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Sonic hedgehog Signaling Regulates Gli2 Transcriptional Activity by Suppressing Its Processing and Degradation†

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Gli2 and Gli3 are the primary transcription factors that mediate Sonic hedgehog (Shh) signals in the mouse. Gli3 mainly acts as a transcriptional repressor, because the majority of full-length Gli3 protein is proteolytically processed. Gli2 is mostly regarded as a transcriptional activator, even though it is also suggested to have a weak repressing activity. What the molecular basis for its possible dual function is and how its activity is regulated by Shh signaling are largely unknown. Here we demonstrate that unlike the results seen with Gli3 and Cubitus Interruptus, the fly homolog of Gli, only a minor fraction of Gli2 is proteolytically processed to form a transcriptional repressor in vivo and that in addition to being processed, Gli2 full-length protein is readily degraded. The degradation of Gli2 requires the phosphorylation of a cluster of numerous serine residues in its carboxyl terminus by protein kinase A and subsequently by casein kinase 1 and glycogen synthase kinase 3. The phosphorylated Gli2 interacts directly with βTrCP in the SCF ubiquitin-ligase complex through two binding sites, which results in Gli2 ubiquitination and subsequent degradation by the proteasome. Both processing and degradation of Gli2 are suppressed by Shh signaling in vivo. Our findings provide the first demonstration of a molecular mechanism by which the Gli2 transcriptional activity is regulated by Shh signaling.

The family of secreted Hedgehog (Hh) signaling molecules plays fundamental roles in the patterning of many embryonic structures of animals ranging from flies to humans (21). In *Drosophila* species, Hh signaling is transduced through Cubitus Interruptus (Ci), a transcription factor with a centrally localized zinc-finger DNA-binding domain. In the absence of a Hh signal, a significant fraction of the full-length Ci (Ci155) undergoes proteolytic processing at its C terminus near the zinc-finger DNA-binding domain to create a transcriptional repressor, Ci75 (4). Ci155 processing requires the phosphorylation of a cluster of at least three serines at its C-terminal region by protein kinase A (PKA) (11, 24, 44), which primes the further phosphorylation of adjacent casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) sites (22, 45). It is also dependent on Slimb, the fly homolog of the vertebrate βTrCP protein which is a component of the SCF complex, and on proteasome activity (10, 24, 58). In the presence of Hh signaling, Ci proteolysis is inhibited by an unknown mechanism and Hh targets are activated. Thus, Ci acts both positively and negatively in regulating Hh targets depending on the presence or absence of Hh signaling.

In vertebrates, the role of Ci has been expanded into three Gli proteins: Gli1, Gli2, and Gli3 (27, 48). Reporter gene assays and analysis of marker gene expression in transgenic and mutant animals have demonstrated that Gli1 functions as a strong transcriptional activator (6, 13, 20, 26, 32). In agreement with its role, Gli1 protein has not been found to contain any repressor domain and to be proteolytically processed (13, 25, 49). Nevertheless, while Gli1 gene is required for Hh signaling in zebra fish (26), loss of *Gli1* gene function in the mouse does not appear to result in obviously abnormal phenotypes (5, 42). Therefore, Gli1 is not essential for initial Sonic hedgehog (Shh) signal transduction in the mouse and its expression is a readout of Shh signaling.

In contrast to *Gli1* results, loss-of-function mutations in *Gli3* result in phenotypes that largely resemble those produced by activation of Hh signaling in both mice and humans (19, 55). Analysis of expression of Hh signaling marker genes has shown that Gli3 mainly functions as a transcriptional repressor (1, 17, 33, 43, 46, 52, 53), even though recent studies have also shown that Gli3 can act as a weak activator in vivo (7, 32a, 40, 54). Consistent with this, we and others have previously shown that the majority of full-length Gli3 protein (Gli3-190) is processed to generate a Gli3-83 transcriptional repressor in the absence of Shh signaling and that Shh signaling blocks Gli3 processing through an unknown mechanism (18, 34, 56). Like Ci, Gli3 processing is dependent on PKA activity, although it has not yet been shown whether GSK3, CK1, βTrCP, and proteasome are required.

Mouse Gli2 appears to exhibit transcriptional activity dis-

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tinct from that of Gli3, even though they share a high degree of sequence similarity, including activator and repressor domain sequence similarity. A cell-based reporter assay has shown that Gli2 exhibits a stronger transcriptional activity than Gli3, albeit Gli2 is a weaker activator than Gli1 (49). Several studies have also suggested that Gli2 may normally function only as a transcriptional activator. For example, loss-of-function mutations in mouse *Gli2* result in loss of most ventral cell types in the developing neural tube (14, 36). Furthermore, *Gli1* is capable of rescuing *Gli2* mutant phenotypes when it is inserted into the *Gli2* locus (6) but Gli3 is not (7). However, recent genetic analysis of *Gli2* mutants on a *Gli3* null background has also indicated that Gli2 could have repressor activity in addition to its primary function as an activator (9, 37). In zebra fish, Gli2 may act as a transcriptional activator or a repressor, depending on expression of different marker genes in different tissues (26). As some of marker genes analyzed in both mouse and zebra fish may not be the direct targets of Gli2, a definitive answer to the question of whether Gli2 functions only as a transcriptional activator or as both an activator and a repressor certainly requires understanding of the molecular nature of Gli2 protein activity.

At the molecular level, little is known about how Gli2 transcriptional activity is regulated in response to Shh signaling. Although Gli2 and Gli3 share an overall amino acid identity of 44%, including conserved PKA sites, it remains questionable whether Gli2 activity is regulated through a proteolytic processing mechanism. On the one hand, PKA stimulation failed to induce Gli2 processing when Gli2 was overexpressed in cultured cells (47, 56). On the other hand, overexpression of frog Gli2 in *Xenopus* embryos produces several protein fragments smaller than full-length Gli2 protein (47). In agreement with processing of Gli2, flies carrying a myc-tagged frog *Gli2* transgene produce not only the full-length Gli2 protein but also a smaller protein fragment (3). Although these observations have been interpreted as evidence for Gli2 processing, it cannot be ruled out that the presence of the smaller Gli2 protein fragments is simply the result of nonspecific proteolysis caused by protein overexpression and/or expression in an abnormal context. This could especially be the case since the injected *Xenopus* embryos expressed several smaller Gli2 protein fragments and the level of the one smaller Gli2 fragment in flies did not appear to be repressed by Hh signaling. Therefore, it remains unclear whether, and how, Gli2 activity is regulated by a proteolytic processing mechanism involving Shh signaling.

In this study, we investigated processing of endogenous Gli2 protein in its normal context. We show that although Gli2 protein is proteolytically processed in vivo, the processing is extremely inefficient. As a result, the vast majority of mouse Gli2 protein is present in the full-length form and only a small fraction exists in the processed form. Interestingly, we have also found that in addition to being processed, Gli2 is readily degraded. Gli2 degradation is dependent on the phosphorylation of a cluster of four PKA sites as well as the PKA-primed phosphorylation of multiple adjacent CK1 and GSK3 sites within the Gli2 C-terminal region. The hyperphosphorylation of Gli2 protein creates binding sites for βTrCP, which in turn conjugates the multiple ubiquitin molecules onto the Gli2 protein and triggers the proteasome-mediated protein degradation. Both Gli2 processing and degradation are inhibited by Shh signaling in vivo. Our study thus has provided a molecular mechanism to explain how Gli2 transcriptional activity is regulated by Shh signaling.

MATERIALS AND METHODS

DNA constructs. The expression construct for mouse Gli2 (mGli2) has been described previously (56). PCR-based mutagenesis was used to alter S residues to A in mGli2 at four PKA sites (residues 789, 805, 817, and 848), either individually or in combination, designated Gli2P1, Gli2P2, Gli2P3, Gli2P4, and Gli2P1-4, respectively; at four putative CK1 phosphorylation sites (residues 792, 808, 820, and 851), either individually or at all the sites, designated Gli2C1, Gli2C2, Gli2C3, Gli2C4, and Gli2C1-4, respectively; and at three GSK3 phosphorylation sites (residues 801, 813, and 844), either individually or all the sites, designated Gli2N2, Gli2N3, Gli2N4, and Gli2N2-4, respectively. An alanine was also introduced to individual serines at positions 795, 796, 831, 835, and 840 of Gli2 to generate Gli2(S795A), Gli2(S796A), Gli2(S831A), Gli2(S835A), and Gli2(S840A) constructs. Gex2T-Gli2PR (residues 781 to 864), Gex2T-Gli2PR-P1-4 (the first four PKA sites mutated), Gex2T-Gli2PR-C1-4 (four CK1 sites mutated), and Gex2T-Gli2PR-N2-4 (three GSK3 sites mutated) were generated by PCR amplification of the region indicated using wild-type Gli2, Gli2P1-4, Gli2C1-4, and Gli2N2-4 as templates and insertion in frame into BamHI and EcoRI sites of pGex2T vector (Pharmacia). A myc-tagged human ubiquitin construct, myc-Ub, was cloned into pRK expression vector by reverse transcription-PCR (RT-PCR) using RNA isolated from HEK293 cells. All amplified sequences by PCR were verified by sequencing analysis for unwanted mutations. myc-mβTrCP and PKA* (constitutively active PKA) constructs were described previously (41a, 51). myc-mβTrCPΔWD was generated by a truncation from the BglII site to the stop codon of mβTrCP.

