Small Ubiquitin-like Modifier (SUMO) Modification Inhibits GLI2 Protein Transcriptional Activity in Vitro and in Vivo**

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Background: How the activity of GLI2 transcriptional factor is regulated is not well understood. Results: Loss of SUMO modification in GLI2 results in an increase in GLI2 transcriptional activity in cultured cells and in vivo. Conclusion: SUMO modification inhibits GLI2 transcriptional activity. Significance: The activation of GLI2 by Hedgehog signaling may be through the inhibition of GLI2 sumoylation.

The Gli transcription factors are key downstream mediators of the Hedgehog (Hh) signaling pathway. How the activities of Gli transcription factors are regulated by upstream Hh signaling events and protein modifications are not fully understood. Here we show that GLI2 is conjugated by small ubiquitin-like modifier (SUMO) at lysine residues 630 and 716 in the cell. The level of GLI2 sumoylation is reduced by either mutations in six serine residues that are normally phosphorylated by protein kinase A (PKA) or stimulation by Hh. This suggests that PKA phosphorylation enhances GLI2 sumoylation, whereas Hh signaling inhibits it. In addition, mutation of these two lysines into arginine residues significantly increases GLI2 transcriptional activity in a cell-based reporter assay. The same mutations in the GLI2 locus also result in an increase in GLI2 activity in the mouse. Interestingly, GLI2 can interact with HDAC5 (histone deacetylase 5), but the GLI2 mutant cannot. Taken together, our results suggest that SUMO modification inhibits GLI2 transcriptional activity by recruiting HDAC5.

The family of secreted Hedgehog (HH) molecules plays an important role in embryonic development of both vertebrates and invertebrates. Loss of function in HH signaling is the cause of many birth defects, whereas an inappropriate activation of the HH pathway is also associated with several types of human cancer (1). Thus, a thorough understanding of the molecular mechanism of HH signal transduction is essential not only for the elucidation of the molecular mechanism of the developmental process of embryos, but also for the prevention and treatment of HH-related cancers.

The molecular mechanism of HH signal transduction has been studied extensively. In vertebrates, HH ligands bind to their cell surface 12-transmembrane receptor, Patched (PTCH) (2, 3). This binding prevents PTCH from inhibiting a seven-transmembrane protein known as Smoothened (SMO), allowing the HH pathway to be activated. SMO then transduces signals to activate the downstream GLI transcription factors. Of the three GLI family members, GLI2 and GLI3 act as transcriptional repressors (GLI2Reip and GLI3Reip) in the absence of HH signaling because the C-terminal activation domains of the full-length proteins (GLI2FL and GLI3FL) are proteolytically processed, and GLI2FL and GLI3FL are not activated (4–6). GLI2 and GLI3 processing is dependent on the phosphorylation of four serine residues in their carboxyl termini by protein kinase A (PKA). The phosphorylation then triggers proteasome-mediated limited degradation of their C termini. HH signaling inhibits the proteolytic processing and converts the latent full-length proteins into activators. However, the molecular mechanisms of inhibition of GLI2 and GLI3 processing and activation of the full-length GLI2 and GLI3 proteins are unknown. Once they are activated, GLI2 and GLI3 activate the downstream transcriptional targets. One of these targets is GLI1, which serves to further enforce the HH pathway activation, whereas PTCH is another target to establish a negative feedback regulation of the pathway (7, 8). So far, little is known about how full-length GLI2 and GLI3 are activated.

The small ubiquitin-like modifier (SUMO) is a 100-amino acid polypeptide that is covalently attached to target proteins in a way similar to the ubiquitination process. SUMO attachment occurs through an e-amino group of a lysine residue in the target protein. The consensus sequence for SUMO modification is ΨKXE/D, where Ψ is a large hydrophobic residue (9). SUMO1 is attached to the target proteins as a single molecule because it lacks the consensus sequence, whereas SUMO2 and SUMO3 can form SUMO chains in the target proteins as they contain the sumoylation consensus sequence (10). Like ubiquitination, sumoylation is catalyzed by three enzymes: E1 activating enzyme, E2-conjugating enzyme, and E3 ligase. SUMO modification has various effects on target protein function. For example, sumoylation of transcription factors mostly results in repression of the transcription, although it is occasionally found to activate the transcription. The repression of transcription factor activity by sumoylation has to do with the fact that
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sumoylated transcription factors can recruit histone deacetylase (HDAC), which reduces transcription by removing acetyl group on histones or transcription factors (11, 12).

In the present study, we show that GLI2 is modified by SUMO at lysine residues 630 and 716. The GLI2 sumoylation is reduced by either mutation in six PKA phosphorylation sites or HH stimulation. Mutating the two lysine residues into arginine residues dramatically increases GLI2 transcriptional activity in cultured cells. The same mutations in the GLI2 locus also result in a slight increase in GLI2 activity. In addition, wild type GLI2 can interact with HDAC5, whereas the GLI2 mutant cannot. Therefore, our results indicate that SUMO modification inhibits GLI2 transcriptional activity through the recruitment of HDAC protein(s).

