Kinetics of Hedgehog-Dependent Full-Length Gli3 Accumulation in Primary Cilia and Subsequent Degradation

Xiaohui Wen
Cary Lai
*University of San Francisco, cklai2@usfca.edu*

Marie Evangelista
Jo-Anne Hongo
Frederic J. de Sauvage

*See next page for additional authors*

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Authors
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Departments of Molecular Biology and Antibody Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080

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Hedgehog (Hh) signaling in vertebrates depends on intraflagellar transport (IFT) within primary cilia. The Hh receptor Patched is found in cilia in the absence of Hh and is replaced by the signal transducer Smoothened within an hour of Hh stimulation. By generating antibodies capable of detecting endogenous pathway transcription factors Gli2 and Gli3, we monitored their kinetics of accumulation in cilia upon Hh stimulation. Localization occurs within minutes of Hh addition, making it the fastest reported readout of pathway activity, which permits more precise temporal and spatial localization of Hh signaling events. We show that the species of Gli3 that accumulates at cilium tips is full-length and likely not protein kinase A phosphorylated. We also confirmed that phosphorylation and βTrCP/Cul1 are required for endogenous Gli3 processing and that this is inhibited by Hh. Surprisingly, however, Hh-dependent inhibition of processing does not lead to accumulation of full-length Gli3, but instead renders it labile, leading to its proteosomal degradation via the SPOP/Cul3 complex. In fact, full-length Gli3 disappears with faster kinetics than the Gli3 repressor, the latter not requiring SPOP/Cul3 or βTrCP/Cul1. This may contribute to the increased Gli3 activator/repressor ratios found in IFT mutants.

The Hedgehog (Hh) signaling pathway is important for establishment of left-right asymmetry and formation of various organs during vertebrate embryonic development (30, 44, 49, 86), but it is mainly quiescent in adults. Inappropriate reactivation, however, contributes to various cancers, thus providing an impetus for further research (for recent reviews, see references 33 and 73).

In vertebrates, the Hh signal transduction cascade is initiated by the Hh ligand binding to its receptor Patched 1 (Ptc1), which abolishes Ptc1’s repression of Smoothened (Smo), enabling this seven-transmembrane G-protein-coupled receptor-like protein to transduce the Hh signal via a complex of cytoplasmic proteins (53, 62). This culminates in activation of a set of transcription factors termed Gli1, Gli2, and Gli3 (69) which modulate Hh pathway target gene transcription in the nucleus (34, 68). Unlike in Drosophila melanogaster, where the single Gli homolog Cubitus interruptus (Ci) functions as a transcriptional activator when intact (CiFL) and as a repressor (CiR) when cleaved (2, 4), Gli1 is thought not to be processed and to function solely as a transcriptional activator to enhance signaling (3, 15), being upregulated by pathway activity (45). By contrast, Gli2 and Gli3 can be processed, acting as activators when intact and as N-terminal repressors when cleaved (15, 41, 72, 76, 87).

Gli2 and Gli3 processing is phosphorylation and proteasome dependent: in the absence of Hh, they are phosphorylated at four to six sites by protein kinase A (PKA), which primes them for further phosphorylation by glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) (56, 81, 87, 88). The phosphorylated residues provide a high-affinity binding site for βTrCP, which in turn recruits the SCF (Skp1/Cullin1/F-box) ubiquitin ligase complex to target full-length, ~190-kDa Gli3 (Gli3FL) and ~185-kDa Gli2 (Gli2FL) for cleavage via the ubiquitin-proteasome pathway (81, 88) to generate the ~83-kDa Gli3 (Gli3R) and ~78-kDa Gli2 (Gli2R) N-terminal repressors, respectively, while the C termini are assumed to be completely degraded (57). Hh stimulation represses this processing and is thought to result in a predominance of full-length (presumably activator) forms of Gli2A and Gli3A. Regulation of Gli3 processing is especially important in limb development, with the appropriate ratio of Gli3FL and Gli3R being essential for proper digit number and identity (42, 82) and a diminishing gradient of Gli3R resulting in derepression of Hh target genes from the anterior to the posterior of the limb bud (26).

The paradigm of vertebrate Hh signaling has shifted considerably with the seminal discovery that components of primary cilia exert crucial roles during mouse tissue patterning and development (29), which has been expertly reviewed in references 16, 66, and 94. The eukaryotic primary cilium is a microtubule-based membrane protrusion that is assembled and maintained by the bidirectional intraflagellar transport (IFT) machinery (39). Mutations in IFT subunits Ift88, Ift172, and Ift122 and motors Kif3a and Dyn2h1 all result in polydactyly due to impaired Gli3 processing (14, 25, 28, 29, 43, 47). Moreover, all three Glis and their binding partner Suppressor of Fused (SuFu) have been found in primary cilia (25), while Ptc1 is only there under unstimulated conditions, with Smo replacing it upon Hh stimulation (13, 65).

Although Gli2 and Gli3 localize to the tips of primary cilia...
(25, 36) in an Hh-dependent manner in some cell lines (18), it is not well understood how quickly this happens or what modifications occur in the cilia. By generating antibodies recognizing endogenous Gli2 and Gli3, we show here that full-length Gli2 and Gli3 accumulate at cilium tips within 5 min of Hh stimulation. This rapid Hh response is useful for investigation of ciliary events in the Hh pathway, permitting us to discover, for example, that PKA stimulation with forskolin (FSK) inhibits its Gli3 accumulation. Furthermore, by Western blotting, we unexpectedly found that while Hh signaling does inhibit endogenous Gli3 processing, this does not result in accumulation of the full-length Gli3 precursor, instead promoting its degradation via SPOP, Cul3, and the proteasome, analogous to the degradation of CiA by Hh-induced MATH and BTB domain-containing protein (HIB) and Cul3 (Cul3) in Drosophila (32, 35, 55, 97). Moreover, we confirmed that IFT is required for efficient Gli3 processing and found it is also required for degradation of Gli3FL.

