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Proteostasis in the Hedgehog signaling pathway

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Abstract

The Hedgehog (Hh) signaling pathway is crucial for the development of vertebrate and invertebrate animals alike. Hh ligand binds its receptor Patched (Ptc), allowing the activation of the obligate signal transducer Smoothened (Smo). The levels and localizations of both Ptc and Smo are regulated by ubiquitination, and Smo is under additional regulation by phosphorylation and SUMOylation. Downstream of Smo, the Ci/Gli family of transcription factors regulates the transcriptional responses to Hh. Phosphorylation, ubiquitination and SUMOylation are important for the stability and localization of Ci/Gli proteins and Hh signaling output. Finally, Suppressor of Fused directly regulates Ci/Gli proteins and itself is under proteolytic regulation that is critical for normal Hh signaling.

Keywords

Hh; Shh; Ptc; Ptch1; Smo; Ci; Gli1; Gli2; Gli3; Sufu; ubiquitin; SUMO; phosphorylation; PKA; GSK3; CK1; Grk2; Gprk2; Smurf; Slimb; Cul1; Cul3; Cul4; Usp8; Uchl5; proteasome; lysosome

1. Introduction

The Hedgehog (Hh) family of signaling proteins plays crucial roles in the development of all vertebrate and many invertebrate animals, and aberrant activation of the signaling pathway downstream of Hh proteins account for many malignancies in humans [1]. More than three decades of research into this pathway has culminated in the FDA approval of Vismodegib, a specific inhibitor of the Hh pathway, for the treatment of basal cell carcinoma, the most common form of skin cancer in the western world, whereas more potential drugs targeting various components of the pathway are being tested for many other types of cancers [2]. Given the great scientific and clinical importance of this signaling pathway, it is critical to understand how it is regulated.

1.1. The Hh signaling cascade

Hh was named after the spiny appearance of the *Drosophila* mutant cuticle due to the formation of denticles throughout each body segment in the absence of Hh [3]. Secreted Hh proteins bind their signal-transducing receptor Patched (Ptc), a twelve-span transmembrane protein similar to the bacterial proton-driven RND family of transporters [4–7] (Fig. 1A). Ptc inhibits the function of Smoothened (Smo), a seven-pass transmembrane protein similar to G-protein coupled receptors [8, 9]. Hh binding to Ptc relieves Smo from the Ptc inhibition, allowing it to activate the downstream transcription factor Cubitus interruptus (Ci) [10, 11]. In the absence of Hh, Ci is sequestered in a cytoplasmic complex comprising Costal2 (Cos2)/Fused (Fu)/Suppressor of Fused (Sufu), and is proteolytically processed into a shorter transcriptional repressor [10, 12–15]. Smo activation inhibits Ci processing and allows the full-length Ci to enter the nucleus and activate transcription of *Ptc, Engrailed(En)* and many tissue-specific targets [16–19].

In vertebrates, the Hh pathway is more complex due to gene duplication and the involvement of the primary cilia. For example, the mammalian Hh family has three members, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), which have partially overlapping functions in development and diseases [20–25]. These Hh family members bind two Ptc homolog proteins (Ptch1 and Ptch2) on the target cells, relieving their inhibition on Smo [26–29] (Fig. 1B). There are three Ci homologs in mammals, the Glioma-associated oncogene family that includes Gli1, Gli2 and Gli3 [30]. Similar to Ci, Gli2 and Gli3 can serve as both transcriptional repressors and activators, whereas Gli 1 can only serve as a transcriptional activator [31, 32].

Interestingly, the requirements for Sufu in Hh signaling diverge between fruit flies and mammals. In flies, loss of *Sufu* suppresses the loss of Hh signaling phenotype of *Fu* mutants; however, *Sufu* mutant flies exhibit very subtle phenotype by itself [33]. In striking contrast, *Sufu* mutant mice exhibit aberrant activation of Hh signaling in the entire embryo, suggesting a possibility that mammals may lack the redundant pathway compensating for the loss of *Sufu* in fruit flies [34, 35].

The primary cilia are microtubule-based cell surface organelles present in almost all mammalian cells [36]. Genetic analyses revealed a surprising connection between mammalian Hh signaling and the cilia [37–39]. Subsequent cell biology studies indicated that most Hh pathway components, including Ptch1/2, Smo, Gli1/2/3 and Sufu, are localized to the primary cilia [40–44]. The importance of such localization has been demonstrated by removing some of these proteins from the cilia and examining the consequential disruption of Hh signaling [42, 45, 46].

1.2. The critical roles of Hh signaling in animal development

Hh signaling plays essential roles in numerous developmental processes in vertebrate and invertebrate animals [1]. For the better understanding of this review, I will briefly introduce a few systems in which Hh signaling has been extensively studied. In fruit flies, a well-studied organ is the wing imaginal disc (Fig. 2A). The fly wing disc is divided into the anterior (A) and posterior (P) compartments [47]. Hh is expressed at a high level in the P compartment,

but P cells are not responsive to Hh due to the lack of Ci expression. In the A compartment, Hh secreted from the P compartment forms a gradient such that the cells by the A/P border receive the highest concentration of Hh, and respond by expressing *En* [48, 49], Cells a few cell diameters from the border receive intermediate levels of Hh exposure and express *Ptc.* Cells situated further away from the A/P boundary receive lower levels of Hh and express *Decapendaplegic (Dpp*) [10, 50]. Ectopic expression of *Hh* leads to induction of its target genes ectopically and duplication of the wing structure [51].

In mammals, Shh is the most prevalent Hh family member that regulates, among many others, the dorsal/ventral (D/V) pattern of the neural tube (Fig. 2B) and the A/P pattern of the limbs (Fig. 2C) [22, 52]. In the neural tube, Shh is produced in the ventral most structure, the floor plate, and notochord, a mesodermal rod ventral to the neural tube [20, 21]. Following a ventral to dorsal order, cells of the floor plate, the V3 interneurons, motor neurons, V2 and V1 interneurons are all dependent on Shh, but require different amount of exposure [52]. In the limb buds, *Shh* is expressed in a small posterior-distal domain of mesoderm known as “the zone of polarizing activity” [22]. Both gain-of-function and loss-of-function studies in the chicken and mice showed that *Shh* is important in setting up the A/P polarity of the limbs and promoting the formation of digits [22, 53–55]. Interestingly, numerous digits form in mouse limbs with simultaneous loss of *Gli3* and *Shh,* suggesting that *Shh* regulates digit formation by limiting the Gli3 repressor activity to the anterior regions of the limb buds [56].

The best-studied developmental role of Ihh is the regulation of skeletal development. *Ihh* is required for the proliferation and hypertrophy of chondrocytes [23, 57]. Meanwhile, it is also required for the differentiation of osteoblasts that replace chondrocytes in bones [58]. In addition, *Ihh* is also involved in vascular development and the development of endodermal organs [59, 60]. Dhh plays a crucial role in gonad development and is involved in axon myelination in peripheral nervous system in mice [24, 61].

