

Molecular mechanisms of suppressor of fused in regulating the hedgehog signalling pathway (Review)

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Abstract. Highly conserved throughout evolution, the hedgehog (Hh) signalling pathway has been demonstrated to be involved in embryonic development, stem cell maintenance and tissue homeostasis in animals ranging from invertebrates to vertebrates. In the human body, a variety of cancer types are associated with the aberrantly activated Hh signalling pathway. Multiple studies have revealed suppressor of fused (Sufu) as a key negative regulator of this signalling pathway. In vertebrates, Sufu primarily functions as a tumor suppressor factor by interacting with and inhibiting glioma-associated oncogene homologues (GLIs), which are the terminal transcription factors of the Hh signalling pathway and belong to the Kruppel family of zinc finger proteins; by contrast, the regulation of Sufu itself remains relatively unclear. In the present review article, we focus on the effects of Sufu on the Hh signalling pathway in tumorigenesis and the molecular mechanisms underlying the regulation of GLI by Sufu. In addition, the factors modulating the activity of Sufu at post-transcriptional levels are also discussed.

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1. Introduction

Being a part of the system that specifies pattern formation during animal embryogenesis, hedgehog (Hh) signalling was initially discovered to regulate cuticle patterns in *Drosophila* more than 30 years ago (1); its existence in mammals suggested the strictly conservative roles of the Hh pathway in animal embryogenesis during evolution (2-4). Considering that development and tumorigenesis are essentially inextricable processes and that tumorigenesis may be the product of abnormal development under undesired circumstances, it is quite reasonable that deregulated Hh signalling was discovered as the promoter of human cancers. It was initially involved in basal cell nevus syndrome in 1996 (5,6). Subsequently, aberrantly active Hh pathways are closely linked to various human cancers (7), including ovarian (8), gastric (9), hepatocellular (10) and bladder cancers (11,12), indicating its significant roles in adult tissue homeostasis maintenance. Therefore, Hh pathway-targeted inhibitors have been developed for cancer therapy (13). Though highly conserved, the Hh pathway is much more complex in vertebrates than in *Drosophila*, likely due to adaptations for the functional complexity of higher multicellular organisms throughout evolution. For instance, the mammalian counterpart of the *Drosophila* Hh ligand is the Hedgehog family of secreted proteins, which contains three members: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh); these proteins perform overlapping but quite distinctive functions. Similarly, the function of the terminal transcription factor Cubitus interruptus (Ci) in *Drosophila* is assigned to three glioma-associated factors (GLI)-GLI1, GLI2 and GLI3-in mammals (14). In addition, the protein suppressor of fused (Sufu), a negative regulator of Hh signalling pathway, seems to display a more important function in vertebrates, where Sufu deletion led to embryo lethality in mice (15) but no visible phenotypic alterations in *Drosophila* (16).

Among the three Hh signalling pathways, the most intensively studied at present is the Shh pathway, which is generally delineated as follows. The Hh ligand binding to the twelve-span transmembrane protein Ptc1 (Patched1), relieves the suppressive effect of Ptc1 on another seven-span transmembrane protein, smoothened (Smo). Active Smo transduces Hh signalling across the cytomembrane to activate GLI through incompletely clarified mechanisms that likely involve

dissociating GLI from a suppressive complex containing Sufu. Finally, activated GLI enters the nucleus to transcriptionally activate a specific set of genes that contribute to certain cellular activities. Furthermore, alternative Hh signalling cascades, through which Hh signalling is transduced through Ptc1 and Smo but are not mediated by GLI, were identified to regulate pain perception (17) and cellular metabolism (18). These alternative cascades comprise non-canonical Hh signalling pathways, which will not be described herein (19). This review focuses within the realm of the canonical mammalian Shh signalling pathway, which will be simply described as the Hh pathway in the following sections.

2. General characterization of Sufu

In early studies (1,16,20), *Hh*, *fused (fu)*, *Sufu*, *Ptc* and *Smo* genes, which we now know are core components of the Hh signalling pathway, were determined to be involved in segment polarity establishment during the embryonic development of *Drosophila*. The *Sufu* gene, located in the 87C8 region of the third chromosome in *Drosophila* (16), encodes a 53-kDa protein with no significant homologies with known proteins (21); its inactivation did not display a visible phenotype but was capable of rescuing the 'fused' phenotype caused by the loss-of-function mutation of the *fused* gene (16). Thus, the gene was called suppressor of fused (*Sufu*), and through interactions with Fu and Ci, the Sufu protein suppresses Ci activity to negatively regulate the *Drosophila* Hh signalling pathway (22).