EXPERIMENTAL PROCEDURES

Generation of Gli2K2R Mutant Allele and Mouse Strains—A PAC clone containing mouse GLI2 genomic DNA sequences (Geneservices, Inc.) was used to create a Gli2K2R knock-in targeting construct. The Gli2K2R construct was engineered by mutating Lys-630 and Lys-716 residues into Arg residues and fusing the last third and second coding exons together by routine molecular cloning techniques. The neomycin cassette flanked by loxP sites was then inserted in the intron between the last two coding exons. The linearized construct was electroporated into W4 ES cells, and targeted ES cell clones were identified by restriction enzyme digestion followed by a Southern blot analysis of ES cell DNA using 5′- and 3′-probes (see Fig. 4). Two Gli2K2R targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeric founders, which were then bred with C57BL/6 to establish F1 heterozygotes. The neomycin cassette was excised by crossing the mutant mice with Actin-cre transgenic mice. Polymerase chain reaction (PCR) analysis was used for routine genotyping with the following primers: YP105 forward, 5′-GAGTGGAGGTGTGACAGAC-CGG-3′, and YP106 reverse, 5′-GTAAACCGGCATGTGCT-CATG-3′, which produced a 216-bp fragment for wild type allele and a 248-bp fragment for the mutant allele. Ptc1 mutant mouse that carries the lacZ gene knocked in the locus was previously described (13). Mouse UBC9 and SUMO2 cDNAs were obtained from Dr. Tso-Pang Yao at Duke University. All cDNA sequences amplified by PCR were validated by DNA sequencing.

Cell Culture—HEK293 cells were grown in DMEM with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C. Micromass limb bud cell primary cultures of stage 22–23 chick embryos were prepared as described (4). For luciferase reporter assay, two micromasses were seeded in each well of a six-well plate. For other purposes, monolayer culture (8 × 104 primary cells/well) was used.

Reporter Assay—Reporter assay was performed by transfecting micromasses of chick limb bud primary cells with a luciferase reporter driven by 8× GLI-binding sites (15), TK-Renilla control plasmid, and various GLI2 expression constructs as indicated using FuGENE 6 reagent (Roche Applied Science). Thirty-six h after transfection, cells were lysed, and luciferase activity was assayed using a Dual-Luciferase assay kit (Promega). Luciferase activity was normalized against Renilla luciferase control. Data presented in this study were compiled from three independent experiments.

Immunoblotting, Coimmunoprecipitation, and Immunohistochemistry—HEK293 cells were transfected with 1 μg of DNA for each expression construct by using a calcium phosphate precipitation method. Chick limb bud primary cells were transfected with 1 μg of DNA for each construct using FuGENE 6 reagents (Roche Applied Science). Thirty-six h after transfection, the cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin). GST pulldown and coimmunoprecipitation were performed as described (5). For detection of sumoylation, the cells were lysed in the denaturing buffer (0.5% SDS, 50 mM Tris (pH 7.5), 0.5 mM EDTA, 1 mM DTT). The lysates were boiled and vortexed to shear DNA. After being diluted 10 times with lysis buffer, the lysates were subject to immunoprecipitation using the indicated antibodies as described (6). Mouse monoclonal antibodies against HA, FLAG, and GST were purchased from Covance and Sigma, respectively. Antibodies against GLI2 and GLI3 were described (4, 5).

For immunohistochemistry, mouse embryos at E10.5 were dissected, fixed in 4% paraformaldehyde, PBS for 20–30 min at 4 °C, equilibrated in 30% sucrose, PBS overnight at 4 °C, and embedded in OCT. The frozen embryos were transversely cryosectioned at forelimb areas (10 μm). The tissue sections were subject to immunostaining using antibodies against FOXA2 (concentrated), NKX2.2, HB9, ISL1/2, NKX6.1, PAX6, and PAX7 (Developmental Studies Hybridoma Bank (DSHB),
RESULTS

GLI2 is Modified by SUMO—To understand the molecular mechanism by which GLI2 activity is regulated, we sought to identify the proteins that interact with the GLI2 protein. To that end, we constructed a mouse limb bud (E10.5) cDNA library for the yeast two-hybrid system. E10.5 limb buds were chosen because HH signaling is very active at that stage. When we used a GLI2 C-terminal fragment (584–1024 amino acids) as a bait to screen the library, two of the interactors identified were SUMO2 and PIAS1, one of the SUMO E3 ligases (data not shown). However, the interaction between GLI2 and SUMO2 or PIAS1 could not be verified in the mammalian cells using coimmunoprecipitation assay, suggesting that the interaction might be transient. Similar results have also recently been reported (18).