MATERIALS AND METHODS

Cell culture. COS7, IMCD3 (murine intermedullary collecting duct), NIH 3T3 fibroblast, and S12 (Gli-luciferase stably transfected CHI1012/2 osteoblast [21]) cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 10 mm HEPES, and 2 mm l-glutamine. S12 and 3T3 cells were never allowed to become >70% confluent, to maintain proper ciliation and Hh signaling. Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 10.5 (E10.5) embryos following standard procedures and maintained in the above medium plus 100 μM β-mercaptoethanol (Calbiochem, La Jolla, CA), 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 50 μg/ml penicillin-streptomycin (all from Gibco, Grand Island, NY). Hh stimulation was carried out by serum starving cells in appropriate growth medium with only 0.5% FBS for 16 to 24 h and adding 200 ng/ml Hh agonist via SPOP, Cul3, and the proteasome, analogous to the degradation of CiA by Hh-induced MATH and BTB domain-containing protein (HIB) and Cul3 (Cul3) in Drosophila (32, 35, 55, 97). Moreover, we confirmed that IFT is required for efficient Gli3 processing and found it is also required for degradation of Gli3FL.

Antigen production. N- and C-terminal fragments of human Gli1 (amino acids [aa] 2 to 151 and aa 958 to 1106 of NM_005269); Gli2 (aa 2 to 199 and aa 827 to 1126 of NM_030380, the nonsense-mediated mRNA decay sequence lacking the 324-aa autoinhibitory N-terminal domain [72], which corresponds to aa 330 to 544 [Gli1N] and 1172 to 1471 [Gli2C] of the NM_005270 full-length Gli2 sequence); and Gli3 (conserved aa 2 to 184 and aa 1365 to 1547 of the 1,596-aa M34366 Gli3 variant [70], selected as the most C-terminal region conserved in both reported C-terminal frameshift variants [1,596 and 1,580 aa] of human and mouse Gli3) were cloned into the expression vector pBSC200 (Qiagen, Valencia, CA), in a 1:1 mixture of antigen and incomplete Freund’s adjuvant (Josman Labs, LLC, Napa, CA). Immune sera from the 10th week were affinity purified on the resin (Actigel ALD; Sterogene, Carlsbad, CA) in PBS.

Development of anti-Gli MAbs. Ten BALB/c mice (Charles River Laboratories, Holliston, MA) were immunized six times each with a mixture of 2 μg of a 1:1 mixture of fixed and unfolded Hh-Gli3 antigens in Ribi adjuvant (Ribi Immunochem Research, Inc., Hamilton, MO), a mixture of the three N-terminal antigens being injected into five mice and a mixture of the three C-terminal antigens into another five mice, since the N termini were more immunogenic in rabbits. The best two mice from each group were chosen based on high anti-Gli antibody titers determined using an enzyme-linked immunosorbent assay (ELISA); plates coated with a mixture of unfolded and 1% formalin-fixed Gli antigens, as well as specific staining of transiently individually expressed myc-tagged Gli3 in PFA-fixed COS7 cells by IF (see below for details). B cells from the two selected mice in each group were pooled and fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection, Rockville, MD), in a manner similar to that described previously (27, 38). After 10 to 12 days, the supernatants were harvested and screened for antibody binding by ELISA (discarding any that reacted with an irrelevant HQ-tagged protein) and IF. The positive hybridomas were subcloned twice by limiting dilution and the supernatants purified by affinity chromatography (fast protein liquid chromatography; Pharmacia, Uppsala, Sweden) as described before (27). The entire screen from mouse inoculation to selection of the final clones (of 232 initial positive hybridomas) took less than 11 weeks to yield 15 monoclonal antibodies (MAbs) to six different Gli antigens; thus, our fusion and cocloning strategy was reasonable, as all successful hybridomas were all stable and were immortalized and stored at −80°C in PBS. While all 15 MAbs and all 5 pAbs recognized their respective transfected Gli by IF and Western blotting, only the 4 MAbs and 2 pAbs that additionally recognized endogenous Gli by at least one method are characterized herein.

IF. For antibody screening, COS7 cells were transiently transfected for 48 h with full-length human myc-Gli1 (aa 2 to 1106 [78]), myc-Gli3 (aa 2 to 1596 [78]), or human myc-Gli2C (aa 827 to 1126) subcloned into pRKs via Ascl and Xbal harboring homologies matching the most C-terminal region conserved in the manufacturer’s protocol in 96-well black-walled microplate plates (Whatman, Clifton, NJ). Cells were fixed with 3% PFA for 10 min at room temperature, quenched for 10 min in 50 mM NH4Cl, and permeabilized with 0.4% saponin (Sigma) in PBS containing 1% bovine serum albumin (BSA) and 2% FBS. Mouse sera were diluted 1:200 and 1:50 in saponin buffer; hybridoma supernatants were diluted 1:2. Final purified MAbs were used at 5 μg/ml, pAbs at 2 μg/ml, and 1 μg/ml anti-myc tag 9E10 (19) was a positive control. Plates were imaged with a 20× objective, using a Discovery-1 high-content screening microscope (Molecular Devices, Downingtown, PA).

For detecting cilia and endogenous Gli3 with rabbit pAbs, cells were plated in eight-well LabTekIY microscope slides (Nalg Nunc, Naperville, IL), fixed and permeabilized for 4.5 min at −20°C with 100% methanol to preserve centroacrosomal structures. Slides were blocked in PBS–2% BSA, and stained by incubating anti-acetylated tubulin MAb 6-11B-1 (Sigma) at 1:3,000, centrosomes with 1 μg/ml mouse IgG to block nonspecific binding, and 3 μg/ml mouse anti-Gli3. Antibody staining was detected with fluorescein isothiocyanate (FITC)-labeled donkey F(ab')2 anti-mouse heavy and light chains and the equivalent Cy3–anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA). A four-label-staining protocol was employed to costain cilia with anti-Gli MAbs: (i) 1:2,000 rabbit anti-y-tubulin (AccuSpecs, Westburg, NY); and 3 μg/ml anti-Gli MAb; (ii) FITC–anti-rabbit and Cy3–anti-mouse antibody; (iii) 10 μg/ml mouse IgG to block Cy3–anti-mouse antibody; and (iv) Alexa 488 Zenon Fab anti-mouse IgG2b-labeled 6-11B-1, prepared according to the manufacturer’s instructions (Molecular Probes, Carlsbad, CA). Then samples were postfixed in 3% PFA. For convenience the Gli3N polyclonal 2676 was used in all quantitative experiments after demonstrating similar results to 6F5 (see Fig. S2E in the supplemental material). Slides were coverslipped in 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield (Vector Labs, Burlingame, CA) or ProLong Gold (Invitrogen) and visualized by epifluorescence microscopy using a DeltaVision microscope (Applied Precision LLC, Issaquah, WA) powered by SoftWoRx software (version 3.4.4) and with a 60× objective. Figures were compiled using Photoshop CS software (Adobe Systems, Inc., San Jose, CA).

