary conservation suggests that it may have an important role in Smo regulation, which has yet to be determined. Interestingly, the missense mutation Cys90Ser, localised in the CRD of the extra-cellular domain of *Drosophila* Smo, is associated with a weak Smo loss of function phenotype [50], suggesting that the occurrence of correct disulphide bridges within the CRD are needed for Smo activity. The CRD domain may also be required for the interaction between Smo and Ptc. In this sense, some reports have shown a weak interaction between PTC and the CRD of Smo, and both proteins can be co-inmunoprecipitated when over-expressed [81]. Direct interactions between Smo and Ptc, however, have not been detected under physiological conditions [82], probably reflecting that this interaction is weak and transient. In addition, Ptc and Smo show minimal co-localisation within the cell, and their interaction is not stechiometric, as Ptc is able to inhibit excess of Smo [46,50,82,83]. Other motifs of homology with the Secretin family shared by Smo and Fz are located between the transmembrane regions of these proteins.

3.2. Seven-transmembrane receptors dimerization

GPCRs have traditionally been thought to act as monomers, but now is widely accepted that GPCRs may exist as either homodimers or even higher-order oligomers.

They are also capable of interacting with distantly related receptor subtypes to form hetero-oligomers and, for many

Fig. 2. Alignment of Smo receptors from representative vertebrate and invertebrate orthologs with other members of the secretin/frizzled GPCR family. The putative regions comprising the transmembrane domains are noted in the alignment, as well as the cysteine-rich domain (CRD). The phosphoserine/threonine residues identified in endogenous *Drosophila* Smo after Hh stimulation [99] are indicated by asterisks. Those phospho-residues that could be phosphorylated by GRKs family members and are present in both *Drosophila* and human Smo protein are boxed. Amino acid sequences accession numbers are: Human-Smoothened (gi52032099), *Drosophila*-Smoothened (gi27919934), human-Frizzled-2 (gi736679) and Human-secretin receptor (gi38609719).

members of this receptor family, the dimer may represent the basic signalling unit during their normal intracellular trafficking and function (reviewed in [84–86]. Certain GPCRs seem to have a strict requirement for heterodimerization to attain proper surface expression and functional activity such as the $GABA_B$ receptor [87]. Heterodimerization can also lead to marked changes in receptor pharmacology, signalling, and/or internalisation.

There is not direct data demonstrating Smo dimerization, but several observations concerning Smo and the related Frizzled (Fz) receptors suggest that they also operate as dimers or multimers. Thus, ectopic expression of Smo variants with C-terminal deletions cause dominant-negative effects, and increased expression of full-length Smo results in ectopic activation of the pathway [88]. More recently, it has been hypothesized that the highest level of Smo activity might involve Smo dimerization [89]. Smo dimers would interact with Cos2–Fu–Ci–Su(fu) complexes, activating Su(fu) phosphorylation by Fu, and releasing the inhibition on Ci.

3.3. Coupling of Smo to heterotrimeric G-proteins

There are emerging evidences pointing to a role of heterotrimeric G-proteins as Smo effectors in vertebrates. First, Smo is constitutively active in the absence of Ptc, and the third intracellular loop and the seventh transmembrane region of Smo are required for this function [90]. Both domains are very important in other GPCRs for coupling with heterotrimeric G-proteins. Assays made in frog melonophores expressing human Smo showed a phenotype of persistent pigment aggregation, and this effect can be blocked by pertussis toxin [91], a treatment known to ADP-ribosylate a cysteine residue near the carboxyl terminus of Gαi or Gαo, disrupting receptor-G protein coupling [92]. These results suggest that Smo can signal through heterotrimeric G-proteins. In fact, it has been recently shown that Smo activates all members of the Gαi family, and this effect is an essential component of Gli activation in mammalian fibroblasts [93]. In the same line, injection of Zebrafish embryos with RNA encoding pertussis toxin and

therefore blocking Gαi-mediated actions suggests a possible role for it in Smo signalling [94]. Further support for a role of heterotrimeric G proteins in Smo signal transduction is the reported transcriptional stimulation of a Gli1 promoter in HEK293 and N2a cells over-expressing constitutively active Gα [95]. These data reveal that the $G\alpha_{12/13}$/RhoA/RhoA kinase pathway participates in Smo signalling. Indirect support for the involvement of RhoA in Smo signalling is provided by the analysis of *moesin* mutants in *Drosophila*. *Moesin*, encoding the only member in *Drosophila* of the Radixin/Ezrin/Moesin family, is required for correct epithelial development and its loss-of-function alleles cause inappropriate Smo signalling. These effects can be reverted by reducing the dose of the RhoA GTPase [96].