Subsequently, a series of studies identified Sufu in vertebrates (23), and its roles as a negative regulator were supported. For instance, Sufu in chicken and mice shows relatively high similarity at the amino acid level to *Drosophila* Sufu (24), and analyses revealed that Sufu, primarily expressed in tissues patterned by Hh signalling, interacts with and suppresses GLI isoforms to function as a negative regulator of mammalian Hh signalling (25,26). Furthermore, some remarkable discoveries were made regarding the primary structure, tissue distribution, and biochemical and functional characteristics of human Sufu (27). i) Two isoforms of Sufu resulting from alternative splicing of primary mRNA were discovered, with the shorter isoform being truncated at the carboxy terminus; however, the difference in function between the two splicing isoforms remains to be clarified. ii) Through facilitating the interaction of ubiquitin-ligase Slimb/ β -TrCP with GLI (GLI1, GLI2 and GLI3), human Sufu could regulate the stability and subcellular localization of GLI, ultimately modulating Hh pathway activity. iii) Frequent deletion of the chromosomal region 10q24-q25 spanning the Sufu locus in glioblastoma, prostate cancer and other cancers suggests a tumour-suppressive role of Sufu (28,29). In addition, a structural study of Sufu, which is conducive to illuminating its physical interactions with other proteins such as GLI, defined the crystal structure of the whole Sufu protein. In both human and *Drosophila*, the Sufu protein presents as a monomer consisting of two globular domains with a short linker between them (30,31).

As described above, Sufu acts as a negative regulator of the Hh signalling pathway and plays essential roles in both embryogenesis and adult tissue homeostasis in species ranging from invertebrates to vertebrates. Loss-of-function of Sufu is sufficient to activate the Hh signalling pathway irrespective of

the presence of the Hh ligand (32). In addition, the embryonic lethality in mice due to Sufu deletion (15) implies important functions of Sufu in mammals. These findings strongly suggest a central role of Sufu in the mammalian Hh pathway, which, along with our observation of loss of Sufu in colorectal cancer specimens, intrigued us greatly.

3. Sufu mutation and cancer predisposition

After the Hh pathway was implicated in human cancers by Ptc1 inactivation associated with basal cell carcinomas (5,6), substantial studies have been conducted to explore the tumour-promoting effects and the underlying mechanisms of a hyperactive Hh pathway. Presently, multiple Sufu mutations have been identified (shown in Table I), which are named according to the standard nomenclature recommendations of the HGVS (<http://www.HGVS.org/mutnomen/>) (33). These mutations determine abnormal forms of the Sufu protein among various human cancers, with the medulloblastoma of desmoplastic/nodular subtype being most abundantly studied, which is one of the five histologic subtypes of medulloblastoma and is characteristic of aberrant Hh pathway (34).

In both familial and sporadic desmoplastic medulloblastoma, multiple somatic and germline Sufu mutations that are likely to predispose individuals to tumorigenesis were identified in a series of studies (35-39), with some mutations, such as c.71del and c.71dup, showing incomplete penetrance of the predisposition to medulloblastoma for unknown reasons (40). Brugières *et al* reported that germline Sufu mutations are responsible for over 50% of seemingly sporadic desmoplastic/nodular medulloblastoma cases, especially in patients diagnosed at younger than 3 years of age (35). Although abnormal protein products speculated from the mutated *Sufu* genes are thought to lose their capacity to suppress GLI, only a few of them have been studied with respect to their functional consequences. Studies taking advantage of genetically modified mice were performed to confirm the tumour-stimulating effects of the loss of Sufu. While *Sufu*^{-/-} mice present embryonic lethality with cephalic and neural tube defects, indicating a pivotal role of Sufu in mouse embryonic development, *Sufu*^{+/-} mice displayed characteristic features of human Gorlin syndrome due to enhanced Hh signalling but were less prone to harbour tumour medulloblastoma and rhabdomyosarcoma compared with *ptch*^{+/-} individuals (15,41). However, the absence of the p53 gene could facilitate tumorigenesis in *Sufu*^{+/-} mice. It was shown that *Sufu*^{+/-} p53^{-/-} mice but not p53^{-/-} individuals frequently developed medulloblastoma and rhabdomyosarcoma similar to those observed in *Ptc1*^{+/-} mice (32,42), suggesting that abrogation of Sufu activity remains a critical step to initiate tumours in the context of p53 inactivation. To some extent, this finding is in agreement with the "two-hit" theory of tumor initiation. The observation that *Sufu*^{+/-} mice are less prone than *Ptc1*^{+/-} individuals to develop tumours may be due not only to the differences in functions of Sufu and Ptc1 but also to the genetic background of the experimental mouse model. Taken together, these studies indicate a stimulatory role of Sufu inactivation in medulloblastoma. However, in sharp contrast to the observations of Sufu mutations in the studies above, no somatic or germ line mutations or altered expression in Sufu was revealed in a screen of 145 primitive neuroectodermal

Table I. Germline *Sufu* mutations associated with predisposition mainly to desmoplastic medulloblastoma, not including non-pathogenic *Sufu* variants.