Cell culture, transfection, protein analysis, and reporter assay. Cell culture conditions and methods of transfection for HEK293 cells, mouse embryonic fibroblasts (MEFs) (from embryonic day 13.5 [E13.5] embryos), and chick limb bud cells were as described previously (56). For induction of Gli2 and Gli3 phosphorylation, degradation, and processing, cells were treated with dideoxyforskolin (ddFSK) (40 μM) (a control), forskolin (FSK) (40 μM) overnight, or okadaic acid (OKA) (100 nM) alone or together with cycloheximide (CHX) (10 μg/ml) for the times indicated. For ShhN induction, cells were grown in medium without serum for 24 h followed by the addition of conditioned medium (1/10 [vol/vol]) with or without ShhN protein for 16 to 24 h. To determine Gli2 polyubiquitination, cells were treated with MG132 (50 μM) for 3 h and lysed in a well of a 6-well plate with 100 μl denaturing buffer (0.5% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5], 0.5 mM EDTA, 1 mM dithiothreitol). After incubation for 5 min at 100°C, the lysates were diluted 10 times with lysis buffer and then subjected to coimmunoprecipitation followed by immunoblotting analysis. Coprecipitation of βTrCP with phosphorylated Gli2 protein was performed using affinity Sepharose beads conjugated with double-stranded oligonucleotides containing Gli binding sites (2, 28), which consist of the following oligonucleotide sequences: for the A1 Gli binding site, 5′-TGG GCG AAG ACC ACC CAC AAT GA-3′ (sense) and 5′-ACC ATC ATT GTG GGT GGT CTT CG-3′ (antisense); for the B1 Gli binding site, 5′-GAT CCG TGG ACC ACC CAA GAC GAA ATT-3′ (sense) and 5′-GAT CAA TTT CGT CTT GGG TGG TCC ACG-3′ (antisense); and for the nonspecific Gli binding site, 5′-GAT CAC AGA TAC ATC TCT CAG ACT GC-3′ (sense) and 5′-GAT CGC AGT CTG AGA GAT GTA TCT GT-3′ (antisense). The immunoblotting (anti-Gli2 antisera) (1:1,000 dilution) and the reporter assay whose results are presented in Fig. 1 were done as described previously (56). The reporter assays using $Gli3^{Xt}$ MEFs were performed in 24-well plates by transfecting the cells with both 0.25 μg of 8× Gli-binding site luciferase reporter and 12 ng of TK-*Renilla* luciferase and either 0.25 μg of pSRα-Gli1, a mouse Gli1 expression construct, or 0.25 μg of pSRα-Gli1 and 0.5 μg of a pSuper-Gli1 RNA interference (RNAi) or pSuper-GFP construct control. Shh induction was done by incubating cells with Dulbecco's modified Eagle's medium-N2 supplement for 24 h followed by incubation with ShhN-conditioned medium (1/10 [vol/vol]) overnight or for the indicated amount of time. Oligonucleotides used to create the pSuperRNAi constructs were as follows: Gli1 RNAi-2 (sense, 5′-GAT CCC CCT CCA CAG GCA CAC AGG ATT TCA AGA GAA TCC TGT GTG CCT GTG GAG TTT TTA-3′; antisense, 5′-AGC TTA AAA ACT CCA CAG GCA CAC AGG ATT CTC TTG AAA TCC TGT GTG CCT GTG GAG GGG-3′) and green fluorescent protein (GFP) RNAi (sense, 5′-GAT CCC CGC TAC CTG TTC CAT GGC CAT TCA AGA GAT GGC CAT GGA ACA GGT AGC TTT TTA-3′; antisense, 5′-AGC

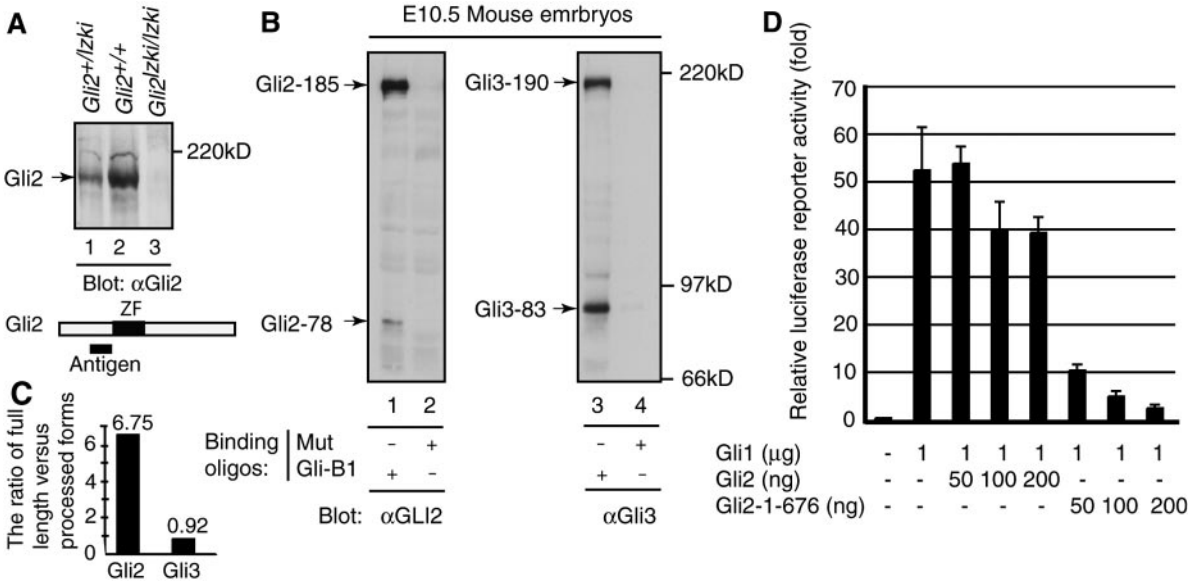

FIG. 1. Differential processing of Gli2 and Gli3 proteins. (A) Characterization of Gli2 antibody. Protein extracts were prepared from E10.5 mouse embryos with indicated genotypes and immunoblotted with an anti-Gli2 antibody (αGli2) raised against the antigen shown below the panel. The αGli2 antibody detected Gli2 protein only in wild-type or *Gli2*$^{lzki/+}$ heterozygous embryos but not *Gli2*$^{lzki/lzki}$ homozygous embryos. (B) Gli2 and Gli3 are differentially processed in vivo. Protein extracts prepared from E10.5 mouse embryos were incubated with a biotinylated double-stranded oligonucleotide containing a specific (Gli-B1) or mutated (mut) Gli-binding site. Proteins bound to the oligonucleotides were pulled down by streptavidin-conjugated magnetic beads. After the reaction mixture was divided into two halves, the proteins were detected by immunoblotting with the αGli2 and αGli3 antibodies. Full-length and processed forms of Gli2 and Gli3 proteins (indicated by arrows) were pulled down only by Gli-B1 but not Mut beads. (C) A histogram shows the ratio of full-length versus processed forms of either Gli2 or Gli3 proteins. (D) The Gli2-78 is a transcriptional repressor. Chick limb bud cell micromasses were transfected with a Gli-dependent luciferase reporter, TK-*Renilla* (a transfection control), and effector constructs as indicated. The data represent the normalized luciferase activities from three independent experiments.

TTA AAA AGC TAC CTG TTC CAT GGC CAT CTC TTG AAT GGC CAT GGA ACA GGT AGC GGG-3′).

Antibody production and detection of endogenous Gli2 and Gli3 proteins. Gli2 antiserum was raised by immunizing rabbits with GST-Gli2-328-430 (Covance, Inc.). The antibody was affinity purified using a GST-Gli2-328-430 fusion column after passing the antiserum through a glutathione *S*-transferase (GST) column.

Shh mutant (12) and wild-type mouse embryos at E10.5 were lysed in either radioimmunoprecipitation buffer (for straight blotting) or lysis buffer (for enrichment of Gli proteins). To enrich Gli proteins, 2 μg biotinylated double-stranded oligonucleotides containing either Gli-B1 or mutated Gli-B1 sequence (28) were first bound to streptavidin conjugated with magnetic beads (New England Biolabs). The biotinylated Gli-B1 sequences were 5′-biotin-GAC GCG TGG ACC ACC CAA GAC GAA ATT CAC A-3′(sense) and 5′-TGT GAA TTT CGT CTT GGG TGG TCC ACG CGT C-3′ (antisense), and mutated Gli-B1 sequences were 5′-biotin-GAC GCG TGG ATT ACA TAA GAC GAA ATT CAC A-3′(sense) and 5′-TGT GAA TTT CGT CTT ATG TAA TCC ACG CGT C-3′ (antisense). The protein extracts from two to three embryos were incubated with the magnet-streptavidin-bound oligonucleotides at 4°C for 1 h. The magnetic beads were washed three times with lysis buffer, and proteins were eluted with the same buffer containing 1 M NaCl. Immunoblotting with the affinity-purified anti-Gli2 or anti-Gli3 antibodies (1:200) was performed as described previously (56) except for Gli2 immunoblotting biotin-conjugated anti-rabbit immunoglobulin G secondary antibody and horseradish peroxidase-conjugated antibiotin tertiary antibody being used (Jackson ImmunoResearch Laboratories).

RNA interference experiments using βTrCP. Small interfering RNA (SiRNA) experiments using human βTrCP were performed as described previously (16).