1.3. The importance of proteostasis in Hh signaling

The levels, localizations and various post-translational modifications (PTMs) of the Hh pathway components are critical for the proper activation of the pathway and the developmental processes it regulates. At the cell surface, Hh activates downstream signaling partly by stabilizing Smo and promoting the internalization and degradation of Ptc. Inside the target cells, partial degradation of Ci, Gli2 and Gli3 through a proteasome-based mechanism leads to the production of their repressor forms. Additional proteasome and lysosome-based mechanisms regulate the stability of all Gli family members. The proteolytic control of Sufu also started to be revealed that could have important implications in Hh signaling regulation in mammals. Finally, Smo and Gli family members are also modified with Small Ubiquitin-like Modifier (SUMO), which generally stabilizes and activates the target proteins. Carefully designed in vivo studies have revealed the physiological significance of such regulations, and have suggested surprising complexities that were not expected from in vitro observation. In the following sections, I will summarize what we know about these processes and some remaining questions.

2. Proteostasis of Smo

2.1. The activation of Smo involves phosphorylation of its carboxyl-terminal tail and change in localization

Smo consists of an extracellular amino-terminal Cysteine-Rich Domain (CRD), seven transmembrane domains, and a cytoplasmic carboxyl-terminal tail (C-tail) (Fig. 3A) [8, 9]. Extensive studies on the biochemistry of Smo indicated that phosphorylation of the C-tail of Smo is an essential step of Smo activation [49, 62–66]. In the absence of Hh, clusters of Arginine residues in the Smo C-tail interact with acidic residues on the same molecule, leading to a closed, inactive conformation [64]. cAMP-dependent protein kinase (PKA) and Casein Kinase 1 (CK1) phosphorylate multiple Serines and Threonines in the Smo C-tail, abrogating the intramolecular electrostatic interaction and changing Smo into an open, active conformation [64]. On the other hand, protein phosphatase 1 (PP1), PP2A and PP4 inhibits Smo activity by removing PKA and CK1-mediated phosphorylation [67, 68]. Increasing Hh concentration leads to progressive phosphorylation of the Smo C-tail by activating the kinases and blocking the phosphatases, resulting in graded downstream responses.

G protein coupled receptor kinase 2 (Gprk2) is also involved in the activation of Smo, partly by directly phosphorylating the C-tail of the Smo [69]. Interestingly, Gprk2 promotes Smo oligomerization independent of its kinase activity. In addition, Gprk2 enriches phosphatidylinositol 4-phosphate, which promotes Smo activation [70].

Hh and Ptc also regulate the dynamic localization of Smo. Ptc promotes the internalization of Smo, whereas Hh promotes Smo surface localization by inhibiting Ptc [62, 71]. Forced Smo localization to the plasma membrane activates Hh signaling, while trapping activated Smo in the endoplasmic reticulum blocks Hh signaling, indicating that surface localization is critical for its activation. Interestingly, blocking lysosomal degradation of Smo activates Smo without significantly increasing its cell surface localization, suggesting Smo activation does not result directly from its localization per se [72].

In *Drosophila,* β-arrestin Kurtz (Krz) promotes Smo internalization by recruiting Clathrin to Smo [73]. Gprk2 facilitates this process as β-arrestin specifically binds Smo phosphorylated by Gprk2.

CK1-mediated phosphorylation of the C-tail also underlies the activation of mammalian Smo [74]. In mammals, however, Smo needs to be in the primary cilia to be active [42]. In the absence of Hh, Smo is kept out of the cilia by Ptch1, which is localized to the cilia (Fig. 3B) [40]. The presence of a ligand, such as Shh, leads to the internalization of Ptch1, hence the ciliary translocation and activation of Smo [40]. Smo enters the cilia by lateral diffusion, and β-arrestin 1/2 mediate Smo interaction with a microtubule motor Kif3a [75, 76]. A Septin 2-based diffusion barrier at the base of the cilia keeps Smo from leaving the cilia and is required for cilia-dependent activation of Hh signaling [77].

Despite its structural similarity to the G-protein coupled receptor family, a conventional ligand for Smo has yet to be found. The prevailing view in the field is that the endogenous direct regulator of Smo is likely a small hydrophobic molecule similar to cholesterol. Many

synthetic small molecule antagonists and agonists regulate Smo activity by targeting a site in the transmembrane domain [78–81]. In contrast, cholesterol and its derivatives bind the CRD of Smo, which is essential for Hh-induced Smo activation [79, 82–87]. In line with cholesterol or its derivatives being the endogenous Smo regulator, recent structural analyses suggested that Ptc could function as a steroid pump [88–90]. Ptch1 also pumps 7-dehydrocholesterol, a cholesterol precursor, out of the cell, and 7-dehydrochosterol and its derivative vitamin D3 bind and inhibit Smo activity [91].

2.2. The ubiquitination of Smo

In *Drosophila,* Ptc promotes, and Hh inhibits, the internalization and degradation of Smo [62]. Ubiquitination plays a key role in Smo internalization and degradation as targeting E1 ubiquitin activating enzyme Uba1 increased Smo at the cell surface [92]. Smo is both multi-monoubiquitinated and polyubiquitinated, and is degraded in both the lysosome and proteasome [92, 93]. Hh treatment, PKA and CK1-mediated Smo phosphorylation inhibit its ubiquitination, internalization and degradation, whereas Ptc promotes Smo ubiquitination. The deubiquitinating enzymes UBPY/Usp8 and Uchl5 interact with the C-tail of Smo and inhibits its ubiquitination [92–94]. Interestingly, Hh enchances the interaction between Uchl5 and Smo [94]. Finally, Krz acts in parallel to ubiquitination to promote Smo internalization and degradation [72]. The mammalian Smo is similarly multi-ubiquitinated and Shh treatment reduces its ubiquitination.

Endosomal Sorting Complexes Required for Transport (ESCRT) was known for its roles in mediating endosomal sorting of ubiquitinated membrane proteins [95]. RNAi knockdown of multiple ESCRT components, including Vps25, Vps28, Vps32 and Vps36, in S2 cells and wing imaginal disc, showed that ESCRT is involved in Smo internalization and degradation [72, 96]. Furthermore, the ESCRT complexes interact with ubiquitinated Smo C-tail via Vps36. As expected, Hh, phosphorylation of the Smo C-tail and deubiquitinating enzyme Usp8 inhibit the Smo/Vps36 interaction.