Author, year	Location	Nucleotide change	Speculated protein change	(Refs.)
Spurrell <i>et al</i> , 2016	Exon 1	c.71delC	p.Pro24ArgfsX72	(46)
Spurrell <i>et al</i> , 2016	Exon 1	c.71delC	p.Ala25GlyfsX23	(46)
Svärd <i>et al</i> , 2009; Koch <i>et al</i> , 2004	Exon 1	c.143insA	p.Gln48ProfsX25	(42,43)
Koch <i>et al</i> , 2004	Exon 1	c.44 C>T ^a	p.Pro15Leu	(43)
Heby-Henricson <i>et al</i> , 2012	Intron 1 ^b	c.182 + 3A>T	p.?	(41)
Svärd <i>et al</i> , 2009; Koch <i>et al</i> , 2004	Intron 1 ^b	c.183 - 2A>T	p.?	(42,43)
Heby-Henricson <i>et al</i> , 2012	Exon 2	c.294_295dupCT	p.Tyr99SerfsX23	(41)
Heby-Henricson <i>et al</i> , 2012	Intron 2 ^b	c.318-10delT	p.?	(41)
Heby-Henricson <i>et al</i> , 2012	Exon 3	c.318-?_454+?dup	p.Glu106-?_Glu152+?dup	(41)
Heby-Henricson <i>et al</i> , 2012	Exon 3	c.422T>G	p.Met141Arg	(41)
Scott <i>et al</i> , 2006	Exon 3	c.544G>T	p.Asp182Tyr	(44)
Scott <i>et al</i> , 2006	Exon 3	c.550 C>T	p.Gln184X	(44)
Goetz and Anderson, 2010	Intron 6 ^b	c.756+1G>A	p.?	(59)
Zabidi and Stark, 2016	Exon 7	c.846insC	p.Glu283ArgfsX3	(45)
Koch <i>et al</i> , 2004	Exon 7	c.1018G>T	p.Ala340Ser	(43)
Koch <i>et al</i> , 2004; Zabidi and Stark, 2016; Endoh-Yamagami <i>et al</i> , 2009	Intron 8 ^b	c.1022+1G>A ^a	p.?	(43,45,61)
Heby-Henricson <i>et al</i> , 2012	Exon 9	c.1123C>T	p.Gln375X	(41)
Heby-Henricson <i>et al</i> , 2012	Exon 9	c.1149_1150dupCT	p.Cys484ProfsX14	(41)
Koch <i>et al</i> , 2004	Exon 9	c.1129_1135delTCCGGAG ^a	p.Ser377ProfsX7	(43)
Heby-Henricson <i>et al</i> , 2012	Intron 10 ^b	c.1297-1G>C	p.?	(41)

^aMutations identified in tumours. ^bMutations in introns mainly occur at splice sites, likely leading to large fragment alterations. The nucleotides are numbered and the mutations are named according to the standard nomenclature recommendations of the HGVS (<http://www.HGVS.org/mutnomen/>). *Sufu*, suppressor of fused.

tumours, suggesting that genetic alteration of *Sufu* is a relatively rare event in human medulloblastoma (43). In addition, in a study to discover critical mutational targets in region 10q23.3-10q25.3, which contains *Sufu* and displays deletion in medulloblastoma, no tumour-specific sequence variations or DNA hypermethylation of promoter CpG islands of *Sufu* and other known genes in that region were discovered (44). One explanation for these observed disparities in *Sufu* mutations among medulloblastoma tumours includes the heterogeneous characteristics of tumours. Moreover, it is also possible that in cancers harbouring hyperactive Hh pathways with intact *Sufu* loci, the function of *Sufu* is debilitated through epigenetic modifications of some unidentified regulatory sequences or translational and post-translational modifications of the *Sufu* protein. Nonetheless, these modifications cannot be detected with techniques involving the screening of only genetic mutations or by simply analysing putative regulatory genomic sequence (45,46) or protein expression levels.

In addition to medulloblastoma, *Sufu* mutations have also been found in other human cancers. Applying genome-wide linkage analysis and exome sequencing, Aavikko *et al* revealed a germline *Sufu* mutation (c.367C>T) in meningiomas encoding the protein mutant p.Arg123Cys. As a result,

an altered tertiary structure and compromised function of *Sufu* occurred (47). Genetic mutations in *Sufu* and other Hh pathway components were also detected in mesothelioma (48) and rhabdomyoma (49). Some other studies have linked *Sufu* mutations to basal cell carcinoma (BCC) (50-52) and skin hamartomata (53). It was also reported that the Hh pathway hyperactivation attributed to the loss of *Sufu* or that overexpression of the Hh ligand may participate in the progression and metastases of prostate cancer (54), while the germline splicing mutation c.1022+1 G>A generated a *Sufu* protein with a deletion of exon 8 instead of the general *Ptc1* mutation that was found in a family with atypical Gorlin syndrome (55).

In summary, consistent with the fact that *Sufu* is a critical suppressor of the Hh pathway, which promotes tumorigenesis when aberrantly activated, *Sufu* inactivation has been implicated in various human cancers. Subsequently, the molecular mechanisms underlying the regulation of Hh signalling activity by *Sufu* will be illustrated in detail.

4. Mechanisms underlying the regulation of GLI by *Sufu*

Within the realm of the Hh signalling pathway, substantial research focusing on *Sufu* has revealed multiple mechanisms by