In vitro phosphorylation and binding assays. The PKA-primed phosphorylation of GST-Gli2PR and its mutants was done as described previously (45). myc-mβTrCP and myc-mβTrCPΔWD were translated in vitro in the presence of [^{35}S]methionine by use of a TNT system (Promega). In vitro interaction between βTrCP and phosphorylated fusion proteins was performed by incubating the phosphorylated fusion protein (~5 μg) with 5 μl in vitro-translated and [^{35}S]methionine-labeled myc-mβTrCP or myc-mβTrCPΔWD in 150 μl of lysis buffer containing 0.1% Triton X-100 for 1 h at 4°C with rotation. After glutathione beads were washed extensively with lysis buffer, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The bound βTrCP was detected by fluorography.

RESULTS

Gli2 and Gli3 are differentially processed in mouse embryos. To investigate whether Gli2 is processed in vivo, we raised an antibody against an N-terminal portion of Gli2 protein (Fig. 1A). This antibody showed a particularly high affinity to Gli2 protein overexpressed in HEK293 cells (data not shown). To test whether the Gli2 antibody was able to recognize endogenous Gli2 protein, we resolved protein extracts prepared from wild-type, *Gli2*$^{+/lzki}$ heterozygous, and *Gli2*$^{lzki/lzki}$ homozygous mutant mouse embryos at E10.5 by use of 5% SDS-PAGE and performed immunoblotting using the Gli2 antibody. *Gli2*^{lzki} is a *Gli2* null allele resulting from knock-in of *LacZ* into the *Gli2* locus and is thus expected not to express Gli2 protein (6). Indeed, a specific band of the predicted Gli2 full-length molecular weight was detected only in wild-type and *Gli2*$^{+/lzki}$ heterozygous embryos but not in *Gli2*$^{lzki/lzki}$ homozygous embryos (Fig. 1A). This antibody was also able to recognize a *Gli2*^{zfd} mutant protein with a predicted size (39) (data not shown).

However, using this antibody we were unable to detect a processed form of Gli2 protein when the straight protein lysates from mouse embryos were used for immunoblotting (data not shown). This failure in the detection could be due to either a very low level of processed Gli2 protein or a complete

lack of Gli2 processing. To test the first possibility, we increased the sensitivity of detection by enriching for Gli2 protein before immunoblotting. Protein extracts made from E10.5 mouse embryos were incubated with a biotinylated double-stranded oligonucleotide containing either a specific or mutated Gli-binding site. The proteins bound to the oligonucleotides were pulled down by use of streptavidin-conjugated magnetic beads (see Materials and Methods for details). This specific Gli-binding oligonucleotide was expected to pull down all three Gli proteins, as the zinc finger DNA-binding domain is conserved among them. The precipitated proteins were then equally divided into two halves, resolved by 7% SDS-PAGE, and immunoblotted with either the anti-Gli2 or an anti-Gli3 antibody (56). As shown in Fig. 1B, it is only when the specific Gli-binding oligonucleotide was used that each of the two antibodies detected two forms of proteins corresponding to the predicted size of full-length and processed forms of Gli2 and Gli3. The two specific Gli2 antibody-reactive bands must represent full-length and processed forms of Gli2 protein, since the Gli2 antibody does not cross-react with Gli1 and Gli3 (data not shown). The full-length and processed Gli2 proteins will be referred to as Gli2-185 and Gli2-78 based on their electrophoretic migration. Since Gli2 has a shorter N terminus than Gli3, the fact that the processed form of Gli2 migrates slightly faster than the processed Gli3 protein suggests that Gli2 is processed at a site equivalent to that of Gli3. To compare the efficiency of processing between Gli2 and Gli3 proteins, we calculated the ratio of the full-length to processed forms for both Gli2 and Gli3 proteins. As shown in Fig. 1C, the Gli2-185/Gli2-78 ratio is more than 6 to 1, whereas the Gli3-190/Gl3-83 ratio was about 1 to 1. From these results we conclude that although Gli2 is processed in vivo, the processing is extremely inefficient compared to that of Gli3.

We next wanted to determine whether Gli2-78 acted as a transcriptional repressor. Since the actual processing site of Gli2 is unknown, we created a C-terminally truncated Gli2 construct, Gli2-1-676 (amino acid residues 1 to 676), which expresses a protein similar to Gli2-78 in size. To determine the transcriptional activity of Gli2-1-676, we examined its ability to antagonize Gli1 in a Gli-based reporter assay, using a transfected chick limb bud micromass culture (56), because it is able to respond to Shh signaling, and Gli1 expression can activate the reporter to a very high level so that Gli2-1-676 repressive activity would be readily detected. As shown in Fig. 1D, expression of Gli1 alone activated the reporter activity more than 50-fold, and coexpression of full-length Gli2 had little or no effect on the ability of Gli1 to activate the reporter, but coexpression of Gli2-1-676 led to a 5- to 20-fold decrease in reporter activity, depending on the amount of the constructs transfected. The slight decrease in reporter activity resulting from the coexpression of higher levels of full-length Gli2 is likely due to the fact that Gli2 is a milder transcription activator (see Fig. S2D in the supplemental material) which competes Gli-binding sites with Gli1. From these results, we conclude that Gli2-78 acts as a transcriptional repressor.

Phosphorylation induces Gli2 degradation. We have previously shown that Gli3 processing is dependent on the phosphorylation of six PKA sites (56). Two recent studies have shown that Ci processing requires the PKA-primed phosphorylation of multiple CK1 and GSK3 sites adjacent to a cluster of PKA sites (22, 45). We found that like Gli3 and Ci, Gli2 was also hyperphosphorylated in both transfected cells and embryos (Fig. 2B to D), even though the extent of its phosphorylation might be different, since Gli2 phosphorylation in cultured cells was induced by FSK, an agent that stimulates PKA activity, or OKA, a phosphatase inhibitor. The phosphorylation was dependent on the first four PKA sites, which prime the phosphorylation of adjacent GSK3 and CK1 sites, as mutating those sites abolished slowly migrating species of Gli2-185 and the extent of phosphorylation in vitro (Fig. 2E and F).

To determine the significance of Gli2 phosphorylation, we induced Gli2 phosphorylation by incubating primary cultures of transfected chick limb bud cells with either FSK or OKA. The primary chick limb bud cell culture was chosen for the following reasons. First, these cells are able to respond to Shh signaling and express Gli2 RNA (reference 56 and data not shown). Second, even though we cannot directly show that endogenous Gli2 is processed due to the inability of our Gli2 antibody to recognize chick Gli2 protein (data not shown), it is still likely that Gli2 is processed in these cells because Gli3 is also processed (56). Lastly, these cells are readily transfected. FSK treatment did not result in a detectable level of processed Gli2 (Fig. 3A; compare lane 2 to lane 3), even though it readily induced Gli3 processing (compare lane 5 to lane 6). Surprisingly, OKA treatment resulted in a rapid reduction in the level of Gli2-185 within a 4-h treatment (Fig. 2B). It should be noted that the reason that Gli2 degradation was not detected under conditions of FSK treatment is probably that FSK treatment can somehow increase the levels of Gli2 RNA (see Fig. S1 in the supplemental material). To determine how quickly Gli2-185 was reduced, we treated the primary cultures with OKA and CHX (an inhibitor for protein synthesis) at different time points. By 1 h of treatment the level of Gli2-185 protein was reduced by nearly half (Fig. 3B; compare lane 3 to lane 2 and the graph), and as treatments continued, the level of Gli2-185 was reduced even more (Fig. 3B, lane 4). The OKA-induced reduction in the level of Gli2-185 appeared to be unique to Gli2, since the same treatment only slightly reduced the levels of Gli3-190 protein (Fig. 3B, right panel, and graph). Interestingly, even when the level of the full-length Gli2 protein was reduced by more than half, processed Gli2 protein was still not detected (Fig. 3B, left panel). This result can be explained by two potential mechanisms. One explanation is that the full-length Gli2 protein underwent degradation following its hyperphosphorylation; the other explanation is that full-length Gli2 was processed but the processed Gli2 peptide was too unstable to be detected. To distinguish which of these two is the more likely, we examined the stability of Gli2-1-676 in primary chick limb bud cells and found that it was more stable than full-length Gli2 protein (Fig. 3D). We thus conclude that phosphorylated Gli2-185 protein undergoes rapid degradation.

