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# Ciliary smoothened-mediated noncanonical hedgehog signaling promotes tubulin acetylation

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ABSTRACT

Hedgehog (Hh) signaling plays key roles in animal development and tissue homeostasis. Binding of the secreted ligand to its Ptch1 receptor triggers Hh signaling through distinct canonical or noncanonical signaling pathways. Canonical Hh signaling leads to the activation of Gli transcription factors to induce Hh target-gene expression. In contrast, noncanonical Hh signaling regulates cytoskeleton rearrangement and apoptosis. Recently, it has been shown that primary cilia are important for canonical Hh signaling, but the ciliary role for signaling through the noncanonical pathway remains unresolved. Here, we examine the role of primary cilia in noncanonical Hh signaling in cultured mammalian cells. We found that Hh pathway activation in mouse embryonic fibroblast cells (MEFs) increases microtubule acetylation via smoothened (Smo), and suppression of Hh signaling by a Smo antagonist abrogates the microtubule acetylation. Using genetically engineered MEFs, we revealed that the increase in microtubule acetylation by Hh is dependent on Smo, but not on Sufu or Gli. In *Kif3a*$^{-/-}$ MEFs, which cannot form primary cilia, we observed that primary cilia were required for transducing noncanonical Hh signaling. Furthermore, we revealed that an increase in intracellular calcium is important for Hh-dependent tubulin acetylation at the downstream of Smo. Collectively, these findings suggest that Smo and primary cilia-dependent noncanonical Hh signaling leads to post-translational regulation of microtubules and may be important for modulating cell behaviors.



## 1. Introduction

Hedgehog (Hh) signaling is a key factor in animal development and tissue homeostasis. It regulates body axis determination throughout embryonic organ formation, and disruption of Hh signaling leads to embryonic lethality with abnormal brain, skeleton, heart, and gastrointestinal tract development [1,2]. Although Hh signaling activity is restricted to stem cell niches in renewable tissues in adults, it plays an important role in tissue homeostasis at multiple stages, including cell differentiation, proliferation, and cell cycle progression [3]. However, abnormal reactivation of Hh signaling induces oncogenic transformation and promotes tumor progression in various human cancers, including basal cell carcinoma and medulloblastoma [4].

Hh pathway regulatory circuits begin with binding of the secreted Hh ligand to its receptor patched 1 (Ptch1), which releases a GPCR-like protein, smoothened (Smo). Signaling downstream of Smo diverges into two distinct pathways: canonical and noncanonical Hh signaling. In canonical signaling, Smo regulates suppressor of fused (Sufu) to activate Gli transcription factors. Interestingly, recent evidence suggests that canonical Hh signaling is mediated through a cellular organelle, the primary cilium [5]. The primary cilium is a microtubule-based organelle emerging from the cell surface of most vertebrate cells to detect extracellular environmental changes or process diverse cellular signals important for animal development, such as Hh, Wnt, Notch, and PDGF [5]. In contrast, Smo-dependent noncanonical Hh signaling controls cell motility via cytoskeleton rearrangement, independent of Gli-mediated *de novo* gene expression [6,7]. For example, transcription-independent Hh signaling promotes cellular morphological changes and migration through Rac1 and RhoA GTPase-mediated remodeling of the actin network [8,9]. In addition, Hh can function as a cellular chemoattractant that guides pathfinding axons to target neurons in the developing nervous system [10,11]. However, aberrant activation can lead to tumorigenesis via cell proliferation and metastasis, as multiple coordinated changes in the cytoskeleton are a prerequisite for cell

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migration in cancer cells, as well as in development. Nevertheless, it is unclear to what extent Hh signaling impinges on cytoskeletal regulation.

Microtubules are composed of two distinct subtypes of tubulin, which form heterodimers that can undergo many types of post-translational modification, including polyglycylation, polyglutamylation, detyrosination, and acetylation [12]. Acetylation of tubulin lysine 40 (K40) plays roles in cell differentiation, microtubule stability, and intracellular trafficking. Regulation of tubulin K40 by the acetyltransferases GCN5, α-TAT, and HDAC6 influences kinesin motor protein function [13]. In addition, hyperacetylation of α-tubulin is observed in ciliopathies such as polycystic kidney disease [14].

Here, we examine whether Hh signaling is involved in post-translational regulation of α-tubulin using genetically engineered mouse embryonic fibroblast cells (MEFs). We observed that α-tubulin acetylation is upregulated by Hh ligand treatment in NIH3T3 cells. Furthermore, high levels of acetylation with constitutive Hh pathway activation in *Ptch1*$^{-/-}$ MEFs were reversed by inhibition of Smo with GDC-0449. Hh-mediated tubulin acetylation was not abolished with suppression of canonical signaling via removal of Gli transcription factors nor gain of Hh signaling in Sufu mutant cells. In contrast, tubulin acetylation was regulated by calcium-dependent noncanonical Hh signaling. Moreover, we showed that upregulation of α-tubulin acetylation by Hh is dependent on the presence of primary cilia. Collectively, these findings suggest that Smo and primary cilia-dependent noncanonical Hh signaling leads to post-translational regulation of microtubules, which may be important for modulating cell behaviors.