Recent studies through RNAi screen have identified two sets of E3 ligases for Smo-the Smurf family of E3 ubiquitin ligases and the Cul4-DDB1-Gß complex-that act in parallel to regulate Smo ubiquitination and cell surface expression [97, 98]. Gprk2 phosphorylates and activates HECT domain-containing E3 ubiquitin ligase Smurf, which catalyzes the ubiquitination of Smo [98]. Interestingly, in the presence of Hh, Smurf dissociates from Gprk2 and Smo, and associates with Ptc and promotes Ptc internalization. Both PKA and Gprk2 phosphorylate Smo C-tail, leading to the dissociation of Smurf from Smo. These data suggest that Gprk2 plays complex roles in Hh signaling.

A recent study implicated a Cul4-based ubiquitin ligase complex Cul4-DDB1-Gß in the ubiquitination of Smo and Gprk2 [97]. Again, Hh induces the dissociation of Cul4-DDB1 from Smo-Gß, allowing stabilization and surface localization of Smo.

In mammalian cells, Grk2 promotes Smo ciliary localization and Shh signaling, but Smo localization is not affected in *Grk2* mutant cells, likely due to functional redundancy with other GRK family members or CK1 [74, 99, 100]. Grk2 acts genetically downstream of Smo but upstream of Gαs and Sufu [99]. Although the molecular mechanisms of its function

remain incompletely understood, Grk2 may act, at least in part, by phosphorylating Smo [74].

2.3. The SUMOylation of Smo

Smo is also modified by the small ubiquitin-like modifier (SUMO) proteins. Hh promotes the SUMOylation of Smo, which leads to its stabilization [101, 102]. SUMOylation of Smo is not dependent on, and acts in parallel to, its phosphorylation in the activation of Smo. Furthermore, SUMOylation of Smo recruits the deubiquitination enzyme Usp8. This mechanism appears to be conserved as SUMOylation of mouse Smo leads to its ciliary localization [103].

Excess Krz recruits ubiquitin like protease 1 to prevent the SUMOylation of Smo, adding another mechanism by which it promotes the internalization and degradation of Smo [102].

3. Proteostasis of Gli transcription factors

3.1. Cul1 and βTRCP-based proteolytic processing of Gli family members

3.1.1. Slimb converts phosphorylated Ci into a transcriptional repressor—

The roles of Ci in Hh signaling is complex as both loss of Ci and overexpression of Ci results in ectopic activation of Hh signaling in the wing disc [10]. The answer to this complex puzzle lies in the fact that Ci is proteolytically processed into a shorter, repressor form, and Hh inhibits this process (Fig. 4) [104]. As a result, Ci exists as a transcriptional activator in cells exposed to high levels of Hh, such as those near the A/P boundary of the wing disc, converting the Hh signal into a transcriptional response (Fig. 2A) [105]. On the other hand, Ci is efficiently processed into a repressor in cells not being exposed to Hh, such as those in the A compartment of the wing disc far away from the boundary, leading to the silencing of the Hh target genes. Reflecting this molecular complexity, the loss of Ci in *Drosophila* leads to upregulation of genes that are normally repressed by Ci repressor, and simultaneous downregulation of genes that are normally dependent on Ci activator [106].

Although PKA phosphorylation of Smo C-tail plays a critical role in Smo activation (see above), genetic analyses in *Drosophila* wing discs showed that PKA inhibited the expression of Hh target genes, suggesting an additional negative role of PKA downstream of Smo [107–110]. Later it was shown that PKA phosphorylates Ci and promotes its proteolytic processing into repressors [111–113]. Mutating 4 or 5 Serines targeted by PKA renders Ci resistant to proteolytic processing. Furthermore, PKA phosphorylation of Ci primes it for subsequent phosphorylation by Gsk3 and Ck1 at adjacent Serines or Threonines, which is also essential for Ci processing [114, 115].

Slimb, part of the SCF (Skp1/Cullin1/F-box) E3 ubiquitin ligase complex, is required for the proteolytic processing of Ci [116]. The phosphorylation of Ci by PKA, CK1 and GSK3 leads to Slimb binding and subsequent proteolytic processing of Ci [117–119]. Cos2 serves as a scaffold protein to bring the above kinases to Ci, leading to Ci phosphorylation and subsequent degradation [120].

A unique feature of Slimb-mediated Ci processing is that only the C-terminal region of the protein is degraded, whereas the N-terminal and DNA binding regions are spared. The DNA binding Zinc finger domain and a Lysine residue at position 750 together form a protection signal to limit proteasomal degradation of Ci to the C-terminal region [121]. Mutations to this signal prevent the formation of Ci repressor through partial degradation.

3.1.2. β TRCP mediates efficient processing of Gli3 and degradation of Gli2

—In mammals, gene duplication adds another layer of complexity to the complex regulation of the Gli protein activities. Genetic studies indicated a primarily positive role of Gli2 and a negative role of Gli3 in Hh signaling, implying a potential difference in their proteolytic processing [122–124]. Indeed, when mammalian Gli proteins are expressed in the fly wing, Gli3 is efficiently processed into a repressor, whereas Gli2 processing is inefficient and Gli 1 is not processed into a repressor at all [125]. More direct evidence for the differential processing of Gli2 and Gli3 came from the examination of these proteins in vivo with antibodies against their N-termini. Gli3 is efficiently processed into a repressor form in the anterior half of the limb buds, whereas Shh limits both the overall expression level and proteolytic processing of Gli3 in the posterior half of the limb buds [31]. In contrast, Gli2 repressor is barely detectable in vivo, and Shh treatment primarily stabilizes full-length Gli2 rather than inhibiting the formation of a repressor [32].

PKA, CK1 and GSK3β phosphorylate Gli2 and Gli3 at multiple sites, and phosphorylation at these sites are critical for Gli3 processing and Gli2 degradation [31, 32]. The phosphorylation of Gli2 and Gli3 allows them to interact with βTrCP, the mammalian homolog of Slimb, which promotes their ubiquitination [32, 126].

The fact that Gli3 is proteolytically processed into a repressor whereas Gli2 is degraded in the proteasome results from their structural difference. A processing determinant domain (PDD) in the central region of Gli3 (residues 648–844) accounts for the much higher proteolytic processing efficiency in Gli3 than Gli2 [127]. Replacing the PDD of Gli3 with the corresponding region of Gli2 (residues 585–780) blocks Gli3 processing, whereas introduction of PDD to Gli2 leads to efficient processing.

The importance of PKA-mediated phosphorylation and subsequent degradation of Gli2 and Gli3 has been investigated genetically by gene replacement in mouse genome. Replacing the four Serines critical for Gli3 processing with Alanines in mouse genome results in the disruption of Gli3 processing and severe polydactyly similar to that of Gli3 null [128]. Replacing the four PKA sites in Gli2 in vivo (in $Gli2^{P1-4}$ mice) leads to stabilization of the protein [129]. Unfortunately, The $Gli2^{P1-4}$ heterozygotes in which the mutant version of *Gli2* is presumably expressed at the wild type level die prematurely, preventing further characterization. The analysis of a different allele, $Gli^{P1-4neo}$, which transcribes the mutant *Gli2* at a much lower level, showed an increase in Hh pathway activity, suggesting that PKA phosphorylation of Gli2 is a critical regulatory mechanism of Hh signaling in development.