To rule out the possibility that OKA-induced Gli2 phosphorylation and degradation is due to Gli2 overexpression and to determine that Gli2 degradation is specific, we examined the fate of endogenous Gli2 and Gli3 proteins in primary cultures of MEFs upon treatment with OKA and CHX. Since the level of endogenous Gli2 protein in MEFs is low, we enriched for both Gli2 and Gli3 (control) proteins by a pull-down experiment using Gli-binding beads prior to immunoblotting. As was seen with overexpressed full-length Gli2 and Gli3 proteins in

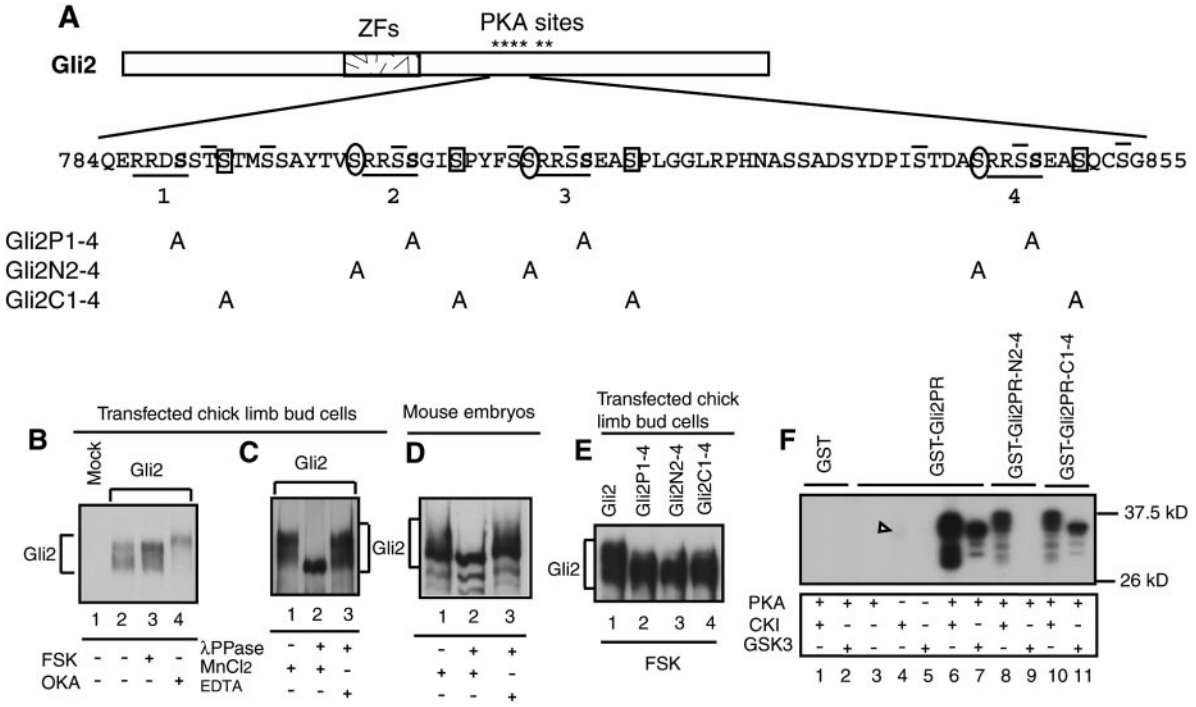

FIG. 2. Gli2 protein phosphorylation in vitro and in vivo. (A) A diagram shows a schematic drawing of Gli2 protein and a region of amino acid sequence including S residues for PKA (bold) and primary GSK3 (circled) and CK1 (boxed) phosphorylation. Ser residues that may become the optimal sites for GSK3 and CK1 phosphorylation, once the primary GSK3 and CK1 sites are phosphorylated, are indicated with lines over the characters representing the residues. Three Gli2 mutants shown below the Gli2 sequence contain S-to-A changes in PKA sites 1 to 4 (Gli2P1-4), GSK3 sites 2 to 4 (Gli2N2-4), or CK1 sites 1 to 4 (Gli2C1-4). (B) An immunoblot showing that Gli2 protein overexpressed in primary chick limb bud cells displays a shift in its migration upon FSK or OKA treatment. (C and D) Gli2 is phosphorylated. λ Protein phosphatase (λPPase) treatment revealed that the slowly migrating species of Gli2-185 protein from either transfected cells (C) or E10.5 mouse embryos (D) represent phosphorylated forms. (E) An immunoblot showing that Gli2 migrated more slowly than its mutants Gli2P1-4, Gli2N2-4, and Gli2C1-4, which contain point mutations as shown in panel A. (F) PKA-primed phosphorylation of Gli2 in vitro. The affinity-purified GST or fusion proteins were incubated with or without PKA in the presence of nonradioactive ATP. After PKA was removed, the proteins were incubated with CK1 or GSK3 or incubated without treatment as indicated in the presence of [γ-^{32}P]ATP and detected by autoradiography (a 3-h exposure). An arrowhead points to a weak signal for GST-Gli2PR phosphorylated by CK1 alone, which can be readily seen after a 6-h exposure (data not shown).

chick limb bud primary cultures, the level of Gli2-185 protein was rapidly reduced but that of Gli3-190 was not (Fig. 3C; compare the left panel to the right panel). No Gli2-78 protein was detected even though the OKA and CHX treatment reduced the level of Gli2-185 protein to about a quarter of the initial amount of Gli2-185 (Fig. 3C, left panel, and graph). The level of processed form of Gli3 was not noticeably changed (Fig. 3C, right panel). We thus conclude that phosphorylation specifically induces Gli2 degradation and that phosphorylation of Gli2 is also likely required for processing Gli2-185 into Gli2-78 in vivo, based on what is known about the requirement of phosphorylation for Gli3 processing.

To determine whether PKA, CK1, and GSK3 sites in Gli2 are involved in Gli2 degradation, we compared the rate of Gli2 degradation with that of three Gli2 mutants, Gli2P1-4 (first four PKA sites mutated), Gli2N2-4 (three GSK3 sites mutated), and Gli2C1-4 (four CK1 sites mutated) (Fig. 2A). OKA and CHX treatment of transfected cells resulted in a marked reduction in the overall levels of wild-type Gli2 and a significant accumulation of the phosphorylated Gli2 species but had little effect on the levels of and extent of phosphorylation of these Gli2 mutants (see Fig. S2 in the supplemental material). The degradation of Gli2 must be caused by phosphorylation but not by an inadvertent pharmacological treatment, since pulse-chase analysis of Gli2 protein degradation without OKA and CHX treatment also showed that wild-type Gli2 was less stable than Gli2P1-4, Gli2N2-4, and Gli2C1-4 mutants (see Fig. S2 in the supplemental material).

Phosphorylation reduces Gli2 transcriptional activator function. To test whether the phosphorylation-induced Gli2 degradation and/or processing affects the activity of Gli2 as a transcriptional activator, we compared the transcription activator function of wild-type Gli2 with that of the Gli2P1-4, Gli2C1-4, and Gli2N2-4 mutants by use of a chicken limb bud micromass-based reporter assay (56). The transfected wild-type Gli2 protein activated a Gli-dependent luciferase reporter to an almost 8-fold increase in activity, whereas each of the three Gli2 mutants activated the reporter to about a 12-fold increase in activity (see Fig. S2 in the supplemental material). Such an increase in Gli2 transcriptional activity would be significant in vivo, since endogenous Gli2 is degraded and/or processed in a physiological condition. Our data thus suggest that Gli2 phosphorylation has a significant effect on its transcriptional activity as a transcription factor.

Gli2 degradation is dependent on βTrCP activity. The SCF$^{\beta TrCP}$ complex, which has been shown to be involved in

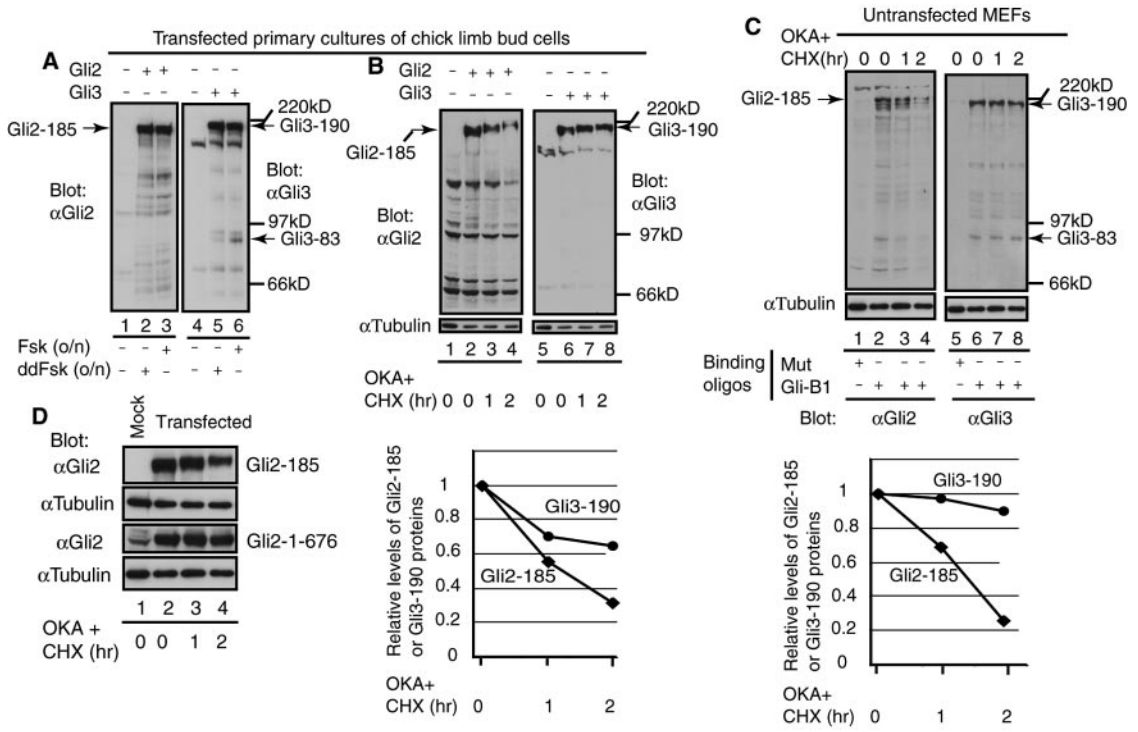

FIG. 3. Phosphorylation-induced Gli2 degradation. Chick limb bud cells were transfected with Gli2, Gli3, or Gli2-1-676 as indicated (A, B, and D). The transfected cells and untransfected MEFs (C) were incubated with FSK or the control drug ddFSK overnight (A) or with OKA and CHX for various time points (B, C, and D). Protein extracts were prepared from the treated cells and directly separated using 7% SDS-PAGE for immunoblotting with the indicated antibodies. The levels of Gli2-185 and Gli3-190 proteins in panels B and C were quantified and are plotted below the gels.