Measurement of ciliary Gli3 signals. Qualitative assessment of the strength of Gli3 staining at cilium tips was monitored by scoring a positive signal as one that was clearly distinct from the surrounding cytoplasmic and nuclear staining in the same cell (see Fig. S2 and S3 in the supplemental material for examples). Around 200 cilia were counted for each sample, and the results were expressed as a percentage of the total cilia counted (average of three independent data sets ± SEM).
standard deviations [SDs], unless otherwise specified). P values for n independent replicate experiments were calculated online using the unpaired t test with n − 1 degrees of freedom (GraphPad).

Quantitative analysis of Gli signal per cilium was performed using ImageJ software (version 1.41; W. Rasband, NIH) by drawing an eight-by-eight circle around the cilium tips in the acetylated tubulin channel and measuring the integrated density in the Gli channel within the same area. The mean fluorescence intensity of all the cilia in one image was averaged with that of the other images in the same experiment, and SDs across all images were calculated.

siRNA transfection. Pools of predesign On-Target-Plus small interfering RNAs (siRNAs) for murine Gli2, Gli3, Smo, SPOP, Cul1, Cul3, IFT88, and Dynch21 or siGenome siRNAs for mouse βTC and Ki67a were purchased from Dharmacon Inc. (Lafayette, CO). S12 cells were seeded into eight-well LabTek II microscope slides at 3 × 10^5 cells/well or into six-well plates at 3 × 10^6 cells/well and reverse transfected with 100 nM final siRNA pools, following a 20 min preincubination of 1 nmol siRNA and 30 μl DharmaFECT-2 (Dharmacon) in OptiMEM (Gibco) at room temperature. After 48 h, cells were shifted into 0.5% FBS medium for an additional 16 h to promote ciliation (with or without Hh for the indicated times).

Immunoprecipitation and Western blot assays. Cells were serum starved to induce cilation for 16 to 24 h with or without 10 μM MG132 (CalBiochem 474790), 100 mM HhAntag (21), 20 μM cell permeant PKA inhibitor (myristoylated 14-22 amide; CalBiochem 476485), 10 μM H-89 (Calbiochem 371963), 10 μM KT5720 (Calbiochem 420320), or 40 μM forskolin (Calbiochem 344270), all with or without Hh. Cell extracts were prepared by lysing EDTA-detached cells with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing freshly added phosphatase inhibitor cocktails I and II (P-2850 and P-5726; Sigma), Complete protease inhibitor cocktail (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) on ice for 30 min. Insoluble debris was removed by centrifugation at 16,000 × g for 20 min at 4°C.

Lysate concentrations were determined by the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL) and compared to BSA standards in RIPA buffer, and the samples were equalized with RIPA buffer. Two-milligram aliquots of lysates were separated on a 4 to 12% Tris-glycine SDS-PAGE (unless otherwise specified) with or without Hh. Cell extracts were prepared by lysing EDTA-detached cells with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing freshly added phosphatase inhibitor cocktails I and II (P-2850 and P-5726; Sigma), Complete protease inhibitor cocktail (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) on ice for 30 min. Insoluble debris was removed by centrifugation at 16,000 × g for 20 min at 4°C.

Lysate concentrations were determined by the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL) and compared to BSA standards in RIPA buffer, and the samples were equalized with RIPA buffer. Two-milligram aliquots of lysates were precleared with 50 μg of 50% (vol/vol) protein G-Sepharose beads, then incubated with 2 μg of pAb or 5 μg of pAb for 1 h at 4°C, and then overnight with 50 μl preequilibrated 50% protein G-Sepharose beads (GE HealthCare) on a rotator. Immunoprecipitates (washed five times) and lysates were boiled in Lane Maker sample reducing buffer (Thermo Scientific, Waltham, MA) for 5 min at 94°C, and half the immunoprecipitates or 30-μg aliquots of the lysates were separated on a 4 to 12% Tris-glycine SDS-PAGE (unless otherwise specified) with Precision Plus protein standards (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (all reagents from Invitrogen, Carlsbad, CA). Membranes were incubated with 2 to 5 μg/ml anti-Gli antibodies in 5% (wt/vol) milk in Tris-buffered saline-0.05% Tween 20 followed by horse radish peroxidise (HRP)-conjugated secondary antibodies. Endogenous Gli bands were visualized with ChemiGlow substrate (Alpha Innotech, San Leandro, CA), while overexpressing Gli antibodies to full-length human Gli2-myc-FLAG (Origene RC217290), Fugene6-transfected into 293 cells as above) and tubulin loading controls (1: 10,000 dilution of 1A2 MAb; Sigma T9028) were visualized with ECL reagent (GE HealthCare). Except where otherwise labeled (in Fig. 1 below and Fig. S1 in the supplemental material), MAb 6F5 was used for all Gli and Western blot assays.

Real-time qPCR. Total RNA was extracted from cells using the RNeasy Protect minikit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. On-column genomic DNA digestion was performed with an RNase-free DNase set (Qiagen). cDNA synthesis from total RNA was conducted using the High Capacity reverse transcription kit (Applied Biosystems, Foster City, CA) with random hexamer primers. Quantitative PCRs (qPCRs) were performed in triplicate on a Prism 7500 sequence detection system (Applied Biosystems) using murine ribosomal protein L19 (mRPL19) as the endogenous control. Gene expression was calculated using the relative quantification (2^(-ΔΔCT)) method. PCR primers and TaqMan probes are listed in Table S1 of the supplemental material.