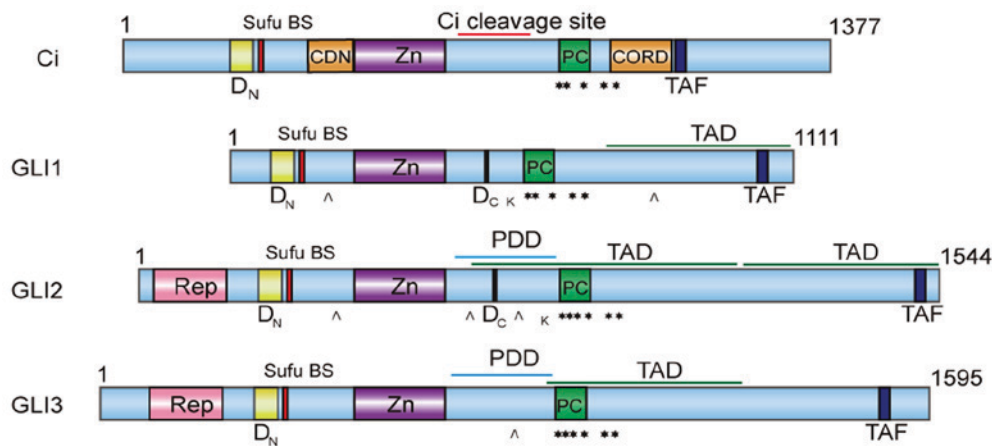


Figure 1. Schematic representation of domains, motifs and modification sites of *Drosophila* Ci and mouse GLI1, GLI2 and GLI3 (14). Ci and GLIs in the figure are aligned against the zinc-finger DNA-binding motif. In mammals, both GLI2 and GLI3 in full-length form can function as transcription activators, whereas under the influence of the processing determinant domain, GLI3 and GLI2, to a lesser extent, will be partially degraded by truncating their C-terminal TADs, resulting in transcription repression. In contrast, GLI1, lacking the processing determinant domain, and the N-terminal Reps in GLI2 and GLI3 can be activated by GLI2, resulting in constitutive transcription activation and indicating Hh pathway activity. In addition, the various motifs and modification sites may imply the high complexity of GLIs in terms of their function and regulation. Ci, Cubitus interruptus; GLI, glioma-associated oncogene homologue; Hh, hedgehog; Zn, zinc-finger DNA-binding domain; TAD, transcription activation domain; TAF, TAF-binding site acidic activation motif; Rep, repressor domain; CDN, CORD, Cos2-binding sites; Sufu BS, Sufu-binding site; PC, phosphorylation cluster; PDD, processing determinant domain; D_N, D_C, degrons; *protein kinase A site; ^sumoylation site; ^acetylation site. Reproduced with permission (14).

which it regulates the transcription activity of GLI, supporting its critical regulatory role in the Hh pathway. As a suppressor of the Hh pathway in both invertebrates and vertebrates, the regulation modes of GLI by Sufu in vertebrates are much more intricate than the regulation of Ci by Sufu in *Drosophila* (56), in part due to different regulation patterns of GLI1, GLI2 and GLI3 because of differences in structure (as shown in Fig. 1) and functionality among GLI proteins (14). As primary mediators of the Hh pathway, GLI2 mainly functions as a potential activator of the pathway, while GLI3 acts mainly as a suppressor (57,58), although they both cooperate to modulate the expression of GLI1 and other target genes. Once generated, GLI1 functions as a constitutive activator to amplify Hh signalling due to the lack of an N-terminal suppressor domain in GLI, which exists in GLI2 and GLI3 (14).

Hh signalling transduction is realized by alterations in the distributions of Hh pathway components, such as Ptc1, Smo, GLI and Sufu, within the primary cilium, a microtubule-based projection of the cell membrane that generally functions in vertebrates as a sensor of various extracellular signal pathways, including the Hh pathway (59). When the Hh signalling pathway is quiescent in the absence of functional Hh ligand, the binding of Sufu to GLI2 and GLI3 sequesters them in a cytoplasmic complex containing a cilium-associated kinesin Kif7 (60-62), known as the vertebrate homologue of Cos2 in *Drosophila*. This sequestration directly prohibits full-length GLI proteins from entering the nucleus and functioning as transcriptional activators. A study indicated that the binding of Sufu to GLI1 impeded the nuclear accumulation of GLI1 (63), consequently inhibiting proliferation, invasion and vascular mimicry of glioma cancer cells, while Sufu knockdown reversed these suppressive effects. Later, Sufu was reported to compete with importin β 1 to bind to the same site on GLI1, thus retarding the importin β 1-mediated nuclear translocation of GLI1 (64). Similarly, by masking the NLS (nuclear localization sequence), Sufu precludes Transportin-mediated

nuclear import of Ci in *Drosophila*, as well as the nuclear accumulation of GLI2 and GLI3 mediated by Kap β 2 (65), the mammalian homologue of Transportin. Recently, a study revealed that Sufu can sequester Ci/GLI proteins in the cytoplasm by binding to their N-terminal regions, while in the nucleus, the suppression of Ci/GLI activity is dependent on their C-terminal regions (66). Moreover, binding of Sufu to GLI2/3 in the cytoplasmic complex facilitates the production of repressor GLI3 (GLI3R) and, to a lesser extent, GLI2R, which result from the partial degradation of the full-length GLI2/3 and function as transcription suppressors (67). The hyper-phosphorylated full-length GLI2/3 resulting from sequential phosphorylation by protein kinase A (PKA), GSK3 β and CK1 within the complex are partially degraded through the ubiquitin-proteasome pathway mediated by SCF^{TrCP} ubiquitin E3 ligase, truncating the C-terminal transcriptional activator domain of GLI2/3 proteins (68,69). In *Drosophila*, a cytoplasmic complex composed of fu, Sufu, Cos2 and Ci similarly regulates Ci activity, suggesting some conserved scenario of Hh pathway regulation by Sufu during evolution. GLI2R and GLI3R likely compete only with full-length GLI2/3 for the same regulatory sequences to inhibit the otherwise activated transcription or may cooperate with co-repressors recruited in a GLI2/3R-dependent manner. Nevertheless, the inhibitory cytoplasmic complex described above would be transformed by Hh signalling (70,71). Following Smo activation, the cytoplasmic complex containing Sufu and GLI2/3 is recruited to the primary cilium tip, where dissociation of the complex releases full-length GLI2 and GLI3 (72), which are subsequently translocated into the nucleus as potential transcriptional activators to take the place of GLIR, while Sufu is induced to degrade (73,74). Recently, it was reported that responding to Hh signalling, Sufu accompanies GLI1 to the primary cilium, which is coupled to the subsequent nuclear transport of GLI1 (75). The necessity of the primary cilium for GLI derepression is supported by the report of a novel