degradation and processing of many proteins, is a ubiquitin E3 ligase that only recognizes phosphorylated protein substrates through a βTrCP subunit (59). Since Gli2 degradation is dependent on phosphorylation and Ci processing requires Slimb activity, we asked whether βTrCP is involved in Gli2 degradation. HEK293 cells were chosen to address this question, since βTrCP-siRNA has been successfully used to knock down βTrCP RNA expression in these cells (16). As shown in Fig. 4A, FSK treatment induced an accumulation of hyperphosphorylated Gli2 species when only Gli2 was expressed but failed to do so when Gli2 and βTrCP were coexpressed (compare lane 4 to lane 3). Consistent with this finding, when βTrCP was coexpressed in cells with the Gli2 phosphorylation site mutant Gli2P1-4, Gli2C1-4, or Gli2N2-4, βTrCP had little effect on the expression levels of any of the mutant proteins (lanes 5 to 10). These results suggest that βTrCP overexpression can promote Gli2 degradation in a phosphorylation-dependent manner.

To further verify that βTrCP was indeed required for Gli2 degradation, we cotransfected HEK293 cells with an siRNA of either βTrCP (βTrCP siRNA), which targets both βTrCP1 and βTrCP2, or GFP (GFP siRNA), along with Gli2. Our RT-PCR analysis confirmed the effectiveness of the βTrCP RNAi, as previously reported (Fig. 3B) (16). Immunoblotting results showed that βTrCP RNAi, but not GFP RNAi, caused an increase in the levels of Gli2 protein (Fig. 3B), indicating that βTrCP is indeed required for Gli2 degradation.

βTrCP interacts directly with phosphorylated Gli2 protein. Although it has been shown that Ci processing requires Slimb (24), the interplay between Slimb and phosphorylation in Ci processing is still not known. It remains possible that Slimb may be indirectly involved in Ci processing via an unidentified factor(s) (35). Studies of many other protein substrates of the $SCF^{\beta TrCP}$ complex have demonstrated that βTrCP recognizes

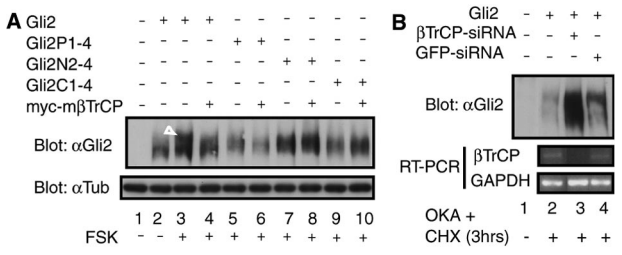

FIG. 4. βTrCP is required for Gli2 degradation. (A) Overexpression of βTrCP promotes Gli2 degradation. Following transfection with constructs as indicated above the panel, HEK293 cells were incubated without or with FSK overnight. Gli2 proteins and tubulin were detected by immunoblotting with the αGli2 and αtubulin antibodies, respectively. Note that coexpression of βTrCP abolished hyperphosphorylated species of Gli2 (indicated by a white arrowhead; compare lane 4 to lane 3) but had little or no effect on the levels of Gli2 mutants. (B) βTrCP RNAi inhibits Gli2 degradation. HEK293 cells were transfected with Gli2 alone (lane 2) or together with either βTrCP siRNA or GFP siRNA (control) (lanes 3 and 4) and were treated with OKA and CHX. Gli2 expression was determined by immunoblotting with the αGli2 antibody (upper panel). The effectiveness of βTrCP RNAi was determined by RT-PCR (two lower panels).

phosphorylated substrates through a DSpGX2-4Sp motif, where Sp refers to phosphoserine and X to any residues (8, 30, 31, 60, 61). The binding of the $SCF^{\beta TrCP}$ complex to its substrates results in the polyubiquitination and degradation of the substrates by the ubiquitin enzymes and proteasome.

The fact that Gli2 protein is phosphorylated at multiple S residues by PKA, CKI, and GSK3 and that βTrCP is required for Gli2 degradation led us to hypothesize that βTrCP may directly bind phosphorylated Gli2 protein. To test this hypothesis, we examined the protein-protein interaction between myc-mβTrCP and either Gli2 or each of the Gli2P1-4, Gli2N2-4, and Gli2C1-4 mutants in transfected HEK293 cells, since these cells expressed high levels of the Gli2 constructs used for transfection. Sepharose beads conjugated with double-stranded oligonucleotides containing Gli-binding sites (Gli-binding beads) were able to coprecipitate myc-mβTrCP only when Gli2 and myc-mβTrCP were coexpressed and not when myc-mβTrCP was expressed alone (Fig. 5A, bottom panel; compare lane 4 to lane 3). Similarly, a myc antibody was able to coimmunoprecipitate only wild-type Gli2 protein (Fig. 5A, second panel from the bottom; compare lane 4 to lanes 6, 8, and 10), indicating that βTrCP interacts with Gli2 in a phosphorylation-dependent manner.

To determine whether the interaction between Gli2 and βTrCP is direct, we examined the ability of phosphorylated GST-Gli2PR (a fusion between GST and a Gli2 segment containing first four PKA sites and adjacent CK1 and GSK3 sites) to pull down βTrCP protein in vitro. βTrCP only interacted with GST-Gli2PR phosphorylated by CK1 alone or by PKA, CK1, and GSK3 together but not by PKA or GSK3 alone (Fig. 5B, lanes 1 to 4). The observation that GST-Gli2PR phosphorylated by CK1 alone can also pull down βTrCP in vitro was not surprising, since we found that CK1 can weakly phosphorylate GST-Gli2PR without priming by PKA in vitro (Fig. 2F, lane 4, and data not shown).

To gain more insight into the interaction between Gli2PR and βTrCP, we performed the same binding experiments using GST-Gli2PR-P1-4, GST-Gli2PR-C1-4, and GST-Gli2PR-N2-4 mutants that were incubated with all three kinases. Surprisingly, only GST-Gli2PR-N2-4 failed to bind βTrCP whereas both GST-Gli2PR-P1-4 and GST-Gli2PR-C1-4 were able to bind βTrCP (Fig. 5B, lanes 5 to 7). This result is in agreement with our in vivo binding results showing that N2 or S801 and S840 residues are directly involved in βTrCP binding whereas the PKA sites and CK1 sites play a priming role (see below). It should be pointed out that the recognition motif for CK1 is SpX1-3S, with two residues between the Sp and the acceptor S being much more effective than one or three residues (15). The mutated four CK1 sites belong to the optimal motifs. When the serines that are located one or three residues apart from Sp are counted, even N2 and N4 could then be phosphorylated by CK1 in vitro. Therefore, it is not surprising that GST-Gli2PR-P1-4 and GST-Gli2PR-C1-4 can still bind βTrCP because CK1 can phosphorylate many S residues, including N2-4 sites that do not conform to the optimal CK1 consensus sequence, and because phosphorylation of some of those sites is not dependent on the phosphorylation of PKA or GSK3 sites in vitro (Fig. 2F, lanes 4 and 8, and data not shown). Therefore, our in vitro binding data indicate that βTrCP directly binds phosphorylated Gli2.

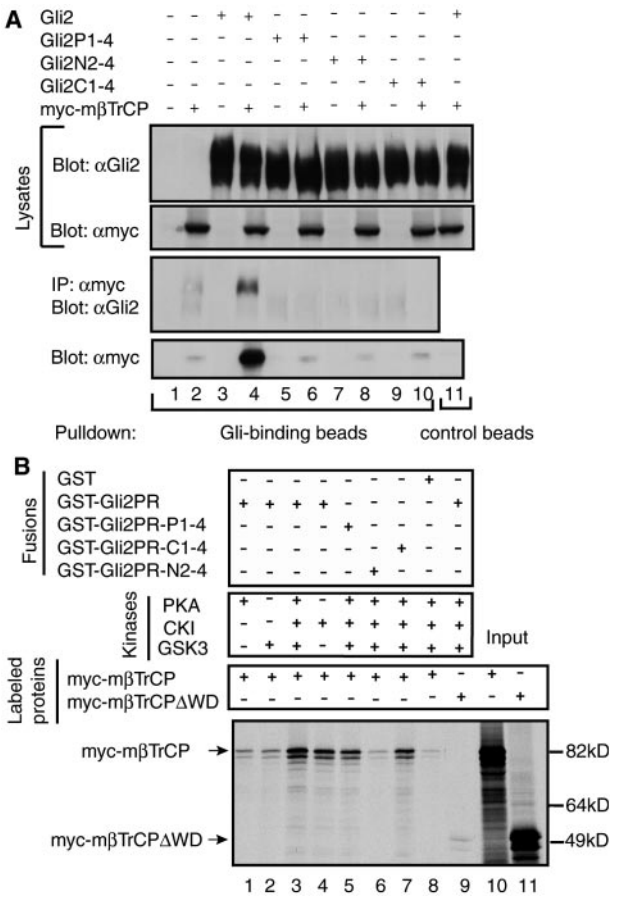

FIG. 5. βTrCP interacts directly with phosphorylated Gli2. (A) βTrCP binds phosphorylated Gli2 in vivo. HEK293 cells were transfected with constructs as shown above the panels. A day after the transfection, the cells were incubated with FSK overnight and with MG132 for 3 h right before cells were lysed. The two upper panels show straight lysates immunoblotted using the indicated antibodies. The lysates were subjected to immunoprecipitation with anti-myc antibody followed by immunoblotting with anti-Gli2 antibody (second panel from the bottom) or to precipitation with a double-stranded oligonucleotide containing specific or nonspecific Gli-binding sites conjugated with Sepharose beads followed by immunoblotting with anti-myc antibody (bottom panel). (B) βTrCP interacts with phosphorylated Gli2 in vitro. Following the in vitro phosphorylation by the indicated kinases, GST fusion proteins (see Fig. 2A and F) were used to pull down the radiolabeled in vitro-translated myc-mβTrCP or myc-mβTrCPΔWD, which was then detected by autoradiography.