RESULTS

Generation of antibodies recognizing endogenous Gli2 and Gli3. To detect endogenous Gli3 and to differentiate between full-length protein and the N-terminal repressor, we raised rabbit pAbs and mouse MAb to the N and C termini of Gli1, Gli2, and -3. We identified three antibodies that detected the N terminus of Gli3 (Gli3N), pAb 2676 and MAb 6F5 and 2087, two antibodies to the Gli3 C terminus (Gli3C), pAb 2438 and MAb 18C3, and one antibody to the Gli2 C terminus (Gli2C), MAb 1H6. All six Abs specifically detected their cognate translocated antigens by IF and Western blotting (see Fig. S1 in the supplemental material).

We found that Gli3 immunoprecipitated from ciliated S12 cell extracts with either Gli3N or Gli3C pAbs was readily detected by MAb 6F5 as an ~190-kDa full-length band (Fig. 1A), as seen in mouse embryos with other anti-Gli3 pAbs (41, 87). As expected, the Gli3N but not the Gli3C pAb pulled down the processed N terminus of Gli3 (Gli3R) at ~83 kDa only in the absence of Hh. Immunoprecipitation using Gli3C MAb or pAb pulled down Gli3FL but not a cleavage product of the expected size for the Gli3 C terminus (~107 kDa), suggesting that the C terminus is rapidly degraded in the absence of Hh signaling, as is that of Ci in Drosophila (4), as previously hypothesized (57, 83).

Furthermore, Gli3N MAb 6F5 and 2087 could detect endogenous Gli3 in extracts of ciliated S12, 3T3, and MEF cells without prior enrichment by immunoprecipitation (Fig. 1B and
Gli3 and Gli2 accumulate at ciliary tips in response to Hh in multiple cell lines. IMCD3 (A, B, and G to I), 3T3 (C and D), primary E10.5 MEF (E and F), or S12 (J to L) cells were treated for 16 h in the absence or presence of Hh and costained with various antibodies to Gli3 or Gli2 in the red channel (left), acetylated tubulin (cilia) and γ-tubulin (centrosomes) together in the green channel (middle), and merged with DAPI (blue) on the right. (A and B) Gli3N MAb 6F5 (A) and 20B7 (B) staining of IMCD3 cells with Hh. (C to F) Gli3N pAb 2576 staining of 3T3 (C and D) and MEF (E and F) cells with or without Hh treatment; pAb 2676 also nonspecifically stains the centrosome (see Fig. S2C and D in the supplemental material). (G and H) Anti-Gli3C pAb 2438 staining of IMCD3 cells with or without Hh. An additional Gli3 spot (arrowhead) along the cilium (I), here in IMCD3 cells in the presence of Hh. (J to L) Anti-Gli2C MAb 1H6 staining of S12 cells without Hh (J), with Hh (K), and with Hh after Gli2 siRNA transfection (L). pAbs are labeled alongside in blue, and MAbS are in red, with + or - indicating the presence or absence of Hh, respectively. White arrows indicate cilia tips.

data not shown), with a stronger signal and fewer background bands than all published antibodies (1, 11, 15, 28, 42, 51, 81, 87). As previously observed (42, 87), in the absence of Hh Gli3R sometimes appears as a doublet (p83 and p75), possibly reflecting the higher levels of endogenous Hh detected in these cells (see Fig. S4A to C). Since Ptch exits and Smo enters cilia within 1 h of Hh stimulation in MEFs (13, 65), we expected that Gli2 and Gli3, as downstream effectors of Smo, would follow a similar temporal trend of ciliary accumulation in S12 cells. Surprisingly, however, Gli3 accumulated at the ciliary tip as quickly as 5 to 10 min after Hh addition, peaking at ~80% cilia in 30 to 60 min and declining somewhat thereafter, reaching a plateau of 50 to 60% (Fig. 3B; see also Fig. S3A in the supplemental material). We also measured the average fluorescence intensity of Gli3 per cilia in the same set of images and found good correlation at each time point, except at 0 to 5 min and at 18 h, when the fluorescence intensity measurements were slightly higher (Fig. 3C). This validates our less-time-consuming qualitative scoring method, which was used in all further experiments. Gli2 was similarly first enriched within 5 min, peaked at 30 min in ~50% of cilia, and then declined with similar kinetics to Gli3, except it increased again after 2 h (Fig. 3B and D), probably due to Hh-dependent stabilization (56), as Gli2 mRNA was not upregulated until ~16 h (Fig. 3E).

To determine if Smo follows the same kinetics, we generated a rabbit pAb that recognizes endogenous ciliary Smo (see Fig. S5 in the supplemental material) and confirmed that, as in MEFs, Smo is not significantly enriched in S12 cilia until ~60 min after Hh addition, continuing to accumulate over 18 h to ~70% of cilia (Fig. 3B). This is likely due to insufficient sensitivity of the anti-Smo antibody, because knocking down or inactivating Smo (with siRNAs [20] or the small-molecule inhibitors HhAntag [21, 67] and cyclopamine [9], respectively) prevented Gli3 accumulation following 30 min of subsequent Hh treatment, indicating active Smo as well as Hh is required for Gli3 accumulation (Fig. 3F; see also Fig. S2F to K in the supplemental material). Smo knockdown similarly inhibited Gli2 accumulation in cilia (Fig. 3F). As Smo inactivation also decreased the basal levels of ciliary Gli, endogenous Hh (Ihh or Dhh) (see Fig. S4) is likely responsible for the low levels of ciliary tip staining was also seen with anti-Gli3C (Fig. 2G and H), indicating accumulated ciliary Gli3 is likely full-length, in agreement with the inhibition of Hh processing by Hh and the lack of Gli3R-green fluorescent protein in cilia (25, 51). Occasionally Gli3 appeared as more than one spot, as if fixed in transit to or from the cilium tip (Fig. 2I).
ciliary Gli2 and Gli3 under unstimulated conditions. Thus, it is probable that Smo is translocated at the same time as or prior to Gli2 and Gli3 but is simply more difficult to detect, being spread all along the cilium, compared to the concentrated single spot of Gli at the tip. While we cannot exclude that Smo acts catalytically in cilia, similar to the inhibition of Smo itself by PtcH (79), we think this unlikely, as the ~10% Smo protein remaining after knockdown (Fig. 3F, inset) was insufficient to permit Gli accumulation.