central domain characterized in GLI2 that is essential for both primary cilium localization and transcriptional activity (76). However, the specific mechanisms by which Hh signal is relayed from activated Smo in primary cilium to finally derepress GLI2/3 are far from fully understood. Recently, a primary cilium G protein-coupled receptor, Gpr161 (77), which also regulates the Wnt signalling pathway, was linked to Hh signalling transduction via Smo in primary cilium (78). Several studies have determined that the EVC/EVC2 ciliary complex, whose loss-of-function mutations contribute to Ellis-van Creveld syndrome, interacts with Smo to particularly regulate Sufu/GLI3 dissociation in primary cilium for normal endochondral bone formation, where Evc is needed for Hh pathway activation even when Sufu is deleted (79-81). In contrast, PKA is reported to efficiently suppress ciliary localization of GLI2-Sufu and GLI3-Sufu, while just moderately affecting GLI1-Sufu (72). While essential for antagonizing Sufu activity, the primary cilium is not required for the inhibitory effect of Sufu on the Hh pathway (82), and localization of Sufu in primary cilium is dependent on GLI, which alone is capable of accumulating in primary cilium (83).

However, considering the potentially suppressive effect of Sufu on GLI activity, the finding that the protein levels of GLI2/3 in mice and Ci in *Drosophila* were reduced when Sufu was deleted seems paradoxical (82,84). Sufu could retard proteasome-dependent degradation of GLI2/GLI3 promoted by Spop, a substrate-binding subunit for Cul3-based E3 ubiquitin ligase, by competing with Spop in binding the N- and C-terminal regions of GLI3 and the C-terminal region of GLI2 (67). Notably, the stabilities of the GLI2R, GLI3R and GLI1 proteins are not influenced in this manner. Nevertheless, a recent study reported that Sufu is also essential for maintaining high protein level and nuclear accumulation of GLI1 in response to Hh signalling, sustaining the proliferation of granule neuron precursor cells (75). Mechanistically, Sufu-mediated stabilization of full-length GLI2/3 is of great importance. Without Hh signalling, the binding of Sufu to GLI2/3 preserves a pool of full-length GLI2/3 by preventing complete degradation stimulated by Spop. It also facilitates generation of GLI3R and, to a lesser extent, GLI2R mediated by SCF^{Slimb/β-TrCP}, as mentioned previously (68,69), which restrains gene transcription. As soon as Hh signalling is present, the preserved full-length GLI2/3 are derepressed to produce a transcriptional activator, which counteracts GLI2R and GLI3R, resulting in the rapid response of Hh signalling. Understandably, the activated full-length GLI2/3 are subjected to Spop-promoted degradation and are thus labile and unable to activate the Hh pathway constitutively, which is similar to the situation of Ci seen in *Drosophila* (85).

As in the cytoplasm, Sufu also regulates GLI proteins within the nucleus. For instance, it can recruit the SAP18-mSin3-HDAC co-repressor complex to suppress GLI transcriptional activity (86,87). SAP18 is recruited to GLI3R-Sufu to form a suppressive complex occupying the GLI binding site when Hh signalling is quiescent, and upon Hh pathway activation this suppressive complex will be replaced by GLI1-Sufu (75) to activate the transcription of target genes. Other nuclear proteins mainly involved in basal transcription machinery were identified to interact with Sufu in a yeast two-hybrid screen using mouse Sufu as a bait, indicating crucial functions

of Sufu in the nucleus. Sufu was revealed to interact with those other nuclear proteins and GLI proteins through the N- and C- termini, respectively, to form the suppressive complex (86). Similarly, p66β is the co-repressor that is recruited by Sufu to suppress GLI2/3 activity in the nucleus, whereas Hh signalling can induce Sufu/p66β dissociation from GLI and stimulate GLI activity (88). In addition, binding of Sufu in the nucleus also promotes GLI1 and GLI2 to export from the nucleus to the cytoplasm (89,90). In C3H10T1/2 cells, treatment with leptomycin B, which inhibits nuclear export by binding to the nuclear export receptor CRM1, dramatically shifts the localization of co-expressed GLI1/Sufu from the cytoplasm to the nucleus, suggesting that Sufu stimulates GLI1 export from the nucleus in a CRM1-dependent manner. However, CRM1 is not involved in Sufu-promoted export of GLI2 from the nucleus. It is possible that the inhibition of GLIs in the nucleus by Sufu is a remedy for its failure to sequester them in the cytoplasm.