Slimb-mediated ubiquitination of Ci may also lead to Ci degradation through the lysosomal pathway in the presence of Debra [130]. This lysosomal pathway of Ci degradation appears

to be essential for maintaining gut homeostasis in flies [131]. Similar mechanism has not been reported in vertebrates.

3.1.3. Gli3 processing in vertebrates is dependent on the primary cilia—Gli3 repressor was greatly reduced while full-length Gli3 accumulated in mouse mutants with complete loss of the primary cilia, suggesting that Gli3 processing requires the cilia [37–39, 132]. Consistent with the decrease in Gli3 repressor, these mutants exhibit polydactyly. The localization of Gli3 to the tips of the cilia raises the possibility that Gli3 processing may occur in or near the cilia [41]. Interestingly, increasing cAMP levels by treating cells with Forskolin and/or IBMX blocks the ciliary entry of Gli3, raising the possibility that PKA phosphorylation of Gli3 may inhibit its ciliary entry or promote its ciliary exit [133, 134]. However, using a phospho-Gli3 specific antibody, it was recently found that phosphorylated Gli3 is localized to the ciliary tip and is reduced by Shh treatment [135]. Furthermore, Forskolin and IBMX treatment blocks Gli3 ciliary localization in PKA mutant cells, suggesting that Gli3 localization to the cilia is regulated by a PKA-independent mechanism [136]. Surprisingly, a recent report showed that blocking Gli3 ciliary entry did not affect Gli3 processing, raising the possibility that the roles of the cilium in Gli3 processing may be indirect [46].

3.2. Cul3 based degradation of Gli transcription factors

3.2.1. Hib targets Ci for degradation—In addition to the Slimb-mediated proteolytic processing, Ci is also under the regulation of a Cul3-based E3 ubiquitin ligase. Hh-induced MATH and BTB domain containing protein (Hib, also known as roadkill/rdx), the substrate-recognition subunit of a Cul3-based E3 ligase, is expressed in response to high levels of Hh signaling, and targets Ci for ubiquitination and degradation [137, 138]. The MATH domain of Hib interacts with both the N and C termini of Ci (Fig. 4) [137]. Unlike Slimb, which partially degrades Ci to produce a transcriptional repressor, Hib-mediated ubiquitination leads to complete degradation of Ci. Consistent with this role in Ci degradation, loss of Hib leads to ectopic activation of Hh signaling, making it a negative regulator of the pathway.

Multiple Serine and Threonine-rich motifs in Ci mediate its interaction with Hib [139]. Both Ci and Hib form dimers/oligomers, and the dimerization/oligomerization appears to be critical for efficient ubiquitination and degradation of Ci.

Although loss of *Sufu* does not cause drastic activation of Hh pathway, it does enhance Hh target gene expression on sensitized background, suggesting that Sufu is a negative regulator of the pathway [140]. Paradoxically, the full-length Ci was greatly reduced in the absence of *Sufu,* suggesting that Sufu protects Ci from degradation. Sufu prevents the association between Hib and Ci, implying that Hib may target activated Ci not protected by Sufu [137]. Downstream of Sufu, CK1 phosphorylation of multiple Serines on Ci prevents Hib-mediated Ci degradation, suggesting a surprising positive role for CK1 in Ci activation [141].

Another study found that Hib downregulates Ci in cells with moderate Hh pathway activation, but not in those with maximal Hh pathway activation [142]. Instead, Hib sequesters Ci in the cytoplasm in the presence of Hh. This appears to be inconsistent with the model that Hib specifically targets activated Ci for degradation, and the observation that

Hib expression is enriched in cells with highest Hh signaling activity. Further investigation is thus needed to resolve this controversy.

3.2.2. Spop targets Gli2 and Gli3 for degradation in cultured cells—In striking contrast to *Drosophila Sufu* mutants that exhibit very subtle activation of the Hh pathway, the mouse *Sufu* mutants exhibit widespread ectopic activation of the Hh pathway [33–35]. Interestingly, the levels of Gli2 and Gli3 were drastically reduced in *Sufu* mutants [43, 44, 143]. Treating cultured cells with Shh similarly downregulates the Gli3 protein level [144, 145]. Knocking down a mouse Hib homologue, Speckle-type POZ Protein (Spop), in *Sufu* mutant fibroblasts, restored the levels of Gli2 and Gli3, suggesting that Sufu protects these Gli proteins from Spop-mediated degradation [69, 143]. Spop knockdown leads to a more significant increase in the levels of full-length Gli2 and Gli3 in the presence of Shh, suggesting that Spop preferentially degrades activated full-length Gli2 and Gli3 [145].

Serine and Threonine-rich Spop binding sites are present in all three Gli proteins, although in vitro studies indicated that only Gli2 and Gli3 are ubiquitinated and degraded by Spop [139]. It is worth mentioning that Spop appears to have a higher affinity to Gli3 than to Gli2.

Spop-like (Spopl) shares 81% sequence identity with Spop, but the function of Spopl is poorly understood [146]. Different from Spop, Spopl carries an extra 18-residue insertion in the BACK domain that prevents it from forming higher order assembly (i.e. multimers) and impairs its catalytic activity [147]. By forming heterodimers with Spop, Spopl also shifts Spop into smaller complexes (i.e. dimers), hence impairing the E3 ubiquitin ligase activity of Spop.

3.2.3. *In vivo* studies reveal a more specific function of Spop in Gli3 ubiquitination and degradation—For the in vivo requirement of *Spop* in mammalian development, two apparent null alleles of *Spop* in mice have been characterized [148, 149]. In contrast to *Drosophila* where *Hib* expression was under the regulation by Hh, *Spop* expression is more widespread in mouse embryos [43, 149]. Notably, it is expressed at higher levels in the cartilage and bones. Consistent with this expression pattern, *Spop* mutants exhibit defects in chondrocyte and osteoblast differentiation [149]. Contrary to the *in vitro* results suggesting that both Gli2 and Gli3 are Spop substrates, the level of Gli2 was not significantly affected in *Spop* mutants [43, 143, 145, 149]. On the other hand, both the full-length and the repressor form of Gli3 were significantly increased in the absence of *Spop.* Significantly, the skeletal defects of *Spop* mutants were rescued by reducing Gli3 dosage, suggesting that increased Gli3 repressor underlies these defects. Therefore, these *in vivo* studies reveal an important physiological role for Spop in the regulation of Gli3 repressor.

Despite the important roles of Hh signaling in neural tube patterning, the neural tube appears normal in *Spop* mutants, consistent with a minor role of Gli3 in this process (Fig. 5A) [148]. However, loss of *Spop* suppresses the partial loss of Hh signaling defects of *Gli2* mutants (Fig. 5A and B). As Gli3 is the primary Hh pathway effector in the absence of Gli2, these data indicate that stabilization of Gli3 in the absence of Spop does increase Hh pathway activation.