Interestingly, while cyclopamine enriched Smo in primary cilia in the absence of Hh, consistent with recent findings (64, 91, 93), HhAntag did not (see Fig. S5D in the supplemental material), suggesting that this inhibitor must stabilize a different, nonciliary conformation of Smo than cyclopamine. Since both compounds inhibit signaling un-
nder these conditions (96), accumulation of Smo in cilia per se cannot be sufficient for subsequent Gli2 and Gli3 accumulation; rather, Smo must accumulate in its active form, as occurs during Hh stimulation. Alternatively, it is possible that a minimal level of active Smo protein is required, but its detectable localization to cilia is not a prerequisite for Gli3 accumulation.

Smo knockdown or inactivation also prevented Hh from inhibiting Gli3 processing (Fig. 3G), retaining both Gli3FL and Gli3R at levels similar to those under nonstimulated conditions. Surprisingly, while Hh stimulation in control cells inhibited Gli3 processing as expected (15, 42, 87), this did not result in increased levels of Gli3FL, instead consistently decreasing them, an effect that also required active Smo (Fig. 3G). While Hh inhibits Gli3 transcription in chick limb buds, as seen with in situ hybridization (74) and in micromass cultures by Northern blotting (87), qPCR revealed no changes in S12 cell Gli3 mRNA over 72 h of Hh treatment that could account for the loss of Gli3FL using any of three independent primer/probe sets (Fig. 3E and data not shown). Rather, the Hh-mediated decline in Gli3FL is due to posttranslational degradation, as it was prevented by the proteasomal inhibitor MG132 (Fig. 3G), which also inhibited processing, as expected.

Gli3FL and Gli3R are proteasomally degraded with distinct kinetics. To further understand Gli3 processing and degradation, we compared Gli3 levels over a time course of Hh and/or MG132 treatment, especially since IF revealed that ciliation was disrupted after 16 h of MG132 treatment (data not shown). We found that the levels of Gli3FL decreased as early as 1 h post-Hh treatment (when cells and cilia appeared normal), with an apparent half-life of ~2 h under our blotting conditions (Fig. 4A and C). However, Gli3FL did not completely disappear, reaching a minimum plateau of ~30% after 6 h. Proteasome inhibition with MG132 alone slightly increased the basal levels of Gli3FL, consistent with inhibition of Gli3 processing. Importantly, MG132 also inhibited the Hh-dependent degradation of Gli3FL at all time points (Fig. 4A and C), confirming that this is due to proteasomal degradation and not loss of cilia.

By comparison, the Hh-dependent disappearance of Gli3R was about twice as slow as that of Gli3FL (apparent t½2/3 ~4 h) (Fig. 4A and D; see also Fig. S6A in the supplemental material) but did continue to completion, with no detectable Gli3R remaining after 16 h of Hh treatment at this exposure level (Fig. 4A and D). This was likely a combination of proteasomal degradation and inhibited formation via Gli3 processing, because adding MG132 together with Hh retarded the disappearance of Gli3R at the same rate as MG132 alone. It is possible that Hh and MG132 similarly inhibit the de novo formation of Gli3R (by inhibiting Gli3FL processing). However, it is not possible to distinguish whether Hh has no effect on the degradation of preexisting Gli3R or if it accelerates it via a proteosomal pathway.

To confirm that this was not an artifact of MG132 treatment, we compared Gli3 levels over a time course of Hh treatment with that of the protein synthesis inhibitor cycloheximide (CHX). Gli3FL disappeared much faster upon treatment with Hh than with CHX (Fig. 4B and E), confirming it is posttranslationally degraded upon Hh stimulation. CHX did not increase the rate of the Hh-mediated disappearance of Gli3FL. Gli3R remained stable for ≥6 h under nonstimulated conditions (CHX alone), and interestingly, adding CHX to the Hh treatment actually inhibited the disappearance of Gli3R compared to Hh alone (Fig. 4B and F; see also Fig. S6 in the supplemental material), suggesting that the proteins that mediate Gli3R degradation may themselves be short-lived (turned over within 3 h) and that their identities may be different from those mediating Gli3FL degradation (see below).

Full-length Hh-activated Gli3 is degraded by the SPOP/Cul3 complex. In the developing eye of Drosophila Hh-activated CiFL (CiA) is labile and proteasomally degraded in the nucleus via binding to HIB (97), also known as Roadkill (35), in a complex with the Cul3 ubiquitin ligase (55). Mammalian SPOP (speckle type POZ protein) is 79% identical to HIB and can functionally substitute for it in degrading Ci in vivo, as well as degrading ectopically expressed Gli2 and Gli3 (97). We therefore asked whether SPOP and Cul3 could be the E3 ligase responsible for the lability of Gli3FL in Hh-stimulated S12 cells. Indeed, siRNA-mediated knockdown of either SPOP (by 80% at the mRNA level) or to a lesser extent Cul3 (by 70%) stabilized Gli3FL 5- to 6-fold in the presence of Hh relative to the nontargeting control (Fig. 4G; see also Fig. S8A and B in the supplemental material). Importantly, Gli3FL was stabilized relatively more in the presence than absence of Hh, leading us to speculate that the nonphosphorylated form of Gli3FL may be the preferred substrate of SPOP. (The 1.4-fold increase in Gli3FL without Hh likely reflects the portion of Gli3FL activated by endogenous Hh.) This is consistent with the ability of HIB to degrade a non-PKA-phosphorylatable mutant of Ci and its preference for CiA over CiFL (97). Unlike HIB, however, SPOP itself was not significantly upregulated by Hh stimulation at this time point (see Fig. S8A).