The integrated regulations of GLI by Sufu are at least partially responsible for further modulating the Hh pathway to maintain graded Hh pathway activity (14,91,92), which is primarily represented by the GLI1/GLI3 ratio and is essential for realizing the complex physiological functions of the Hh pathway within multicellular organisms. For example, deletion of Sufu disrupts the gradient of Ptc1 expression, which displays a high-to-low gradient from ventral to dorsal areas of the wild-type mouse neural tube, similar to the Hh level gradient (67). Furthermore, Sufu deletion compromised Hh pathway activity in the ventral area of neural tube in Sufu^{-/-} mice because more full-length GLI2/3 proteins are completely degraded compared with the situation in wild-type mice; conversely, in the dorsal area of the neural tube, Hh pathway activity is enhanced because GLI3R expression is reduced due to the lack of full-length GLI3 in the absence of Sufu (67). Thus, in combination with other factors, such as Cul3-HIB/SPOP and SCF^{Slimb/β-TrCP}, Sufu functions as a sort of a buffer for regulating GLI proteins (as shown in Fig. 2) that makes it possible to alter Hh pathway activity with high sensitivity in response to Hh signalling. Furthermore, Sufu is helpful for the interpretation of the Hh level gradient into graded Hh pathway activity.

Another important aspect of studies of GLI regulation by Sufu is the exploration of the protein structure of Sufu since it is helpful for explaining the physical interactions between Sufu and GLI (93), which is the basis for the regulations described above. As early as 2003, it was reported that a 19-aa C-terminal fragment in Sufu was essential for the interaction with a novel motif SYGH within residues 116-125 in GLI1, which is consistent with the observation that among the three alternatively spliced transcripts of Sufu, two variants lacking the C-terminal fragment failed to interact with and suppress GLI1 (94). In addition, GLI1 with mutations in the SYGH motif is no longer suppressed by Sufu. Following arduous exploration, great progress has been made in the study of the Sufu protein crystal structure (30,31), which in both humans and *Drosophila* presents as a monomer consisting of two globular domains-NTD (N-terminal domain) and CTD (C-terminal domain)-with a short linker in between. An 'open-to-closed' transition mode of Sufu conformation during interaction with GLI was proposed in both humans and *Drosophila*. In the analysis of the crystal structure of hSufu (with 286-345 aa