To define the βTrCP binding site(s) in Gli2, we examined how well Gli2 proteins with a single mutation in an individual PKA (designated P1, P2, P3, and P4), GSK3 (designated N2, N3, and N4), or CK1 (designated C1, C2, C3, and C4) site could bind myc-mβTrCP (Fig. 6A). We consistently found that P1, P2, or P4 mutations dramatically reduced myc-mβTrCP binding, whereas the P3 mutation appeared to have little effect (Fig. 6B, lanes 3, 4, and 6). Consistent with this finding, we also found that C1, N2, or N4 mutations significantly reduced the βTrCP binding (Fig. 6B, lanes 7, 11, and 13) but that C2, C3, C4, or N3 did not (Fig. 6B, lanes 8 to 10 and 12). Taken together, these data suggest that there are at least two βTrCP binding sites in the region, one somewhere between the first

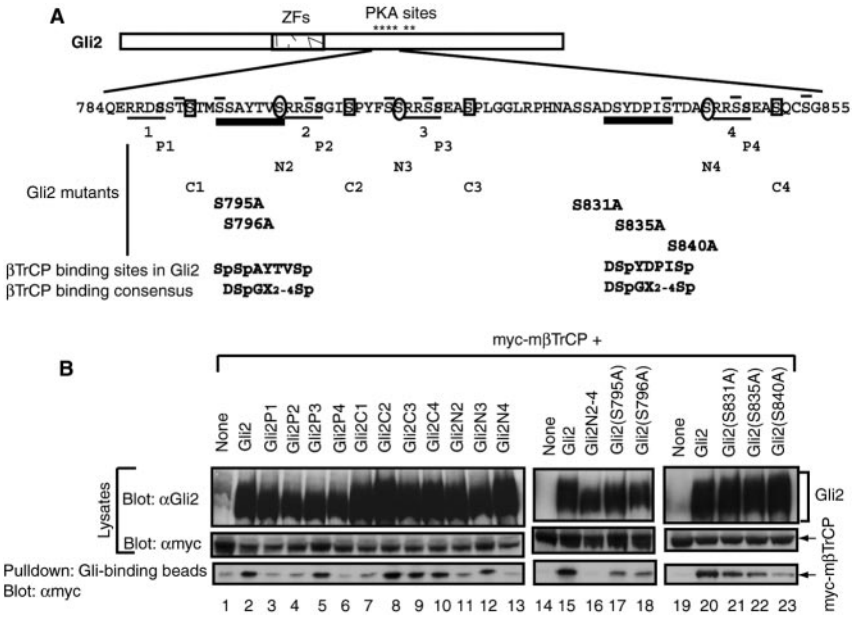

FIG. 6. Identification of βTrCP binding sites in Gli2. (A) A Gli2 diagram (see Fig. 2 caption) representing the Gli2 mutants used for panel B. Each of the Gli2 mutants contains an A residue substitution for the corresponding S residues in the sequence. Two thick lines underline two βTrCP binding sites mapped in panel B. The alignment between βTrCP binding sites in Gli2 and the known βTrCP binding motif is shown. (B) Identification of two βTrCP binding sites in Gli2. HEK293 cells were transfected with each of the indicated Gli2 constructs along with myc-mβTrCP. Interaction between Gli2 proteins and βTrCP was determined as described for Fig. 5A.

and second PKA sites and the other between the third CK1 site (not included) and the fourth PKA site.

To identify the potential βTrCP binding motifs, we carefully inspected the amino acid sequences around the P1, P2, P4, C1, N2, and N4 sites and found two sequences similar to the known βTrCP-binding motif. The first sequence, 792STMSSAYTVS 801, begins with C1 and ends with N2 (Fig. 6A). When C1 has been phosphorylated, both S795 and S796 residues could become the potential CK1 targets, although S796 is less optimal for CK1 phosphorylation (15). After phosphorylated, the sequence becomes 792SpTMSpSpAYTVSp801, in which the underlined sequence resembles the known βTrCP-binding motif, since the Sp795 residue, like the D residue, is negatively charged. The second sequence, which starts with S831 and ends with N4, is 831SSADSYDPISTDAS844, in which the underlined section is the consensus sequence (S/TXXD/E) for CK2 phosphorylation (38). When S831 has been phosphorylated by CK2, S835 may become a site for CK1 phosphorylation, even though it is not optimal, while S840 may be phosphorylated by GSK3 when site N4 or S844 has been phosphorylated by GSK3. As a result, the sequence becomes 831SpSADSpYDPISpTDASp844, in which underlined sequence resembles the known βTrCP-binding motif DSpGX2-4Sp.

To test the prediction detailed above, we introduced an A residue into individual S residues in positions 795, 796, 831, 835, and 840 to create Gli2(S795A), Gli2(S796A), Gli2(S831A), Gli2(S835A), and Gli2(S840A) mutant constructs (Fig. 6A), respectively, and examined the interaction of these mutants with myc-mβTrCP in transfected HEK293 cells. As predicted, the interaction between myc-mβTrCP and Gli2(S795A) or Gli2(S796A) was significantly reduced, suggesting that these two S residues are required for βTrCP binding (Fig. 6B; compare lanes 17 and 18 to lane 15). Similarly, the ability of Gli2(S831A), Gli2(S835A), or Gli2(S840A) mutants to bind βTrCP was dramatically reduced as well (Fig. 6B, compare lanes 21 to 23 to lane 20). Thus, our analysis reveals two βTrCP binding motifs (underlined) in Gli2: 792SpTMSpSpAYTVSp801 and 831SpSADSpYDPISpTDASp844.

Gli2 protein is polyubiquitinated in the cell. Our findings that protein phosphorylation induces Gli2 degradation, which requires $SCF^{\beta TrCP}$ activity, are consistent with the notion that Gli2 is degraded through the ubiquitination and proteasome pathway. We thus tested whether Gli2 is polyubiquitinated in the cell. HEK293 cells overexpressing Gli2 (control), myc-ub (myc-tagged ubiquitin) (control), or both Gli2 and myc-ub were treated with MG132, a specific proteasome inhibitor. Ubiquitination of Gli2 was examined by immunoprecipitating Gli2 with the anti-Gli2 antibody followed by immunoblotting with anti-myc to detect ubiquitinated proteins. myc-ub protein was detected only when Gli2 and myc-ub were coexpressed but not when either Gli2 or myc-ub alone was expressed (see Fig. S3 in the supplemental material; compare lane 4 to lanes 2 and 3). The polyubiquitination of Gli2 was dependent on phosphorylation, since no polyubiquitins were detected for the Gli2P1-4, Gli2C1-4, or Gli2N2-4 mutants (see Fig. S3 in the supplemental material; compare lanes 6, 8, and 10).

Shh signaling inhibits Gli2 degradation and processing. In the fly, Ci processing is inhibited by Hh signaling (4, 10). Likewise, Shh signaling blocks the proteolytic processing of Gli3 in vertebrates (18, 34, 56). To determine whether Gli2 processing is regulated by Shh signaling, we examined the levels of both the full-length and processed forms of Gli2 in wild-type and *Shh* mutant mouse embryos by immunoblotting after Gli proteins were precipitated using Gli-binding beads.