Although *Gli1* is dispensable for neural tube patterning in the presence of *Sufu,* the floor plate and V3 interneurons that require high levels of Hh signaling activities fail to form in *Sufu;Gli1* double mutants, revealing a surprising positive role of *Sufu* in Hh signaling (Fig. 5C) [150]. Interestingly, the formation of these structures are restored in *Sufu;Gli1;Spop* triple mutants, suggesting that *Spop* plays a negative role in Hh signaling in this context (Fig. 5D) [148]. Surprisingly, the levels of Gli2 and Gli3 were not significantly increased in *Sufu;Spop* double mutants compared to *Sufu* mutants, suggesting that Spop may inhibit their activities in addition to targeting them for degradation. It is possible that an alternative mechanism, such as altered subcellular localization as suggested earlier in *Drosophila* [142], may contribute to the difference. Indeed, overexpression of Spop appears to make Gli3 more cytoplasmic (Cai and Liu, unpublished data)[43].

3.3. Deubiquitination of Ci/Gli proteins

The deubiquitination enzyme Usp7 catalyzes the removal of ubiquitin from Ci, antagonizing both Cul1/Slimb-mediated processing and Cul3/Hib-mediated degradation [151]. Hh induces the interaction between Ci and Usp7. Similar function of Usp7 was also observed in zebrafish and mammals, suggesting that it is a conserved regulator of Ci/Gli stability and Hh signaling.

3.4. SUMOylation of the Gli proteins

The E3 SUMO ligase Pias1 mediates mammalian Gli SUMOylation [152, 153]. The roles of Gli phosphorylation in their SUMOylation have been controversial. One report found that phosphorylation by PKA inhibits the SUMOylation of Gli2 and Gli3, but not Gli1, and SUMOylation only happens to the full-length, but not the repressor form, of Gli3 [152]. In contrast, another report suggested that PKA promotes, and Hh inhibits, Gli2 SUMOylation [153]. More importantly, whether SUMOylation promotes or inhibits Gli activity is also under debate. Overexpression of Pias1 in the chicken neural tube induces ectopic Nkx2.2 expression, which can be inhibited by a dominant/negative form of Gli3, suggesting a potential positive role of SUMOylation in Gli activation [152]. On the other hand, Han et al (2012) found that replacing two Lysines critical for Gli2 SUMOylation with Arginines leads to elevated Hh signaling in the mouse neural tube, although the difference appears subtle [153]. The differences may result from different experimental paradigms (chicken vs mouse, overexpression of an enzyme that targets multiple substrates vs mutating one individual substrate), and additional rigorous investigation is needed to resolve this controversy.

SUMOylation also plays a role in regulating Ci activity in *Drosophila* [154]. RNAi knockdown of E2 conjugase Lwr and E3 SUMO ligase Su(Var) leads to reduced Ptc expression and disrupted testis development. It was found that Lwr interacts with Ci, and mutating all SUMOylation sites on Ci abrogates its activity, supporting a positive role of SUMOylation in Ci activation. It should be noted that mutating the SUMOylation sites on Ci reduces its activity in S2 cells but does not drastically change Hh pathway activity in wing discs [103].

4. Proteostasis of other pathway components

4.1. Proteostasis of Patched

Being an important regulator of Smo, the localization and level of Ptc itself is also under regulation. Upon Hh treatment, Ptc is internalized and degraded despite an increase in its transcription [62]. As reviewed above, Smurf promotes Smo ubiquitination and degradation in the absence of Hh; however, mutating Smurf in *Drosophila* or zebrafish leads to decreased Hh signaling, suggesting it must target additional components of the pathway [155]. Indeed, Smurf promotes Ptc ubiquitination and internalization by directly interacting with the C-tail of Ptc, and reducing Ptc dosage rescues the Hh signaling defects in *Smurf1/2* morphant fish (Fig. 3). Interestingly, Smo promotes Smurf-mediated Ptc ubiquitination and degradation.

Combining the results in Smo and Ptc, a model was proposed to explain the complex roles of Smurf in Hh signaling (Fig. 3) [156]. In the absence of Hh, both Smurf and Gprk associate with the Smo C-tail, leading to Smo ubiquitination and degradation (Fig. 3A). In the presence of Hh, the phosphorylation and SUMOylation of the Smo C-tail reduces binding of Smurf to Smo (Fig. 3B). Instead, Smurf binds and ubiquitinates Ptc.

Itch, another E3 ubiquitin ligase, plays a role in regulating the ubiquitination, internalization and degradation of mammalian Ptchl, but not Ptch2 [157]. Reducing Itch or mutating the Itch target (K1413) in Ptchl stabilizes Ptchl. Itch appears to regulate Ptchl only in cells not receiving Hh signal, thus is not involved in Hh mediated downregulation of Ptchl.

4.2. Proteostasis of Sufu

Sufu directly interacts with the Gli proteins and inhibits their transcriptional activities by sequestering them in the cytoplasm [14, 158–163]. Hh signaling alleviates this inhibition, but how it achieves this remains a hotly debated issue. Some believe that Hh separates Sufu from Gli proteins, allowing Gli to enter the nucleus and activate downstream genes [134, 144]. Others believe Sufu enters the nucleus with the Gli proteins and exhibits an inhibitory role inside the nucleus [14, 164–167]. Among these, some believe that Sufu dissociates from the Gli proteins in the nucleus upon Hh signaling [166]. Others posits that Sufu and Gli proteins remain associated upon Hh pathway activation, and Sufu is turned into a positive regulator of Gli in this context [167]. A clear answer to this question awaits more studies.

The dependence on direct association for its inhibitory function on the Gli proteins suggests that a higher level of Sufu is needed to prevent improper activation of the pathway, which has been confirmed by a study showing Sufu is in great excess compared to Ci in *Drosophila* [168]. Recent studies have started to reveal mechanisms by which Hh signaling regulates the levels of Sufu in vertebrates and insects. In mammals, Shh promotes the ubiquitination of Sufu at K257 and its subsequent degradation [169]. Decreased Sufu stability appears to be associated with cancer formation. Interestingly, GSK3β and PKA phosphorylate Sufu at Ser342 and Ser346, respectively, and stabilize Sufu [170]. Sufu phosphorylation promotes its ciliary localization and colocalization with Gli3. The levels of total and phosphorylated Sufu are elevated in the absence of Smo, suggesting that Hh signaling may inhibit Sufu phosphorylation.

Nek2A phosphorylates Sufu at T225 and S352 and inhibits Sufu ubiquitination and degradation, hence negatively regulates Hh signaling [171, 172]. Interestingly, Hh signaling induces the transcription of *Nek2A,* making Nek2A a negative feedback regulator of the Hh pathway.