Gli3 processing in the absence of Hh was not inhibited by loss of SPOP/Cul3 but was inhibited by loss of βTrCP and the SCF complex component Cul1, as expected from earlier results with transfected Gli3 (81, 88). Both Gli3FL and Gli3FL:R ratios (Fig. 4H) increased more with knockdown of Cul1 (by ~75% at the mRNA level) than with βTrCP, which we could not silence to >50% without inducing toxicity (see Fig. S8C and D in the supplemental material). Note that unlike with MG132 (Fig. 4A and D), Gli3R completely disappeared after 16-h Hh treatment in the absence of either SPOP/Cul3 or βTrCP/Cul1 complexes, suggesting that yet another E3 ligase complex promotes its proteasomal degradation. This would be consistent with our above hypothesis that the mediators of Gli3R degradation are short-lived (~3 h), since the half-life of Cul3 is as long as 6 h in MEFs (48) and similar to that of Cul1 in Drosophila larval cells (95). Taken together, these results support the hypothesis (97) that the function of HIB/SPOP is conserved from flies to mammals, degrading unphosphorylated Gli3FL in the presence of Hh, while the βTrCP/Cul1 complex processes phosphorylated Gli3FL into Gli3R in the absence of Hh.

Inhibition of Gli3FL degradation increases the levels of ciliary Gli3. The above results suggest that Hh-activated Gli3 is labile because SPOP degrades presumably unphosphorylated Gli3FL in an Hh-dependent manner. Since Hh also accumulates Gli3FL at cilium tips (Fig. 2H), we speculated that increasing the available pool of Gli3FL by SPOP knockdown would result in accumulation of ciliary Gli3 in an Hh-dependent manner. Indeed, siRNAs to SPOP (and to a lesser extent Cul3) did cause a small but reproducible increase in the per-
percentage of Gli3-positive cilia after 16 h of Hh treatment, compared to control Hh-stimulated cells (Fig. 4J) and, as expected, this depended on the presence of Hh. More strikingly, a 2-fold increase in average Gli3 fluorescence intensity per cilium was observed compared to control cells (see Fig. S7 in the supplemental material), supporting our hypothesis that unphosphorylated Gli3FL may be the accumulated species. As expected from the 2-h half-life of Gli3FL in the presence of Hh (Fig. 4C), 30 min of Hh stimulation was not sufficient to accumulate this extra ciliary Gli3 in SPOP-deficient cells (Fig. 4K).

To confirm that Hh is required for ciliary Gli3 accumulation and that mere accumulation of Gli3FL by inhibiting its pro-
cessing or degradation with MG132 is not sufficient, we compared ciliary Gli3 following Hh or MG132 treatment. At no time point could MG132 substitute for Hh in accumulation of ciliary Gli3 (Fig. 4I); thus, Hh activation is essential, and so we speculate that it is Gli3A rather than Gli3FL that accumulates in cilia.

Enhanced ciliary Gli3 was also observed following enrichment of Gli3FL with siRNAs to CUL1 (and to a lesser extent βTrCP, again likely due to less efficient knockdown) (Fig. 4J and K). However, unlike SPOP knockdown, a slight increase also occurred in the absence of Hh, since Gli3FL already accumulates in the absence of Hh via inhibited processing (Fig. 4H). A small but significant increase in Gli1 levels in the absence of Hh resulted from knockdown of βTrCP/CUL1 as well as with SPOP (see Fig. S8A to D in the supplemental material); thus, these results can likely be explained either by accumulation of Gli3A (activated by endogenous Hh) or by pathway derepression due to loss of Gli3R. However, the enhanced ciliary accumulation of Gli3FL under Hh-stimulated conditions did not lead to significantly greater Gli1 transcription compared to Hh-stimulated cells with intact E3 ligases. This suggests either that Gli3A is not the major transcriptional activator of Gli1 in S12 cells (perhaps because it is degraded too quickly, as Gli1 is not elevated until 3 h of Hh stimulation [Fig. 3E]) or that ciliary Gli3FL, while unphosphorylated, is not the functional equivalent of Gli3A. Nonetheless, the levels of ciliary Gli3 do mirror the subsequent levels of Gli1 transcript, but they are elevated more rapidly in response to Hh.

**PKA-phosphorylated Gli3 does not accumulate in primary cilia.** The βTrCP/SCF complex is specific for Gli3 phosphorylated by PKA/CKI/GSK3β in the absence of Hh (88), while HIB (and presumably SPOP) can bind and mediate degradation of unphosphorylated Cia1 (32, 97). Since Hh stimulation is required for both Gli3FL accumulation in cilia and SPOP-mediated degradation, we hypothesized that ciliary Gli3 should be non-PKA phosphorylated. To test this, we examined whether accumulation or depletion of PKA-phosphorylated Gli3 (using the PKA stimulator FSK or PKA inhibitors) prevented or enhanced Gli3 accumulation in cilia, respectively.

The efficacies of FSK and three different PKA inhibitors, H-89, KT5720, and myristoylated 14-22 amide, were first verified by testing their effects on Gli3 processing. As expected, FSK stimulated Gli3 processing irrespective of Hh treatment, as previously shown (81, 88). The possibility that FSK simply stabilizes preexisting Gli3R rather than stimulating processing per se was ruled out by its lack of effect in CUL1-depleted cells (see Fig. S8G in the supplemental material). Conversely, all three PKA inhibitors inhibited Gli3 processing and accumulated Gli3FL (Fig. 5A; see also Fig. S8H in the supplemental material). Further experiments utilized myristoylated 14-22 amide (hereafter abbreviated as PKAi) due to its higher potency and greater PKA selectivity. Neither FSK nor PKAi affected Gli3 mRNA levels, confirming that these changes are posttranscriptional (see Fig. S8E). Furthermore, consistent with PKA being a known negative regulator of the mammalian Hh pathway (24, 75, 90), the 4-h FSK treatment inhibited Hh-induced Gli1 transcription as well as decreasing basal levels by ~40% (see Fig. S8F). Conversely, PKAi caused a small but significant upregulation of Gli1 mRNA (see Fig. S8F), likely due to pathway derepression induced by loss of Gli3R.