## 2. Materials and methods

### 2.1. Generation of MEFs

MEFs were isolated from *Ptch1*$^{-/-}$ or *Kif3a*$^{-/-}$ E9.5 embryos. The viscera, heart, limbs, and head were discarded from the embryo, and the remaining tissue was dissected into fine pieces in cold Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT). Tissue was dissociated with Trypsin-EDTA at 37 °C and 5% $CO_2$ for 30 min. The dissociated tissue was mixed with 10% fetal bovine serum containing DMEM and passed through a pipette for further dissociation. MEFs were cultured in a 100-mm culture dish for experiments.

### 2.2. Cell culture

NIH3T3 cells obtained from American Type Culture Collection (Manassas, VA) were seeded on 6-well culture plates at $3 \times 10^5$ cells per well and 24-well culture plates at $2 \times 10^4$ cells per well. Sufu and Gli mutants MEFs were previously described [15,16]. Cells were cultured following standard protocols in DMEM containing 10% fetal bovine serum. Subcultured NIH3T3 cells and primary MEFs were cultured overnight in an incubator at 37 °C with 5% $CO_2$.

To induce ciliogenesis, media was replaced with low-serum (0.5%) media for 2 d and Hh signaling was activated by adding ShhN-conditioned media after cilia generation. ShhN-conditioned media was made by first transfecting 293FT cells with ShhN pMT21 plasmid (kindly provided by Dr. Wendy Ingram, Queensland Children's Medical Research Institute, AU). Media was replaced with fresh DMEM 1 d after transfection, and cells were further incubated for 2 d at 37 °C before collection [17].

### 2.3. Immunoblotting

NIH3T3 cells or primary MEFs were lysed in ice-cold modified RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% NP40, and 0.25% Na-deoxycholate) supplemented with 1 × protease inhibitor (Roche, Indianapolis, IN). Protein extracts were clarified by centrifugation for 15 min at 13500 rpm at 4 °C, and the supernatant was transferred to a fresh tube. Protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL). Equal amounts of protein (10 μg) were loaded onto a gel for electrophoresis, then transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA) using a *Trans*-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). Membranes were washed with Tris-buffered saline solution containing 0.2% Tween 20 (TBST) and blocked for 1 h in TBST containing 5% skim milk. Membranes were then incubated overnight at 4 °C with the following primary antibodies: mouse anti-acetylated α-tubulin, mouse anti-detyrosinated α-tubulin, mouse anti-α-tubulin (Sigma, St. Louis, MO), or mouse anti-β actin (Cell Signaling, Beverly, MA). Membranes were washed and incubated for 2 h at room temperature with adequate secondary antibodies. Signals were detected with chemiluminescent reagent (Millipore) following the manufacturer's instructions.

### 2.4. Immunofluorescence staining

Cultured NIH3T3 cells and primary MEFs were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature followed by three washes with PBS. Fixed cells were incubated with blocking buffer (1% horse serum, 0.1% Triton X-100 in PBS) for 30 min at 4 °C. Cells were incubated in a combination of acetylated-α-tubulin (Sigma, St. Louis, MO), Arl13b, or γ-tubulin (Sigma) antibodies overnight at 4 °C. After three washes with blocking solution, cells were simultaneously incubated with FITC- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Stained cells were imaged on a Nikon confocal laser-scanning microscope. To analyze the localization of different antigens in double-stained tissue, multiple images were obtained from the same area and merged using EZ-C1 software (Nikon, Tokyo, Japan).

### 2.5. Statistical analysis

Data analyses were performed with SigmaStat 3.5 software. Student's *t* tests were used for comparisons between two groups, followed by Tukey's multiple comparison tests for grouped samples. $P < 0.05$ was considered statistically significant. All data are presented as mean ± standard error of the mean (SEM) ($n = 3$ for all experiments).

## 3. Results

### 3.1. Hh signaling promotes α-tubulin acetylation

To unveil the role of Hh signaling in tubulin polymerization and stability, we examined the extent of ε-amino K40 acetylation on α-tubulin in cultured NIH3T3 cells stimulated with ShhN-conditioned media. Hh pathway activation significantly elevated (~2.5-fold) the level of tubulin acetylation compared with control ($P < 0.05$; Fig. 1A). In contrast, other type of α-tubulin post-translational modification, such as detyrosination, was not affected.

ShhN-conditioned media-treated NIH3T3 cells were fixed and immunostained to further examine acetylated tubulin. As shown in Fig. 1B, cells treated with ShhN-conditioned media displayed intense staining of elongated acetylated α-tubulin, which is consistent with the immunoblotting results. These results indicate that Hh signaling regulates α-tubulin acetylation and microtubule polymerization.

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