Hh also regulates the level of Sufu in *Drosophila,* but through an indirect mechanism [173]. Hh upregulates Hib, which sequesters a spliceosome factor Crn in the nucleus, and interferes with the translation of the *Sufu* mRNA. However, the direct target of Hib in this context has not been identified.

5. Conclusion

Like all other developmental signaling pathways, Hh signaling is under strict regulation in development and adulthood to avoid developmental errors and malignancies. Proteostasis plays a particularly important role in the regulation of this pathway. Proteasome-based processing of the Ci/Gli proteins and Hh regulation of this process, directly result in the transcriptional output of the pathway. The deubiquitination and cell surface/ciliary translocation of Smo is an integral part of its activation. Although the exact mechanisms by which Hh inhibits the activities of Ptc and Sufu are still actively pursued, the downregulation of these proteins certainly contributes to Hh pathway activation.

New components of the Hh pathway and regulatory mechanisms, including proteostasis, have been revealed at a striking pace. In addition to above proteins, other components of the Hh signaling pathway are also subject to PTMs that change their level, localization and/or activities in the cells. For example, Cos2 is SUMOylated and this modification antagonizes its activity in Hh signaling [102]. Cos2/Kif7 is ubiquitinated by Ubr3, which destabilizes the protein [174].

With nearly three decades of extensive investigation, great progress has been made in understanding the proteostasis in the Hh signaling pathway. However, many outstanding questions remain. As discussed above, several issues remain unsettled, including the roles and regulation of Gli SUMOylation [152, 153], the exact roles of the cilia in Gli3 processing [39, 46, 136, 144] and the exact relationship between Sufu and Gli proteins upon Hh pathway activation [134, 144, 166, 175]. Furthermore, although Smurf family members regulate Ptch1 endocytosis [176], it is not clear whether Smurf-mediated ubiquitination also regulates Smo localization in mammals. It was long known that Hh signaling downregulates Cos2 [19], and a recent study suggested that the E3 ligase Ubr3 regulates the degradation of Cos2 and Kif7 to modulate Hh signaling [174]. However, loss of Ubr3 does not block Hh-induced Cos2 degradation in vivo, suggesting that Hh may promote Cos2 degradation through additional mechanism(s). Finally, although the significance of proteostasis in Hh signaling has been extensively addressed in vivo in *Drosophila,* more in vivo genetics studies are still needed in mammals.

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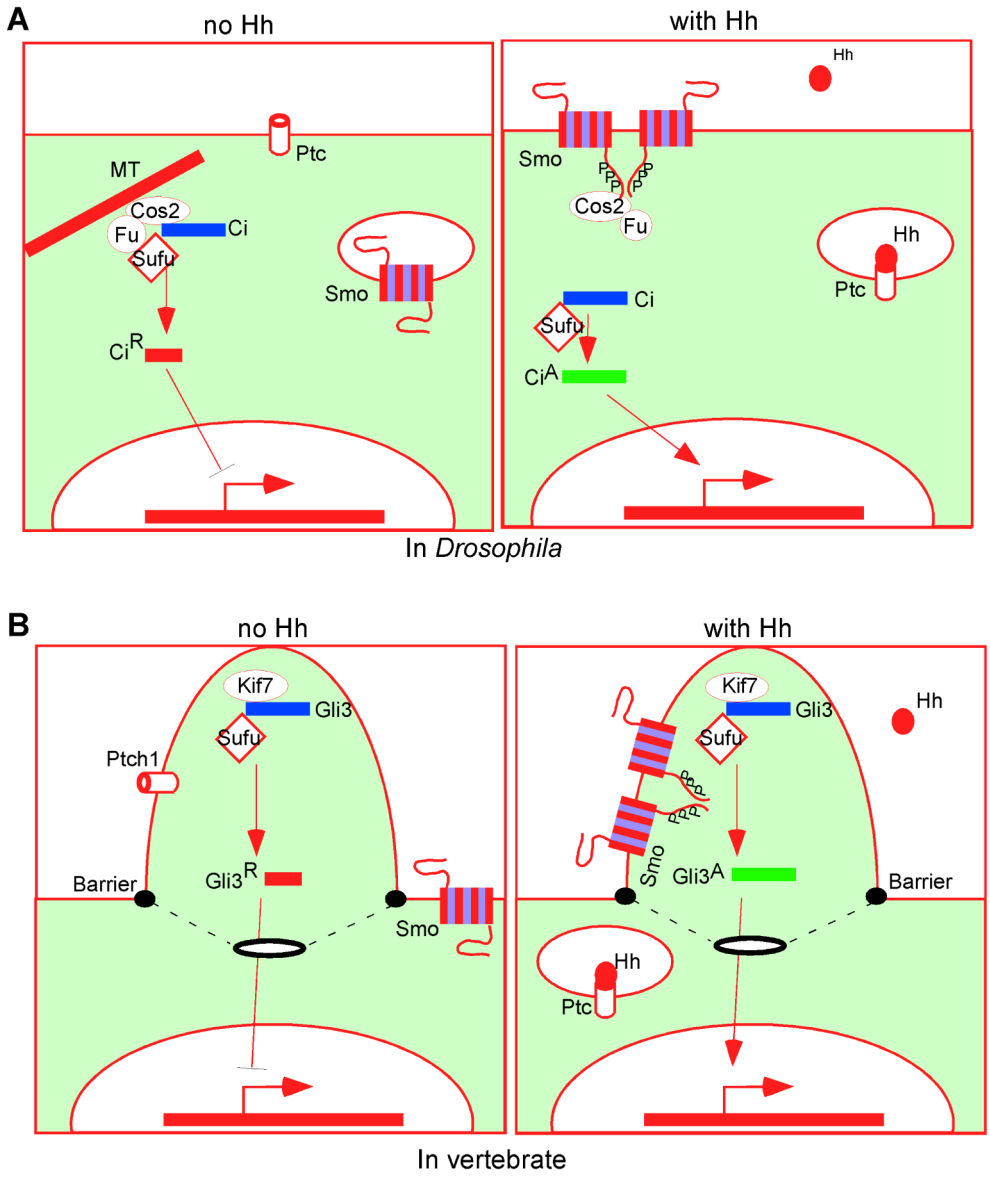

Figure 1.

The outlines of Hh signaling

(A) In *Drosophila,* Ptc keeps Smo inactive, partly by promoting its internalization and degradation. Full-length Ci is sequestered in the cytoplasm in a complex with Cos2, Fu and Sufu, whereas Ci repressor (Ci^R) inhibits target gene expression in the nucleus. Hh blocks Ptc function, allowing the phosphorylation and dimerization/oligomerization of Smo, leading to Ci activation (Ci^A) and target gene expression. MT: microtubule;

(B) In vertebrates, Ptch1 in the primary cilia prevents Smo ciliary localization and activation. Gli3 enters the cilia with Sufu and Kif7 and is processed into $Gli3^R$. Shh binds Ptch1, leading to Smo ciliary translocation, phosphorylation, dimerization/oligomerization and Gli3 activation ($Gli3^A$). A membrane barrier near the base of the cilia prevents free diffusion of Smo.