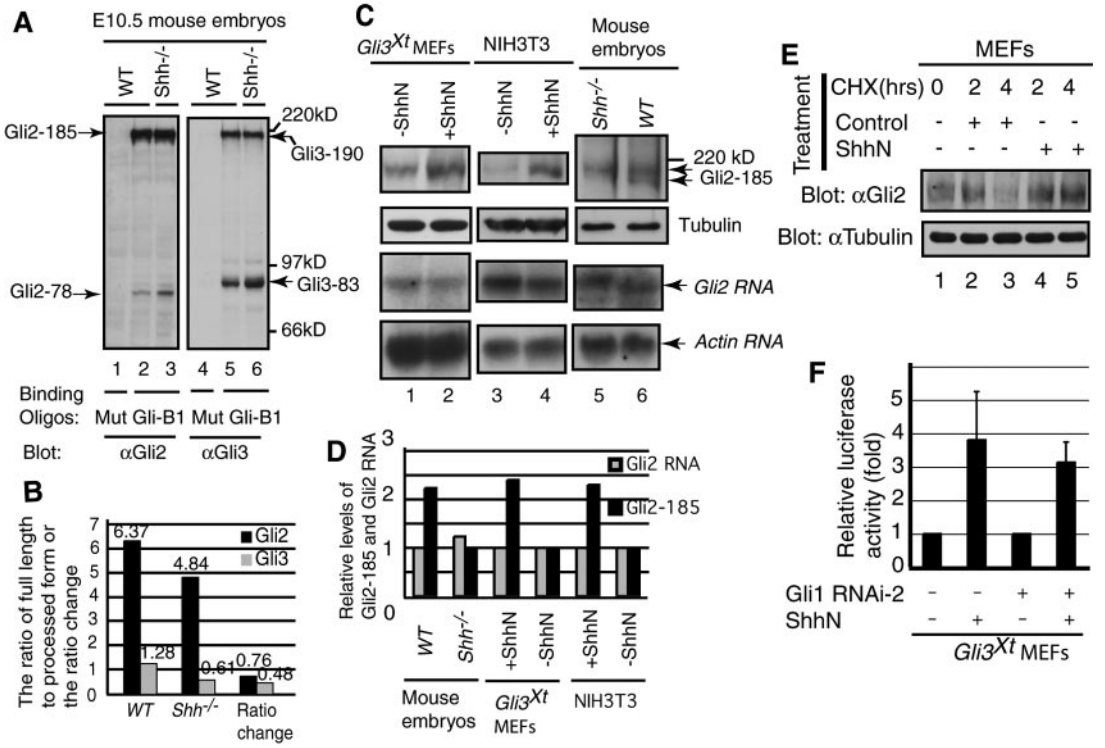

FIG. 7. Hh signaling inhibits Gli2 processing and degradation and induces Gli2 transcriptional activity in vivo. (A and B) Differential regulation of Gli2 and Gli3 processing by Shh signaling. Enrichment and detection of endogenous Gli2 and Gli3 proteins in Shh mutant and wild-type (WT) mouse embryos were performed as described in the Fig. 1 legend. Panel B shows the ratio of Gli2-185 to Gli2-78 (black bars), Gli3-190 to Gli3-83 (gray bars), and ratio changes between *Shh* mutants and wild type (Shh mutant/WT). (C and D) Stabilization of Gli2 protein by Shh signaling. The two upper panels show straight immunoblots of the Gli2-185 protein levels with α-tubulin as loading controls for E10.5 wild-type (WT) and *Shh* mutant ($Shh^{-/-}$) mouse embryos and indicated cells treated with or without ShhN-conditioned medium. The two lower panels show the expression of Gli2 and actin mRNAs in the corresponding cells and mouse embryos. The relative levels of Gli2 protein and RNA were plotted in panel D. (E) ShhN treatment stabilizes endogenous Gli2-185 protein in wild-type MEFs. (F) Shh signaling induces the Gli2 transcriptional activity in vivo. $Gli3^{Xt}$ mutant MEFs were transfected with a Gli-dependent luciferase reporter, TK-*Renilla* (a transfection control), with or without a Gli1 RNAi-2 construct. Cells that received ShhN treatment are indicated. The firefly luciferase activity data were derived from three independent experiments.

As a control, the levels of two forms of Gli3 in the same precipitated Gli proteins were also examined, since Gli3 processing is known to be inhibited in Shh mutant embryos (34). Because Shh mutant embryos are smaller and Gli2 protein levels are lower in Shh mutant embryos than that in wild-type embryos (see below), the amount of protein lysates from Shh mutant embryos was adjusted in order to pull down sufficient amount of Gli2-78 for detection. The level of Gli2-78 in *Shh* mutants was slightly higher than that in wild-type embryos (Fig. 7A; compare lanes 3 and 2), whereas the level of Gli3-83 in *Shh* mutants was significantly higher than that in wild-type embryos (Fig. 7A; compare lanes 6 and 5), which is consistent with what has been reported previously (34). Quantitative analysis of the intensity of Gli2 and Gli3 protein bands revealed that the ratio of Gli2-185 to Gli2-78 was about 6.4 to 1 in wild-type embryos and 4.8 to 1 in Shh mutants, while the ratio of Gli3-190 to Gli3-83 was 1.28 to 1 in wild-type embryos and only 0.61 to 1 in *Shh* mutants (Fig. 7B). From these results we conclude that the processing of both Gli2 and Gli3 proteins is suppressed by Shh signaling but that the extent of suppression for Gli2 processing is significantly lower than that for Gli3 processing.

Since protein phosphorylation induces rapid Gli2 degradation, we next determined whether Gli2 degradation is also regulated by Shh signaling. In the previous experiment, the amount of protein lysates from Shh mutant embryos was adjusted in order to pull down enough Gli2-78 protein for detection. Although using this method of enrichment causes little variation in the ratio of full-length to processed Gli2, since both forms of Gli2 in the same lysates were pulled down at the same time, this method may cause variations in determining the actual levels of Gli2-185 protein in mouse embryonic lysates, as the procedure by which Gli2-185 is pulled down from Shh mutant and wild-type embryos has to be performed in two different tubes. To determine the Gli2-185 level in both Shh mutant and wild-type embryos, we performed immunoblotting directly using protein lysates prepared from E10.5 wild-type and *Shh* mutant mouse embryos. The level of Gli2-185 protein in wild-type embryos (including *Shh* heterozygotes) was more than two times as high as that in *Shh* mutant embryos (Fig. 7C, upper panels, lanes 5 and 6, and Fig. 7D), even though similar amounts of protein lysates were loaded as measured by the levels of tubulin (Fig. 7C, second row of panels from the top, lanes 5 to 6). The difference in the levels of Gli2-185 should have resulted from posttranslational regulation of Gli2 protein, since Northern blot analysis showed that *Gli2* mRNA expression was not affected by *Shh* mutation (Fig. 7C, two lower

panel rows, lanes 5 and 6, and Fig. 7D). It should be noted that the two distinct Gli2-185 bands in wild-type embryonic lysates were not always detected. Although it would be consistent with the idea that Shh signaling may regulate the state of Gli2 phosphorylation, the molecular nature of the potentially different species of Gli2-185 remains to be determined. Since the level of Gli2-185 is more than six times as high as that of Gli2-78 in wild-type mouse embryos and since Shh signaling only slightly reduces the level of Gli2-78 (Fig. 7A and B), the difference in the levels of Gli2-185 between wild-type embryos and Shh mutants cannot be explained solely by the conversion of Gli2-78 to Gli2-185 due to a reduction in Gli2 processing by Shh signaling. Taken together, these results thus indicate that Shh signaling regulates Gli2 protein stability in addition to Gli2 processing.

To determine whether the Gli2-185 protein level is directly regulated by Shh signaling, Shh-stimulated Gli2 stabilization was examined in wild-type MEFs, *Gli3*^{Xt} MEFs, and NIH3T3 cells. We found that Shh stimulation resulted in a more than twofold increase in Gli2-185 protein levels in both *Gli3*^{Xt} MEFs and NIH3T3 cells, even though Gli2 RNA expression was not affected (Fig. 7C and D). However, we also found that Shh treatment somehow led to a slight increase in the Gli2 RNA level in wild-type MEFs in addition to an increase in the Gli2-185 protein level (data not shown). For this reason, we incubated wild-type MEFs with conditioned medium with or without ShhN in the presence of CHX, which blocks protein synthesis. Direct immunoblotting analysis showed that the level of Gli2-185 protein was markedly reduced in the cells treated with CHX and without ShhN, but it remained unnoticeably changed in the same cells treated with CHX and ShhN (Fig. 7E). Taken together, these results indicate that Shh signaling stabilizes Gli2-185 protein.

Shh signaling induces Gli2 protein transcription activity. Our findings that Shh signaling regulates both Gli2 stability and processing provide a molecular mechanism to explain how the function of Gli2 as a transcriptional activator may be activated by Shh signaling. To test directly the hypothesis that Shh signaling stimulates Gli2 transcription activator function, we performed a Gli-dependent luciferase reporter assay using *Gli3*^{Xt} mutant MEFs. Since recent studies have shown that Shh signaling is initially mediated by both Gli2 and Gli3 but not Gli1 (5, 7, 32a), we reasoned that any Shh-stimulated reporter activity in MEFs lacking Gli3 would reflect an initial activation of Gli2. However, it should also be pointed out that since Gli1 is a transcriptional target of Shh signaling, Shh-stimulated reporter activity in *Gli3*^{Xt} MEFs may be, in part, contributed by the possible increase in the Gli1 RNA level. To rule out this possibility, the RNAi approach was taken to knock down Gli1 transcriptional activity in the reporter assay. We tested five different Gli1-RNAi constructs and found that only one, Gli1-RNAi-2, was able to specifically and significantly knock down transcriptional activity of overexpressed Gli1 protein in the reporter assay (Fig. S4 in the supplemental material and data not shown). To determine whether Shh signaling activated Gli2 transcriptional activity in *Gli3*^{Xt} MEFs, the cells were transfected with the Gli-dependent luciferase reporter alone or together with the Gli1-RNAi-2 construct and were subsequently incubated with conditioned medium with or without ShhN. As shown in Fig. 7F, ShhN treatment of cells transfected with the reporter alone resulted in a nearly fourfold increase in reporter activity, and cotransfection of the Gli1-RNAi-2 construct only slightly affected the reporter activity stimulated by ShhN. These results correlate to the ShhN-stimulated increase in the Gli2-185 level (Fig. 7C) and indicate that ShhN stimulation directly activates Gli2 transcriptional activator function.