Because sumoylation is known to play important roles in the regulation of transcription factor activity, we went on to determine whether GLI2 is sumoylated in the cell. HA-tagged SUMO2 (HA-SUMO2) was expressed in HEK293 cells alone or together with GLI2. After cells were lysed, the protein lysates were subject to either immunoblotting to determine GLI2 and GLI3 expression (upper panels) or immunoprecipitation (IP) with GLI2 or GLI3 antibodies followed by immunoblotting with anti-HA antibody to detect sumoylated GLI2 and GLI3 proteins (lower panels). Smearing signals in lane 4 of each panel are the sumoylated GLI2 or GLI3 proteins. B, dominant negative UBC9 (FLAG-UBC9DN) inhibits GLI2 sumoylation. Expression constructs used are shown above the panel. The same methods as those in A were used. The upper panel shows the sumoylated GLI2 protein. The lower panel shows GLI2 expression. C, the Lys-630 and Lys-716 were the sumoylation sites. Wild type or mutant GST-Gli2PDD as indicated was expressed alone or together with HA-SUMO2 in HEK293 cells. Expression levels of GST-Gli2PDD fusion proteins were examined by immunoblotting (lower panel). The SUMO modification was detected by the GST pulldown followed by immunoblotting with an HA antibody (upper panel). Two arrows indicate the sumoylated GST-Gli2PDD as they were absent in the mutants.

FIGURE 1. GLI2 is modified by SUMO. A, both GLI2 and GLI3 are sumoylated. GLI2 or GLI3 were coexpressed with HA-SUMO2 in HEK293 cells. After cells were lysed, the lysates were subject to either immunoblotting to determine GLI2 and GLI3 expression (upper panels) or immunoprecipitation (IP) with GLI2 or GLI3 antibodies followed by immunoblotting with anti-HA antibody to detect sumoylated GLI2 and GLI3 proteins (lower panels). Smearing signals in lane 4 of each panel are the sumoylated GLI2 or GLI3 proteins. B, dominant negative UBC9 (FLAG-UBC9DN) inhibits GLI2 sumoylation. Expression constructs used are shown above the panel. The same methods as those in A were used. The upper panel shows the sumoylated GLI2 protein. The lower panel shows GLI2 expression. C, the Lys-630 and Lys-716 were the sumoylation sites. Wild type or mutant GST-Gli2PDD as indicated was expressed alone or together with HA-SUMO2 in HEK293 cells. Expression levels of GST-Gli2PDD fusion proteins were examined by immunoblotting (lower panel). The SUMO modification was detected by the GST pulldown followed by immunoblotting with an HA antibody (upper panel). Two arrows indicate the sumoylated GST-Gli2PDD as they were absent in the mutants.
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**A**

| Gli2P5-6 | Gli2P1-4 | Gli2P1-6 | Gli2 | HA-Sumo2 |
|----------|----------|----------|------|----------|
| IP: αGli2 | IB: αHA  | IP: αGli2 | IB: αHA  | Ctrl  |

**B**

FIGURE 2. GLI2 sumoylation is inhibited by mutations in PKA sites (A) and stimulation with HH (B). GLI2 or its mutants were expressed alone or together with HA-SUMO2 in chick limb bud mesenchymal cells. IP, immunoprecipitation; IB, immunoblotting. The cells were incubated with medium with or without SHHN conditioned medium overnight (B). Expression levels of GLI2 and its mutant proteins were determined by immunoblotting (lower panel). The SUMO modification was detected by immunoprecipitation with GLI2 antibodies followed by immunoblotting with an HA antibody (upper panel). Ctrl, control.

Previously there were also three additional imperfect SUMO sites in the N terminus (Lys-12, -407, and -447). Because the bait used contains the PDD, we determined whether the two sites in the PDD were sumoylated in the cell. Expression constructs for GST-fused wild type PDD or for PDD with Lys → Arg substitutions in one (K630R, K716R) or both (K630R/K716R) sumoylation sites were created. After they were separately transfected into HEK293 cells or together with HA-SUMO2 expression construct, sumoylation of the fusion proteins was examined by pulling down the fusion proteins and subsequently immunoblotting. The results showed that SUMO modification was detected in the wild type PDD, but significantly reduced or abolished in the single or double SUMO site mutants, indicating that Lys-630 and Lys-716 are sumoylated in the cell (Fig. 1C).