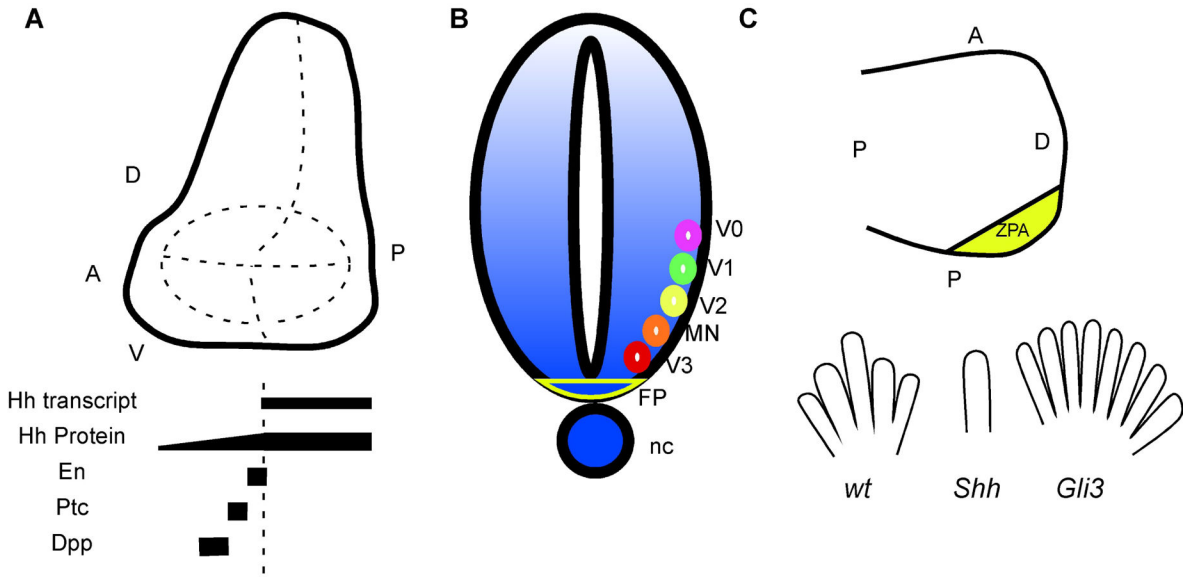

Figure 2.

Most popular systems for the studies of Hh signaling

(A) The wing imaginal disc in *Drosophila. Hh* is transcribed only in the posterior compartment, and form a gradient in the anterior compartment. The highest level of Hh signaling induces *En* expression immediately adjacent to the compartment boundary. An intermediate level of Hh signaling induces *Ptc* expression, and a lower level of Hh signaling induces *Dpp* expression, in cells further anterior in the A compartment. A: anterior; P: posterior; D: dorsal; V: ventral.

(B) The vertebrate neural tube. Shh is produced in the notochord (nc) and floor plate (FP), and forms a ventral-to-dorsal gradient. Decreasing concentrations of Shh are required to determine the fates of the V3 interneurons, motor neurons (MN), V2 and V1 interneurons.

(C) The vertebrate limbs. *Shh* is expressed in the posterior mesenchyme known as the zone of polarizing activity (ZPA), and regulates digit formation by antagonizing Gli3 repressor activity. Wild type (wt) mouse limbs have five digits. *Shh* mutant limbs have only one digit. *Gli3* mutant limbs have 8–9 identical digits. A: anterior; P: posterior; P: proximal; D: distal.

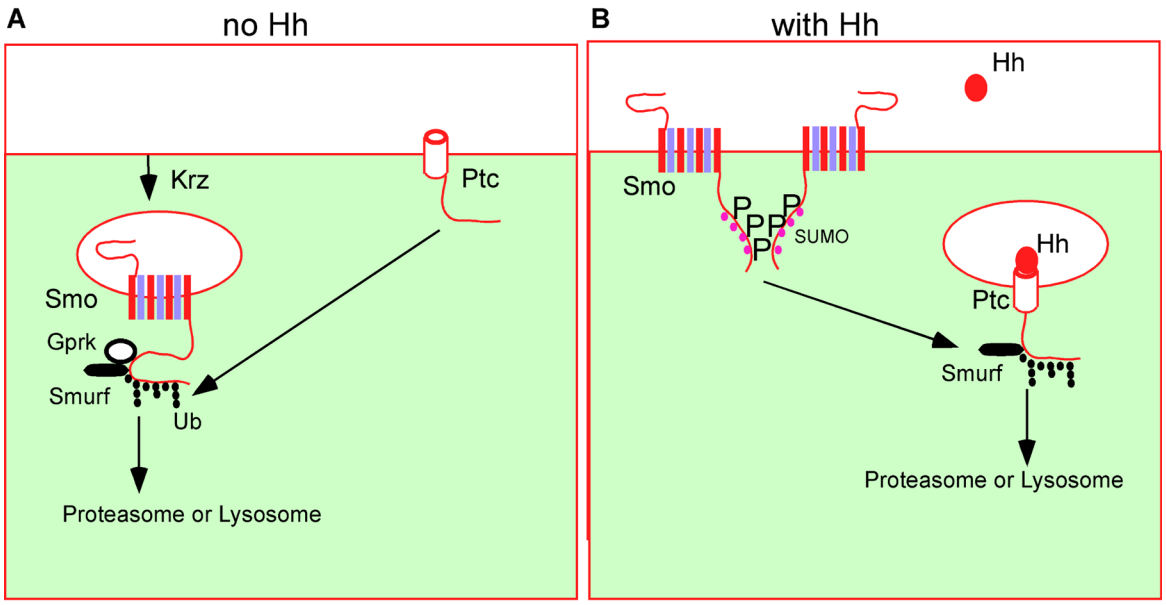

Figure 3.

Proteostatic regulation of Smo and Ptc.

(A) In the absence of Hh, Ptc promotes the ubiquitination of Smo mediated by Smurf, internalization and degradation of Smo. Krz promotes Smo internalization in parallel to Smo ubiquitination. (B) In the presence of Hh, Smo is phosphorylated and SUMOylated, forming dimers/oligomers, dissociating from Smurf, and translocates to cell surface. Smurf associates with and ubiquitinates Ptc, leading to its internalization and degradation.