As predicted, phosphorylation of Gli3 via FSK treatment completely abolished its accumulation in cilia, both with and without concomitant addition of Hh for 30 min (data not shown) or for 4 h (Fig. 5B), even though Gli3FL is still abundant at these time points (data not shown). Conversely, 4-h PKAi treatment led to accumulated Gli3 in cilia even without Hh treatment and to a similar level as Hh treatment alone (Fig. 5C). Hh stimulation was not additive with PKA inhibition with respect to either the percentage of Gli3-positive cilia or the stimulation of Gli1 transcription (Fig.
This confirms that non-PKA-phosphorylated Gli3 preferentially accumulates in cilia and that one role of Hh is to antagonize PKA activity toward Gli3 (87), as previously proposed for Ci activation (90). This does not mean, however, that PKA phosphorylation of Gli3 does not occur in cilia, just that phospho-Gli3 does not accumulate at cilia tips.

Because FSK inhibits Gli3 accumulation as early as 30 min (prior to any detectable changes in Gli3 transcription), we wanted to determine whether this was due to inhibited translocation of Smo. Unlike Gli3, however, Smo did accumulate all along cilia after a 4-h FSK treatment, to a comparable level as with Hh treatment, and was further enhanced by Hh treatment (Fig. 5B). This confirms previous observations that FSK inhibition of pathway activity occurs downstream of Smo translocation: Chuang et al. reported similar results in MEF cilia after overnight FSK incubation (93), although by that time point Smo accumulated only at the base of (not all along) the cilium, which they hypothesized to be inactive Smo. The base of the cilium cannot be the only location of inactive Smo, however, since cyclopamine-inactivated Smo accumulates all along the cilium (64, 91, 93) (see Fig. 5D in the supplemental material).

Furthermore, Smo also showed significant ciliary accumulation in PKAi-treated cells, although to a lesser extent than with Hh or FSK stimulation (Fig. 5C). Thus, while it is unclear whether Smo all along cilia after FSK treatment is active or not, ciliary Gli3 clearly only accumulates when not PKA phosphorylated.

Phospho-Gli3 is dephosphorylated or processed. To examine whether PKA-phosphorylated Gli3 is stable in the absence of Hh, we attempted to enrich it with FSK or the protein phosphatase inhibitor okadaic acid (OKA), both in the presence of MG132 to suppress any processing of phospho-Gli3. Immunoblotting with 6F5 on a low-percentage gel permitted resolution of a doublet of Gli3FL in the absence of MG132, presumptively unphosphorylated and phosphorylated Gli3 (although other posttranslational modifications are also possible), both bands of which were stabilized by MG132 (Fig. 5D, lanes 1 and 2). Addition of OKA caused a marked decrease in electrophoretic mobility (lane 3), while FSK did not unless OKA was also present (lanes 4 and 5). Combined with the knowledge that FSK must effectively stimulate the Gli3 phosphorylation cascade, because it efficiently stimulated Gli3 processing (Fig. 5A), these data imply the existence of robust phosphatase activity (in the absence of OKA). Although we cannot exclude the presence of additional modifications, such as ubiquitination during OKA treatment, these data are consistent with the known accumulation of hyperphosphorylated Ci by OKA (8) and imply that Gli3FL can be similarly hyperphosphorylated but subject to rapid processing or dephosphorylation by an OKA-sensitive phosphatase, such as PP2A (31). The OKA-induced retardation of migration in the presence of MG132 was detectable as early as 3 h of treatment and was not observed in the absence of MG132, due to enhanced processing (Fig. 5E). Taken together, these results suggest that Gli3 is dynamically phosphorylated and either processed or dephosphorylated and that non-PKA-phosphorylated Gli3FL preferentially accumulates in cilia upon Hh stimulation.

IFT is required for efficient Gli3 degradation as well as processing. Gli3 processing is thought to require cilia, or at least intact IFT machinery, as Gli3R formation is impaired in a variety of ift mutant mice, resulting in an increased Gli3FL:R ratio and polydactyly in limb buds (16, 28, 29). However, our discovery of the Hh-induced lability of Gli3FL makes it possible that this increased ratio could be at least partly due to inefficient SPOP-mediated Gli3FL degradation. To determine if this is the case, we knocked down the anterograde IFT component IFT88 (25, 29, 43) and the anterograde and retrograde motors Kif3a and Dyn2ch1 (28, 47), respectively, with siRNA and examined Gli3 levels with or without Hh treatment. Kif3a knockdown was not very effective and so had less of an effect on signaling, as assessed by Hh-stimulated Gli1 upregulation (Fig. 6E), than the ~70% knockdown levels of Dyn2ch1 and IFT88 (see Fig. S8I in the supplemental material). As previously reported, in the absence of Hh disruption of the latter two IFT components decreased production of Gli3R by up to 60% (Fig. 6A and B) and increased Gli3FL levels by ~3-fold (Fig. 6C). As predicted, in the presence of Hh, Gli3FL levels were indeed also elevated, by ~10 times compared to control siRNA-treated cells, consistent with impaired degradation in activated IFT-depleted cells. Thus, both inhibition of Gli3R formation and inhibition of Gli3FL degradation contribute to the increased Gli3FL:R ratios under these conditions (Fig. 6D) and likely also in ift mutant mouse limb buds, given the known expression of Hh there (7).

**DISCUSSION**

We have shown here using four new antibodies capable of detecting endogenous Gli3 that this transcription factor local-
izes to the tips of primary cilia within 5 to 10 min of Hh stimulation in four different cell lines. The ciliary Gli3 is full-length, non-PKA phosphorylated, and depends on active Smo as well as Hh. Full-length Gli2 also accumulates in cilia with similar kinetics in a Smo- and Hh-dependent fashion. The relative Gli2 and Gli3 accumulation levels upon Hh stimulation are greater in cells expressing low levels of endogenous Hh (like S12 cells) than in those with higher levels of Hh (like MEFs), which might explain why ciliary Gli3 appeared to be Hh independent in the Hh-expressing limb bud cultures, where Gli3 was first seen in cilia (25). During review of this report, Chuang and colleagues reported similar Hh-stimulated endogenous Gli3 and Gli2 accumulation in MEF cilia by using independent antibodies (10), corroborating our results.