DISCUSSION

In this report, we provide direct evidence that Gli2 is also proteolytically processed in vivo to generate a transcriptional repressor but that, unlike Gli3 processing, Gli2 processing is extremely inefficient. As a result, the majority of Gli2 exists in its full-length, Gli2-185 form, whereas at least a half of Gli3 protein is processed and is present as a transcriptional repressor. Gli2 processing is also inhibited by Shh signaling in vivo, since the ratio of Gli2-185 to Gli2-78 is slightly reduced in *Shh* mutant embryos (Fig. 7A and B). Significantly, this ratio change is not nearly as great as that of Gli3-190 to Gli3-83 in the presence and absence of Shh. These observations provide a molecular mechanism as an explanation of why Gli3 mainly exhibits a repressor function and why its weak activator function can be detected only when *Gli2* is mutated whereas Gli2 mostly acts as a transcriptional activator in mice and its weak repressing activity can only be revealed when the Gli3 repressor function is removed (9, 37). The same molecular mechanism may also likely explain the possible dual activities of Gli2 in zebra fish revealed by genetic analysis of Gli2 dominant-negative alleles and RNA knockdown (26).

Since the level of Gli2-78 is extremely low in mouse embryos, the processed form of Gli2 protein was able to be detected by immunoblotting in conjunction with precipitation of Gli proteins only by use of Gli-binding beads in this study. Therefore, the Gli2-185 and Gli2-78 proteins that are detected only represent the proteins that can bind Gli-binding oligonucleotide. The results of two recent studies indicate that overexpressed Gli2 or Gli3 in cell culture can dimerize with Zic transcription factors or itself through the zinc finger DNA-binding domain (29, 41). Another study also suggests that PKA might phosphorylate a site within the second zinc finger of Gli1 protein (25). These reports raise the possibility that some of Gli2 protein might be discounted when the ratio between Gli2-185 and Gli2-78 is calculated for this study, even though it is not clear whether Gli2 dimerization and the second zinc-finger phosphorylation ever occur in vivo. Nevertheless, several lines of evidence strongly suggest that the levels of Gli2-185 and Gli2-78 we observed indeed closely represent the actual ratio of Gli2-185 versus Gli2-78 in vivo. First, the majority of Gli2 protein can be pulled down by Gli-binding beads from mouse embryonic lysates, since Gli2-185 protein was undetectable in the embryonic lysates that had been incubated with Gli-binding beads (data not shown). This result also suggests that Gli dimerization and phosphorylation within zinc fingers, if there is any, must be minimal. Second, we were able to detect Gli2-185 protein by Western blotting directly using embryonic lysates (Fig. 1A), which should detect Gli2 monomer and dimer as well as zinc-finger phosphorylated Gli2, if any, but under the same conditions we were unable to detect Gli2-78, indicating that the difference between Gli2-185 and Gli2-78 levels in vivo must be huge. This is in agreement with what we observed

about the ratio between Gli2-185 and Gli2-78 levels in the Gli-binding bead pulldown assay. Third, it is known that a significant fraction of Gli3-190 is processed. When the amounts of full-length and processed forms of both Gli2 and Gli3 pulled down using Gli-binding beads were examined by immunoblotting, at least half of Gli3 protein was found to be in the processed form whereas only a minor fraction of Gli2-78 was detected compared to Gli2-185 (Fig. 1B). Taken together, these results clearly indicate that the ratio between Gli2-185 and Gli2-78 is large as compared to that between Gli3-190 and Gli3-83 since the results were obtained under the same conditions and are thus comparable.

The fact that Gli2 protein is inefficiently processed allowed us to uncover a regulation of Gli2 activity by Shh through protein degradation. We find that OKA treatment of both MEFs and transfected cells leads to a dramatic decrease in the level of Gli2-185 protein. This reduction is likely the result of protein degradation instead of processing, since even when the level of Gli2-185 is reduced by more than half, the processed Gli2 protein, Gli2-78, remains undetectable. Furthermore, a C-terminally truncated Gli2, which mimics Gli2-78, is actually more stable than Gli2-185 (Fig. 3D). The rapid Gli2 degradation appears to be unique to Gli2, since the same treatment of either transfected cells with Gli3 or untransfected MEFs does not induce significant Gli3 degradation (Fig. 3). Consistent with these observations, we also found that the level of Gli2-185 protein in wild-type mouse embryos is approximately two times as high as that in *Shh* mutant embryos (Fig. 7C and D). The increase in the level of Gli2-185 should be the direct result of Shh signaling, since ShhN treatment of both MEFs and NIH3T3 cells leads to either an increase in the level of Gli2-185 protein or stabilization of the protein (Fig. 7C, D, and E). Since the majority of Gli2 protein is present in the Gli2-185 form (six times as high as Gli2-78) and since processed Gli2-78 is found to be more stable than Gli2-185 in cultured cells, the higher level of Gli2-185 in wild-type embryos cannot be explained simply by the conversion of Gli2-78 into Gli2-185 due to an inhibition of Gli2 processing by Shh signaling. Our results thus indicate that in addition to being processed, Gli2-185 protein is readily degraded and that both Gli2 processing and degradation are regulated by Shh signaling.

The Gli2 processing and degradation appear to be regulated through protein phosphorylation and dephosphorylation. In the fly, Ci is phosphorylated by PKA at its C terminus, and the phosphorylation of the first three of the five PKA sites primes the further phosphorylation of adjacent CK1 and GSK3 sites (22, 45). Both Gli2 and Gli3 share these conserved PKA, CK1, and GSK3 sites with Ci; in fact, each of them contains six instead of five PKA sites. The first four of the six PKA sites in the Gli2 and Gli3 C termini are surrounded by the primary optimal GSK3 and CK1 sites. An inspection of the amino acid sequence in the region containing the PKA sites revealed additional S residues that conform to optimal CK1 and GSK3 phosphorylation motifs when the primary GSK3 and CK1 sites are phosphorylated (Fig. 2A and 6A). In principle, phosphorylation of the first four PKA sites can prime the phosphorylation of as many as 15 GSK3 and CK1 sites even without including the nonoptimal CK1 sites (SpX1 or 3 S) (15). Consistent with this, both overexpressed and endogenous Gli2 proteins exhibit a huge shift in electrophoretic migration, and such a shift becomes even greater when the transfected cells are treated with either OKA or FSK. In contrast, mutations in the PKA sites and primary GSK3 and CK1 sites either abolish or significantly reduce the level of Gli2 phosphorylation. Similarly, Gli2-185 protein in wild-type mouse embryos is found to be hyperphosphorylated (Fig. 2B to E). However, we were unable to determine whether the same phosphorylation is required for Gli2 processing, since Gli2 processing was not detectable in transfected chick limb bud cells. Based on what is known about the requirement of phosphorylation for Gli3 and Ci processing, hyperphosphorylation is likely to be essential for Gli2 processing as well.

In addition to phosphorylation, Ci processing also requires Slimb, a subunit that forms part of SCF ubiquitin ligase. Although it is tempting to speculate that Slimb is directly involved in Ci processing, so far there is no evidence indicating that this is the case. Therefore, the interplay between Ci phosphorylation and the role of Slimb in Ci processing remains unknown. The fact that hyperphosphorylation induces Gli2 degradation stimulated us to test whether βTrCP is directly involved in Gli2 degradation. Three lines of evidence demonstrate that this in fact is the case. First, overexpression of βTrCP reduces the level of phosphorylated species of wild-type Gli2 protein but not of Gli2 protein with mutated phosphorylation sites for PKA, CK1, or GSK3. Second, knocking down the endogenous level of βTrCP by RNAi leads to an accumulation of Gli2. Third, βTrCP directly binds phosphorylated Gli2 but not Gli2 with mutations in its PKA and primary CK1 and GSK3 sites. Interestingly, although the phosphorylation of the first four PKA sites and primary CK1 and GSK3 sites is required for Gli2 degradation and likely processing as well, analysis of Gli2 mutant proteins with single-point mutations in either the individual PKA or primary or secondary optimal CK1 or GSK3 sites indicates that only the first GSK3 site (N2) is directly involved in βTrCP binding. All other sites seem just to prime the further phosphorylation of the secondary set of S residues in the region (see Fig. 6A). It is the secondary phosphoserine residues of Gli2 that are apparently involved in directly binding to βTrCP. Furthermore, we identified two βTrCP binding sites in Gli2. Our findings are the first to establish a direct link between βTrCP function and Gli2 degradation and possibly processing as well. The phosphorylation-dependent direct involvement of βTrCP in Gli2 degradation supports the notion that Gli2 degradation is mediated by the proteasome. Indeed, coexpression of Gli2 and ubiquitin leads to polyubiquitination of Gli2 (see Fig. S3 in the supplemental material). Our observations thus indicate that Gli2 degradation, and possibly processing, is mediated by the βTrCP-dependent ubiquitination and proteasome pathway. Shh signaling probably suppresses Gli2 degradation and processing by regulating the extent of Gli2 phosphorylation.

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ADDENDUM

While this paper was being revised, three studies, including one from our group, showed direct interaction between Ci/Gli3 and Slimb/βTrCP (23, 50, 57). However, none of these studies experimentallyidentified Slimb/βTrCP binding sites in Ci/Gli3.

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