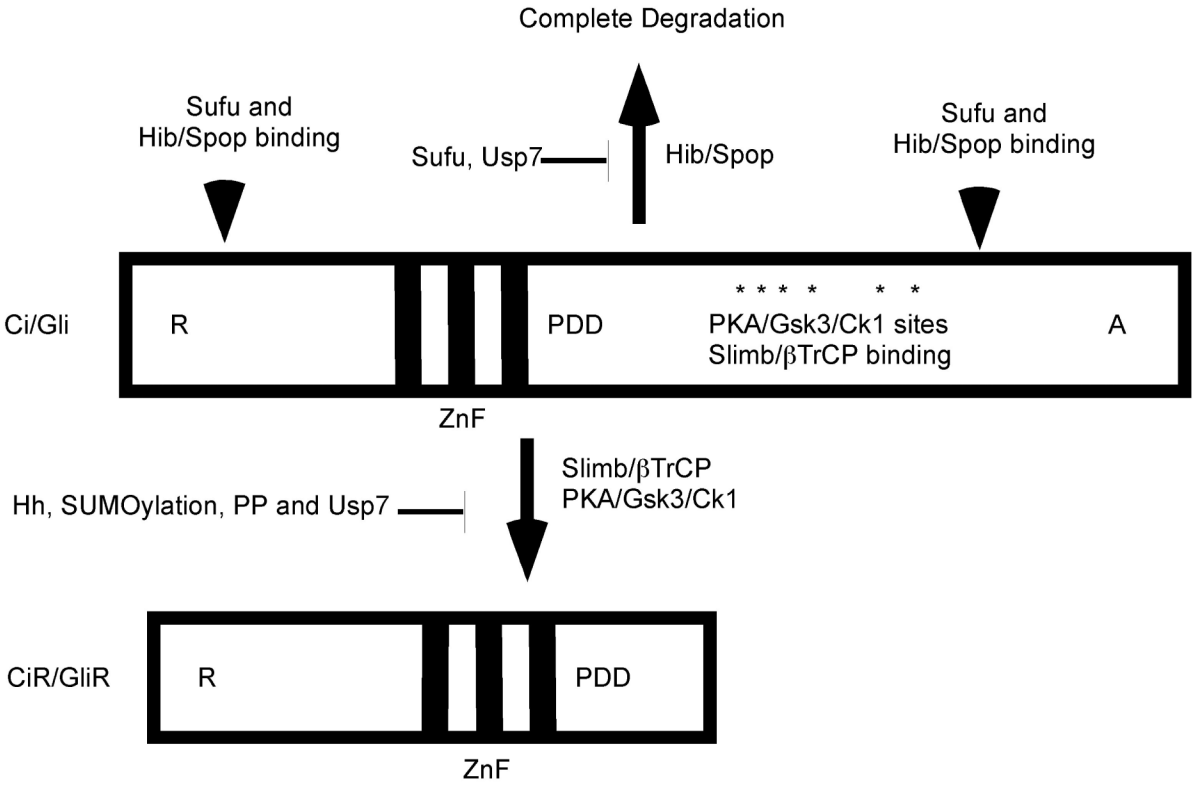

Figure 4.

Proteostatic regulation of Ci/Gli proteins

All Ci/Gli proteins, except mammalian Gli1, have an N-terminal repressor (R), a central zinc-figure (ZnF) and a C-terminal activator (A) domain. PKA/Gsk3/Ck1 phosphorylate a series of Serines/Threonines (asterisks) and promote ubiquitination of Ci/Gli proteins by Slimb/pTrCP and proteolytic processing of Ci/Gli proteins into repressors (CiR/GliR). SUMOylation, protein phosphatases (PP) and deubiquitinating enzymes (Usp8) inhibit Ci/Gli processing. A processing determinant domain (PDD) in Gli3 is required for efficient processing. Hib/Spop catalyzes the complete degradation of Ci/Gli proteins, and Sufu protects Ci/Gli proteins from this degradation. Sufu and Spop bind both the N- and C-termini of the Ci/Gli proteins.

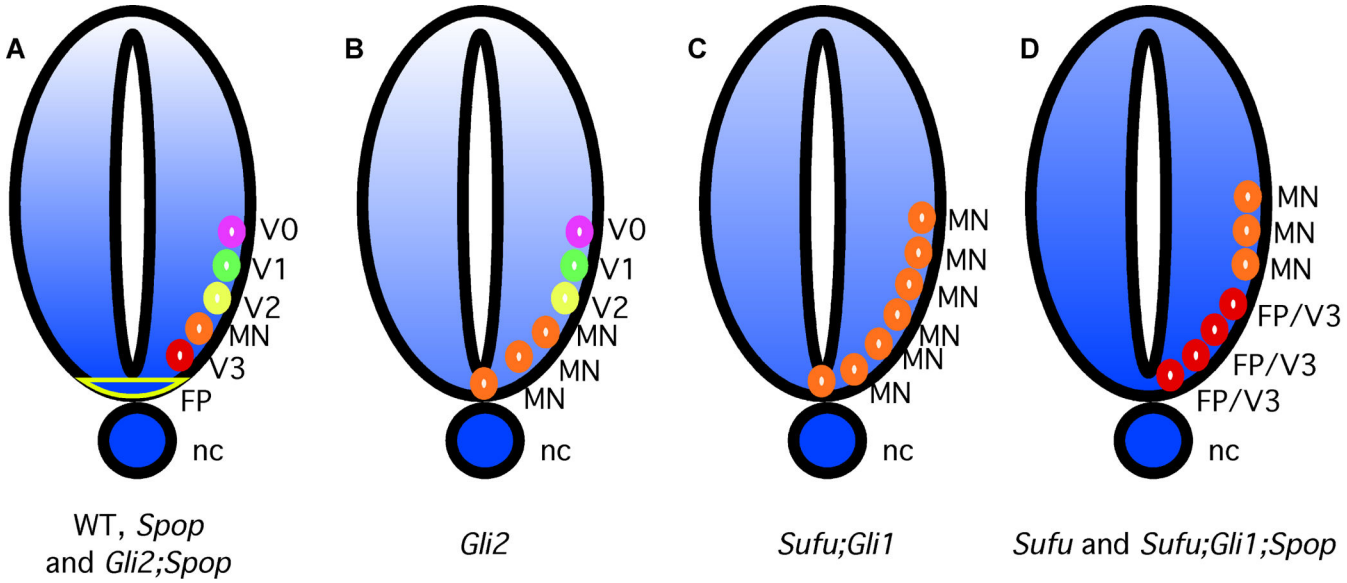

Figure 5.

The importance of Sufu/Spop-mediated proteostatic regulation of Gli3 in neural tube patterning

(A) Gli2A and Gli3A are sufficient for patterning ventral NT properly in the presence (wt) and absence *(Spop)* of Spop. Increased Gli3A is sufficient for patterning ventral NT in the absence of Gli2 and Spop *(Gli2;Spop)*. (B) Gli3A is insufficient for FP and V3 formation in *Gli2* mutants. (C) In *Sufu;Gli1* double mutants, greatly reduced Gli2A and Gli3A are not sufficient for FP and V3. (D) Increased Gli2A and Gli3A activities in *Sufu;Gli1;Spop* triple mutants support FP and V3 interneurons, similar to *Sufu* single mutants.