Our findings that ciliary Gli3 is full-length and requires both Hh and lack of PKA phosphorylation are consistent with previous proposals that activation of Gli3FL into Gli3A occurs via anterograde transport into cilia (28, 84). Full-length Gli2 also accumulates in cilia in an Hh-dependent fashion with similar kinetics and may similarly be activated into Gli2A at cilium tips because in alien mutant mice (mutated in thml1/Jft139) it accumulates there due to impaired retrograde IFT, leading to ventralized neural tubes characteristic of excessive Gli2A activity (84). An activation step other than inhibition of PKA phosphorylation is also likely because although non-PKA-phosphorylatable mutants of Gli2 and Gli3 (P1 to P4) exhibit constitutive activity, both require Hh for full activation in vivo (58, 89).

In the absence of Hh, low basal levels of Gli2 and Gli3 are seen at cilium tips, likely at least partly due to endogenous Hh signaling, since their levels decrease upon Smo inhibition. However, it is also possible that Gli2 and Gli3 constitutively cycle through cilia at low levels, as has been proposed for Smo itself (54), and that the rate of ciliary entry increases or ciliary exit decreases upon Hh stimulation. Alternatively the rate-limiting step in Gli2 and Gli3 translocation could be during the switch from anterograde to retrograde IFT, resulting in transient accumulation that permits an Hh-dependent activating modification. Or, something could specifically tether activated Gli2 and Gli3 at cilium tips. Such a tether is unlikely to be Smo, because it accumulates all along the cilia, not just at tips, in response to Hh.

Since PKA has been localized to cilia (46, 60, 71), while the downstream kinases CK1ε and GSK3β (23, 77) and ubiquitin ligase components βTrCP, Skp1, and Cul1 are all found at centrosomes (22, 63), the simplest working model for Hh-dependent Gli3 accumulation is as presented in Fig. 7. In the absence of Hh, low levels of Gli3FL cycle in and out of cilia via IFT, becoming phosphorylated by PKA but not accumulating at cilium tips (Fig. 7A). Upon returning to the centrosome at the base of the cilia, phospho-Gli3 is further phosphorylated by GSK3β and CK1ε, becoming a substrate for βTrCP/Cul1 ubiquitination and processing into Gli3R by the proteasome, a portion of which is also found at centrosomes (92). Gli3R then translocates to the nucleus to inhibit Hh target gene expression prior to being degraded by an unknown E3 ligase complex. We suspect that the nuclear Gli3 signal detected with anti-Gli3N MAb 6F5 is mainly Gli3R, because it mostly disappears following overnight Hh treatment, when little Gli3R remains (see Fig. S1F in the supplemental material), although it is not yet clear whether the rate of Gli3R degradation per se (as opposed to inhibition formation) is affected by Hh.

In the presence of Hh (Fig. 7B), PKA is inhibited, preventing the Gli3 phosphorylation cascade and permitting unphosphorylated Gli3FL to accumulate at cilium tips, where we propose yet-to-be determined modifications activate it into Gli3A. While Gli3 accumulation can be detected faster than the exchange of Ptc1 for Smo in cilia (65), it does depend on active Smo. We speculate that Gli3A then translocates to the nucleus, consistent with the notion that it can act as a transcriptional activator (76) and is rapidly degraded (t1/2 of ~2 h in S12 cells) by the SPOP/Cul3 complex and nuclear proteasomes (5), perhaps explaining its relatively minor contribution to Gli1 transcription in these cells. Our proposed degradation of Gli3A by SPOP/Cul3 is analogous to that of CiA by the HIB/Cul3 complex in Drosophila. Like HIB, endogenous mamm-
malian SPOP localizes to nuclear speckles (50) and can recruit Cul3 from the cytoplasm along with degradation substrates (40), likely including Gli3. Other scenarios for Gli3 phosphorylation and its inhibition by Hh are also possible, including Hh-regulated relocation to cilia of one or more of the kinase or E3 ligase components themselves.

Our finding that endogenous Gli3FL (or Gli3A) is degraded by endogenous SPOP in an Hh-dependent manner is in partial agreement with recent findings by Chuang and colleagues, who showed that overexpressed Gli3FL (and Gli2FL) could be degraded by overexpressed SPOP (10), albeit in large cytoplasmic “blobs” reminiscent of inclusion bodies rather than in the nucleus. However, they did not find degradation to be Hh dependent, perhaps because overexpression disrupts Hh regulation; indeed, transfected Gli accumulates in cilia without Hh (10, 25). Alternatively, the higher endogenous Hh levels in MEFs may obscure the Hh dependence of SPOP degradation, although exogenous Hh still promotes ciliary accumulation of endogenous Gli3 in these cells.

Our results also have implications for understanding the role of cilia in Gli3 function. While IFT renders Gli3 processing more efficient, perhaps by concentrating Gli3 together with endogenous Gli in these cells.

Since PKA is present in nonciliated organisms and is not required for Gli3 phosphorylation and processing of Gli3 in cilia (although Gli3 could still transit through cilia undetected). GI

It should be noted that the Gli3 spot at the tip of cilia at any one time is only a small fraction (likely less than 0.01%) of the total Gli3 seen on a Western blot. The small fraction of Gli3 in cilia makes it unlikely that Gli3 is simply protected from cential processing by sequestration at cilium tips in the presence of Hh. It may also explain the disconnect between the rapid ciliary accumulation in minutes and prominent Gli3 band intensity changes in hours. It has recently been proposed that SuFu may regulate Gli2 and Gli3 stability by competing for SPOP downstream of cilia altogether (10).

In summary, our novel antibodies recognizing endogenous Gli2 and Gli3 are valuable tools for better understanding the Hh pathway, and the Gli ciliary accumulation assay can be used to decipher Hh signaling events upstream of Gli. We recently found that Ki7, which itself concentrates at cilium tips upon Hh stimulation, is required for maximal levels of Gli accumulation per cilium (18), although it does not affect the overall percentage of positive cilia. However, many questions remain to be addressed, including how Gli2 and Gli3 accumulate at cilium tips upon Hh stimulation, what happens to activate them there, and how their transport to the nucleus from the cilium and their subsequent degradation is regulated.

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