Finally, several mutations in Smo that promote some phenotypic features coincident with those of canonical GPCRs provide additional support for Smo/G-protein coupling. A somatic missense mutation in human Smo, caused by an amino acid substitution in the seventh transmembrane domain (Trp535Leu), a site predicted to disrupt G-protein coupling [97], cause Smo activation. Similarly, *a smo* loss of function phenotype can be generated by the K474C missense mutation in Smo, which changes an Arg residue localised in the third intracellular loop, close to the boundary with transmembrane TM6. This mutation is similar to other found to abolish G-protein coupling in other GPCRs [50], indicating an important role for these domains in Smo function.

However other data suggest that G-proteins are not always required for Smo signalling. In vitro studies using a primary fish myoblast assay system reveal that the response to Shh is insensitive to pertussis toxin treatment. Moreover, there is to date no report of mutations in a *Drosophila* G-protein giving a phenotype reminiscent of Smo signalling disruption [94], and studies using RNA interference to inhibit a full spectrum of G-protein subunits in *Drosophila* tissue culture cells failed to compromise Hh signalling [8]. The observed coupling of Smo to Gαi-proteins occurring in particular cell types could be part of a mechanism used to reduce PKA activity by decreasing the level of cAMP, preventing Gli phosphorylation and amplifying Smo signalling. Additional inputs from Smo to Gli contributing to pathway activation depend on the integrity of the C-terminal domain of Smo and might be mediated by Smo phosphorylation by GRK2 (see below). In addition, the effects on Smo signalling caused by G protein activity modifications could be indirect, based on changes on cytoskeleton architecture or vesicular trafficking mediated by these proteins.

4. Smo phosphorylation

Drosophila Smo activation is accompanied by its phosphorylation, accumulation and translocation to the plasma membrane [46,50,59,98–100]. When Hh is present, the Smo C-terminal tail becomes hyper-phosphorylated by protein kinase A (PKA) and Casein kinase I (CKI) (see Fig. 1B). Loss of these PKA or CK1 sites, using un-phosphorylatable (Ser/Thr → A) forms of Smo renders Smo inactive, whereas changes of multiple serine residues to acidic residues (Ser/Thr → D mutants, phosphorylation-mimicking forms) activate signalling even in the absence of Hh [98–100]. Zhang and collaborators [99] identified 26 serine and threonine residues within the *Drosophila* Smo C-terminal cytoplasmic tail (marked with asterisks in Fig. 2) that become phosphorylated upon Hh stimulation in S2 *Drosophila* cell lines. There are some identified phosphoserine and phosphothreonine residues that lye in consensus kinase recognition motifs for PKA, CKI and GSK3 among others, but there are also phosphoresidues that do not belong to consensus motifs for any of these kinases. Most of these Smo phosphorylated residues are not conserved in vertebrates, suggesting that activation of vertebrate Smo is not triggered by PKA and CKI phosphorylation. Thus, other kinases might play a role in vertebrate Smo activation and, in fact, in vitro approaches using mammalian cell culture models revealed that upon Hh stimulation, Smo protein is phosphorylated by the G protein-coupled receptor kinase GRK2 [101]. As it happens for other GPCRs, phosphorylation of Smo by GRK2 promotes binding of β-arrestin-2 and Smo internalisation (see below). This observation suggests that different sets of kinases might be implicated in regulating Smo activity in vertebrates and *Drosophila*.

Interestingly, a *Drosophila* Smo mutant with acidic residues in place of PKA and CK1-targeted serine residues can be further activated by Hh treatment, indicating that additional changes in Smo can be induced by Hh, conceivably involving additional phosphorylation sites [98–100]. Some of those identified residues are in the vicinity of acidic residues that fit a "GRKs phosphorylation consensus-sequence" observed in both plasma membrane receptors and cytoplasmic substrates of GRK2 [102–106]. Some of these phosphorylated residues are present both in *Drosophila* and in vertebrate Smo C-terminal cytoplasmatic tails (Ser 633, 634, 680, 746; Fig. 2 mark as square) [99], suggesting that GRK phosphorylation of Smo might be conserved in *Drosophila* and vertebrates.