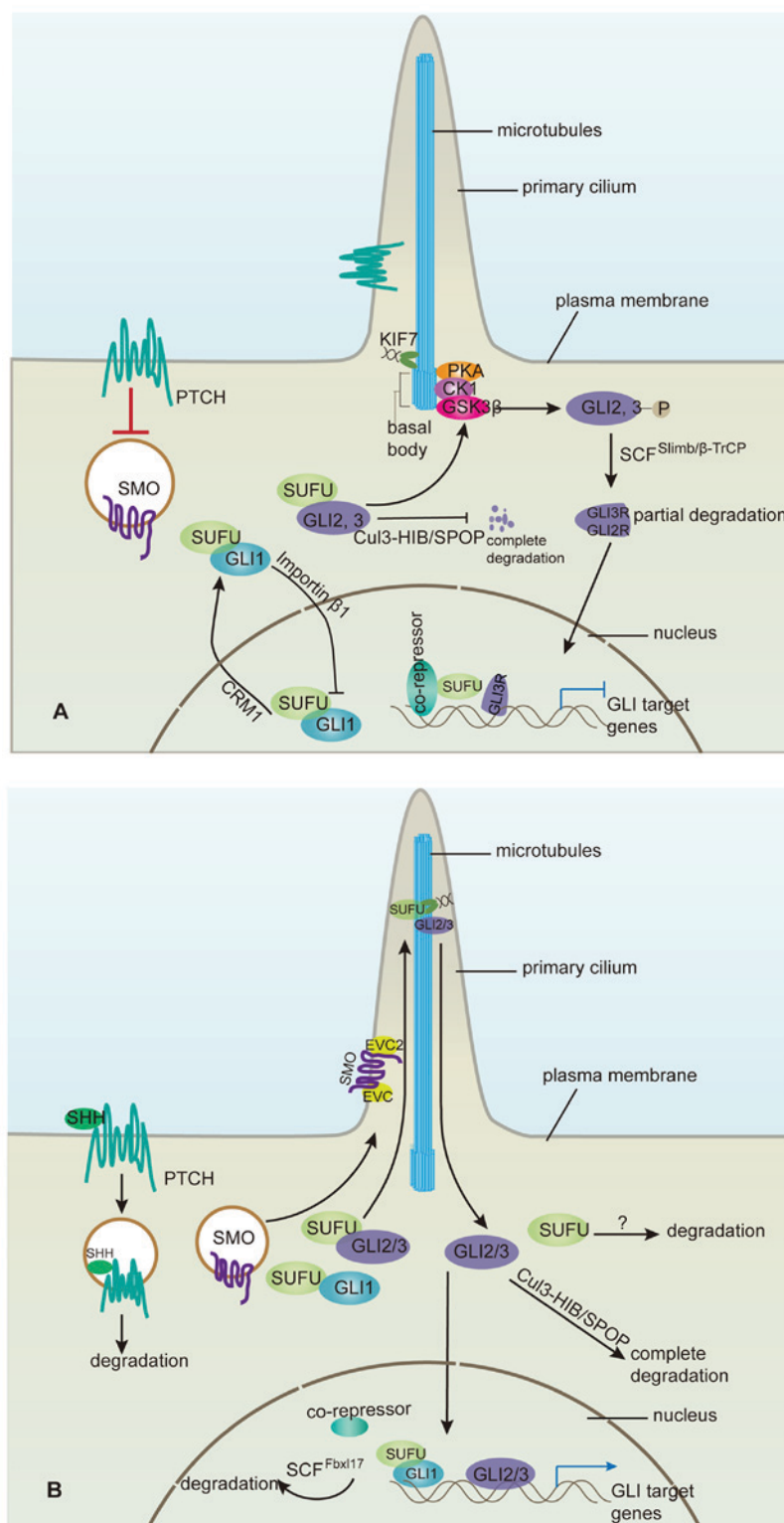


Figure 2. Regulation of GLI by Sufu in the Hh signalling pathway. (A) When the Hh pathway is quiescent without an Hh ligand, the GLI proteins are suppressed by Sufu through multiple mechanisms. In the cytoplasm, Sufu impedes importin β 1-mediated nuclear transport of GLI1, while nuclear Sufu promotes CRM1-mediated GLI1 export from the nucleus. In addition to impacting the nucleus-cytoplasm shuttle of GLI2 and GLI3, binding of Sufu to GLI2 and GLI3 facilitates the SCF (Slimb/ β -TrCP) ubiquitin ligase-mediated partial degradation of GLI2 and GLI3 following sequential phosphorylation by PKA, GSK3 β and CK1. The partially degraded GLI2 and GLI3 are transported into the nucleus to function as transcription repressors (GLI2R, GLI3R), and Sufu can also recruit co-repressor, such as SAP18-mSin3-HDAC and p66 β to suppress GLI activity in the nucleus, which can be reversed by Hh signalling. Meanwhile, Sufu binding can preserve a pool of full-length GLI2 and GLI3 by blocking their Cul3-HIB/SPOP ubiquitin ligase-mediated complete degradation. (B) Following Smo activation by Hh ligand binding to Ptc1, the suppressive cytoplasmic complex containing Sufu, GLI2/3, Kif7 and other proteins is recruited to the primary cilium tip, where dissociation of the complex releases the preserved full-length GLI2 and GLI3, while Sufu is destabilized. Without suppression by Sufu, the full-length GLI2 and GLI3 can function as potential transcription activators; however, they are susceptible to complete Cul3-HIB/SPOP-promoted degradation. Thus, the transcription activators GLI2/3 are labile and cannot continuously activate gene transcription in the absence of Hh signalling, and GLI1 is also transported into the nucleus accompanied with Sufu to stabilize it. In the nucleus, destabilization of Sufu, such as SCF (Fbx17) ubiquitin ligase-mediated degradation of Sufu, results in dissociation of the SAP18-mSin3-HDAC-containing suppressive complex. GLI, glioma-associated oncogene homologue; Sufu, suppressor of fused; Hh, hedgehog; Fbx17, F-box and leucine-rich repeat protein 17; Smo, smoothened.

deleted) in a complex with hGLI1 (112-128aa) containing the SYGHLS motif, Sufu bound to the GLI1 peptide via clamping to the NTD and the CTD, showing the closed conformation of Sufu. This complex could be induced by Hh signalling to dissociate, which most likely occurs by transformation of Sufu from a closed to an open conformation, while the specific mechanism remains to be defined. Moreover, the key residues mediating the association between GLI and Sufu were further investigated through the analysis of the crystal structure in combination with site-specific mutagenesis.

To recapitulate the findings above, depending on the physical interaction between Sufu and GLI in combination with other factors, Sufu is engaged in regulating GLI protein along with the signalling transduction from the cytoplasm into the nucleus, modulating both the subcellular localization and stability of GLI to exquisitely realize the graded activity of GLI proteins in response to the Hh signalling gradient. These sophisticated regulations of GLI by Sufu indicate a central position of GLI-Sufu correlation downstream of Smo in the canonical mammalian Hh signalling pathway.