5. Implication of the canonical GPRC signalling components GRK and β-arrestin in Smo signalling

In the last 2 years a new connection has been established between Smo signalling and some proteins involved in canonical GPCR regulation and signalling: the cytosolic β-arrestin proteins and the G protein-coupled receptor kinase GRK [96,101,107]. Canonical GPCR stimulation promotes the activation of heterotrimeric G proteins, and triggers receptor phosphorylation in Ser/Thr residues by GRKs. β-arrestins then bind to the phosphorylated receptor, leading to (i) impaired communication of the receptor with the G protein even in the presence of stimulus, (ii) induction of clathrin-mediated receptor internalisation, mediating its coupling to the endocytic machinery. Internalised receptors can then be dephosphorylated by the action of specific phosphatases in low pH endosomes and recycled to the plasma membrane (resensitization), or be degraded in lysosomes [101,108–110]. In addition, binding of β-arrestins can recruit additional proteins to the membrane, acting as signal transducers through the formation of scaffolding complexes with accessory effector molecules such as Src, Raf, ERK1/2, JNK3, MAPK4 and p38 [111–116].

The implication of β-arrestin and GRKs in modulating protein activity and localisation is not exclusive to GPCRs. For example, the non-visual arrestin of *Drosophila* encoded by *Kurtz* interacts with the putative E3 ubiquitin ligase Deltex, and promotes ubiquitination and degradation of the Notch receptor [117]. Arrestin also constitutes an essential component in the signalling pathways initiated by the insulin-like growth factor 1 (IGF-1) by promoting ubiquitination of the receptor by the E3 ubiquitin ligase oncoprotein MDM2 [116]. β-arrestin2 can also bind to the single transmembrane-spanning type II TGFβ receptor, and its binding is triggered by receptor auto-phosphorylation [101,105].

In addition to arrestin, GRK proteins have also been involved in the modulation of both non-GPCR receptors and other non-receptor proteins. Thus, GRKs are able to phosphorylate non-receptor substrates such as tubulin, synucleins, phosducin, ribosomal protein P2, the inhibitory γ-subunit of the type 6 retinal cGMP phosphoriesterase, a subunit of the epithelial Na^+-channel and ezrin [68,102,105,106,118–121]. GRKs also participate in several pathways and modulate cellular functions in a phosphorylation-independent manner. These actions of GRKs are due to their ability to interact with a variety of proteins involved in signalling and trafficking, such as Gαq, Gβγ, caveolin, or GIT [122–124]. Finally, arrestins and GRKs also participate in several signalling platforms regulating other receptor families, such as tyrosine kinase receptors [125,126].

The secretin receptor, belonging to the same family of GPCR as Smo and Fz, can be phosphorylated by GRK2 and GRK5 upon binding of agonists, promoting its desensitization [127]. Secretin is not the only member of this family that becomes phosphorylated by GRKs upon agonist stimulation. Smo over-expression in mammalian cultured cells recruits β-arrestin2 to the plasma membrane [101]. A regulatory role for β-arrestin 2 in the Hh signalling pathway has also been described in vivo. Thus, functional knockdown of β-arrestin 2 by morpholino depletion in zebrafish embryos lead to phenotypes similar to those resulting from mutants in the Hh pathway, indicating a functional interaction between β-arrestin2 and Smo [107]. This interaction was demonstrated in mammalian cell culture experiments, which showed that both β-arrestin and GRK2 mediate clathrin-dependent internalisation of active Smo. Furthermore, Ptc, as well as the antagonist cyclopamine, inhibits the association of β-arrestin 2 with Smo, and this inhibition is relieved in cells treated with the agonist Shh [101]. Finally, a direct requirement of mammalian GRK2 in Smo signalling has been identified in C3H10TI/2 cells, where GRK2 promotes coupling of β-arrestin and Smo [128]. Because the integrity of the Smo C-terminal, in addition to G-protein coupling, is necessary for Gli activation [93], it is tempting to speculate that GRK2 activity acting through the Smo C-terminal domain is a key component of Smo pathway activation.

The vertebrate β-arrestins and GRK proteins have homologous genes in *Drosophila*, although their functional characterisation has not yet being reported for most of them. There are two GRKs in *Drosophila*, GPRK1 and GPRK2, which share homology with members of the mammalian subfamilies 2 and 4, respectively. GPRK1 (more similar in sequence to mammalian GRK2 and GRK3) modulates the amplitude of the visual response acting as a Rhodopsin kinase [129]. GPRK2 has higher sequence identity with mammalian members of the GRK4 subfamily (GRK4–6), and its function is required to regulate the level of cAMP during *Drosophila* oogenesis [130,131]. Recent work in our laboratory using the *Drosophila* model revealed that when *Grpk2* levels are lowered the function of Smo is impaired. Thus, flies expressing interference RNA directed against GPRK2 display a phenotype similar to that characteristic of Smo loss-of-function alleles [132]. Furthermore, loss of GPRK2 also affects Smo protein localisation and

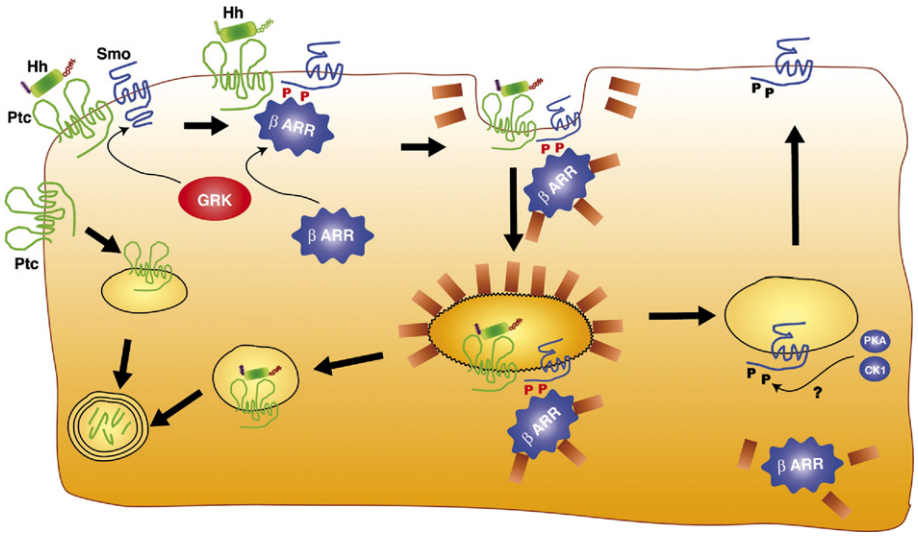

Fig. 3. Putative roles of β-arrestin and GRK2/Gprk2 in Smo regulation. Smo receptor activity is constitutively inhibited by Ptc. Upon Hh binding to Ptc receptor, Smo is released and rapidly phosphorylated by a G-protein coupled receptor kinase (GRK) family member, thus leading to β-arrestin recruitment, and receptor internalisation. Once internalised, Ptc is degraded and Smo can be recycled back to the plasma membrane. It is tentatively speculated that in the endocytic compartment β-arrestin would be released, so Smo can be further phosphorylated by other kinases such as PKA and CKI. As a final step, Smo receptor would be recycled in an active form to the plasma membrane.

stability, suggesting that GPKK2 is required for the correct activation of Smo [132]. So far, no *Drosophila* β-arrestin homolog has been reported to affect Hh signalling. In this regard, in vivo studies in vertebrates also have shown that β-arrestin 2 acts as a positive regulator of the Hh pathway in zebrafish [107]. However, there are no data concerning the role of β-arrestin and GRKs during mammalian development. Mice lacking either β-arrestin 1 or β-arrestin 2 are viable, whereas those lacking both are embryonic lethal [133], although the double-mutant embryos have to our knowledge not been examined for defects in Hh signalling. Mice lacking Grk2 die between embryonic day (E) 9.0 and E15.5 with heart abnormalities [134], but again analysis of a potential Hh/Smo signalling-related phenotype has not been reported.

In sum, these results suggest that Smo regulation by GRK family members and subsequent β-arrestin protein interaction are important in the Hh pathway, both in vertebrates and invertebrates, probably involving modulation of Smo phosphorylation, stability and subcellular localisation (see proposed model in Fig. 3). Further research in both cellular and animal models will help to understand the mechanisms involved in the functional interaction among GRKs, β-arrestins and components of Smo signalling pathway, and its physiological and pathological implications.

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