5. Regulation of Sufu at the mRNA and protein level

In contrast to the relatively extensive accomplishments in the regulation of GLI by Sufu, how Sufu itself is regulated by other factors remains obscure, although there have been some achievements in the study of Sufu regulation at the post-transcriptional level. Given the critical suppressive role of Sufu in the Hh pathway, it is conceivable that Sufu deactivation should be one of the prerequisites of Hh pathway activation, as was previously reported. Hh signalling, especially Shh, is capable of destabilizing the Sufu protein through inducing Sufu polyubiquitination at K257 and its subsequent degradation by the proteasome. In contrast, a K257R mutant of Sufu is, to a large extent, resistant to Hh signalling-induced degradation and exhibits a more potent suppressive function against cell growth (73), suggesting that the much more reduced stability of the Sufu protein is likely responsible for cancer development when there is an excess of Hh ligand. Though ubiquitin-proteasome pathway-mediated degradation is of great importance for Sufu regulation at the post-translational level, the exact ubiquitin ligase involved in this process remains unknown. Further studies revealed that phosphorylation also plays an important role in regulating Sufu stability. When Hh binds to Ptc1, following recruitment and dissociation of the Sufu/GLI complex in primary cilium, GLIs are released to enter the nucleus, while Sufu was transported out of primary cilium and degraded via the proteasome-dependent pathway. Dual phosphorylation at Ser-346 and Ser-342 by PKA and GSK3 β can prolong the stay of Sufu in primary cilium and stabilize the protein (95). Recently, a study showed that the E3 ubiquitin ligase Skp1-Cul1-F-box protein Fbx117 (F-box and leucine-rich repeat protein 17) targets Sufu proteolysis in the nucleus and promotes medulloblastoma (74), thus bridging the above ubiquitination and phosphorylation modifications in Sufu protein regulation. In response to Hh signalling, phosphorylation at Ser-346 and Ser-342 by PKA and GSK3 β is debilitated, while Sufu dephosphorylation facilitates its polyubiquitination at K257 by SCF^{Fbx117} and subsequent degradation, leading to Hh pathway activation.

Given the crucial modulatory effects of Sufu on GLI in both the cytoplasm and the nucleus, and the existence of

different ubiquitin ligases responsible for GLI ubiquitination, such as Cul3-HIB/SPOP and SCF^{Slimb/ β -TrCP}, other ubiquitin ligases for Sufu ubiquitination may exist in the cytoplasm that are distinct from SCF^{Fbx117}, which ubiquitinates nuclear Sufu. Additionally, our team recently identified NIMA-related expressed kinase 2A (Nek2A) as a Sufu-interacting protein by a yeast two-hybrid screen; the enzyme could phosphorylate and stabilize Sufu, consequently dampening Hh/GLI2 signalling (96,97). It is likely that different post-translational modifications, including phosphorylation and ubiquitination, are orchestrated to modulate the stability of the Sufu protein, although additional studies are required to unveil other regulations and the relationships between them.

In addition to regulating the stability of the Sufu protein, Hh signalling also attenuates Sufu activity through suppressing the maturation of Sufu mRNA. In *Drosophila*, the target gene HIB (Hh-induced protein) of the Hh pathway is a substrate recognition component of Cul3-based ubiquitin ligase. In response to Hh signalling, the ubiquitin ligase Cul3-HIB, through poorly clarified mechanisms, stimulates enrichment of the spliceosome factor crooked neck (Crn) in the nucleus, which likely inhibits the formation of functional sufu mRNA and ultimately weakens Sufu activity. Moreover, a similar mechanism may also exist in mammals. Spop, the mammalian homologue of HIB, is also capable of downregulating Sufu levels in *Drosophila* (98). Other factors discovered to impact Sufu mRNA include micro-RNAs. MicroRNA-378 can promote cell survival, tumour growth and angiogenesis through attenuating the translation of both Sufu and Fus-1 (99). Furthermore, microRNA-214 can directly target Sufu mRNA and compromise its function, which facilitates lung adenocarcinoma metastasis and the epithelial-mesenchymal transition (EMT), which are most likely attributed to deregulated Hh signalling (100). Shown here, microRNA-214 and microRNA-378 display tumour-promoting roles, whereas some studies reported tumour-suppressive roles in cervical and breast cancer cells (101,102), implying the intrinsic complexity of multi-cellular organisms and the considerable influence of the microenvironment on gene functions.

Although the mechanism underlying Sufu regulation remains obscure, its exploration is mounting, as the various post-transcriptional regulations that have been identified have begun to delineate the regulatory system focusing on Sufu. These intricate regulatory mechanisms at different levels, through the overall process of gene expression and at the post-translational level, remain to be clarified.

6. Conclusions

While a substantial number of studies have examined the Hh signalling pathway, some mechanisms within the Hh signalling cascade remain elusive. For instance, i) how is Smo inhibited by Ptc1? ii) How does the relay of Hh signalling from activated Smo to GLI occur? iii) How is the specific transcription by GLI proteins realized? and iv) Given the pivotal role of Sufu in Hh signalling, the study on the regulation of Sufu itself seems somewhat inadequate, with no studies reporting on the transcription of the *Sufu* gene. In addition, what has been presented in this review is merely part of the whole picture, especially since Sufu is also implicated in the regulation of the Wnt pathway (103,104),

probably coordinating the Wnt and Hh pathways for their proper functions. In addition, Sufu was first associated with human immunity by its single-nucleotide polymorphism involvement in graft-versus-host disease (105). Furthermore, in a study on non-canonical Hh signalling, Sufu binds to and stabilizes cellular nucleic acid-binding protein (CNBP) to promote polyamine biosynthesis, which supports neuronal and medulloblastoma cell growth (106), suggesting a tumour-stimulating effect of Sufu. This seeming contradiction is likely due to our general inclination to assign definite functions to certain proteins within linear-pattern cellular signalling pathways, which are in actuality more like networks with proteins functioning in context-dependent manners. Perhaps a panoramic view is needed to understand these interconnected networks.

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