**Mutations in PKA Phosphorylation Sites of GLI2 and HH Stimulation Inhibit GLI2 Sumoylation**—Phosphorylation of GLI2 by PKA induces GLI2 proteolytic processing to generate the GLI2 repressor. It may also exert an inhibitory effect on GLI2FL activity. In contrast, HH signaling inhibits GLI2 processing and activates GLI2FL protein (5, 16). Thus, we wanted to know whether GLI2 sumoylation is affected by PKA-mediated phosphorylation and HH stimulation. To address this question, the extent of sumoylation of wild type GLI2 was compared with that of mutant GLI2 proteins containing mutations at different PKA phosphorylation sites in transfected chicken limb bud primary cells. The chick limb bud primary cells were used because they respond to HH stimulation very well (4). Mutations at sites 5 and 6 (Gli2P5–6) only slightly reduced GLI2 sumoylation level; mutations at sites 1–4 (Gli2P1–4) significantly decreased GLI2 sumoylation, whereas mutations of all six sites (Gli2P1–6) reduced GLI2 sumoylation even further (Fig. 2A). Similarly, HH stimulation also reduced GLI2 sumoylation level (Fig. 2B). These results indicate that both mutations at the six PKA phosphorylation sites and HH stimulation inhibit GLI2 sumoylation, suggesting that GLI2 phosphorylation by PKA may positively regulate GLI2 sumoylation.

**Sumoylation Inhibits GLI2 Transcriptional Activity**—To determine the significance of SUMO modification in HH signaling in vivo, we employed the targeted gene knock-in approach and replaced Lys-630 and Lys-716 with an Arg residue at the GLI2 locus (Fig. 4A, indicated by single-letter codes in the figure). Because the two Lys residues are in two consecutive small exons, the exons were fused in the targeting construct to ensure that the GLI2K2R mutant allele carries both point mutations after homologous recombination. Southern blot analysis identified 10 mutant ES cell clones (Fig. 4B), two of which were used to create a GLI2 SUMO site mutant mouse line named GLI2K2R. Because the SUMO sites can also be the sites for ubiquitination, we first determined whether the GLI2K2R mutant protein is more active than wild type GLI2, we examined the neuronal cell specific expression of GLI2K2R in vivo. Following an enrichment of GLI2 protein using a double-stranded oligonucleotide that contains specific GLI binding sites, immunoprecipitation and immunoblotting analysis using GLI2 antibodies showed that the extent of GLI2K2R protein processing is comparable with that of wild type GLI2 protein in mouse embryos, indicating that GLI2 processing is not compromised (Fig. 4D).

To determine whether GLI2K2R mutant protein is more active than wild type GLI2, we examined the neuronal cell specific expression and patterning in the developing neural tube. In wild type embryos, the floor plate, V3 progenitor cells, and motoneurons are specified and patterned in the distinct locations of the ventricle.
tral neural tube. They can be marked by transcription factors FOXA2, NKX2.2, and HB9 and ISL1 expression, respectively. NKX6.1 expression covers from the floor plate to the V1 progenitors. In contrast, PAX6 and PAX7 expression is found in the dorsal neural tube and is absent in the ventral-most area and in the entire ventral area, respectively, because their expression is suppressed by HH signaling (20). In the 

\[ \text{Gli2K}2\text{R} \] homozygous neural tube, the expression and patterning of these transcription factors are indistinguishable from those in the wild type (Fig. 5, A–N). To more directly compare the Gli2K2R transcriptional activity with that of wild type GLI2, [PTCH1] expression in the neural tube was examined using lacZ reporter gene inserted in the [PTCH1] locus (13). We found that Ptc1-lacZ expression in the mutant neural tube was slightly higher and dorsally expanded than that in wild type (Fig. 5, O–P). These results indicate that Gli2K2R is slightly more active than wild type GLI2 in vivo, suggesting that sumoylation inhibits GLI2 activity in vivo.

**HDAC5 Interacts with GLI2 but Not Gli2K2R Mutant**—Histone acetylation and deacetylation have been recognized as a general mechanism by which eukaryotic transcription factors can activate or suppress gene expression. Sumoylation can suppress transcriptional activity by promoting the interaction of transcription factors with HDACs, which remove acetyl groups from histones and thus generally mediate transcriptional repression (21). To elucidate the molecular mechanism by which sumoylation inhibits GLI2 transcriptional activity, we wanted to know whether GLI2 interacts with HDACs, and if it does, whether Gli2K630/716R interacts differently with HDACs. To this end, GLI2 or Gli2K630/716R were coexpressed with either FLAG-tagged HDAC4 or FLAG-tagged HDAC5 in HEK293 cells. Western blot showed that FLAG-HDAC4

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**FIGURE 4. Generation of Gli2K2R allele that expresses a GLI2 protein lacking sumoylation sites at both Lys-630 and Lys-716.** A, the gene targeting strategy used to create Gli2K2R allele and screen for mutant ES cell clones. Open rectangles are referred to as exons, and two lysines (K) indicate the SUMO sites, which were mutated to arginines (RR) in the mutant. PKA sites are indicated. The relevant restriction sites are: B, BamHI; Bg, BglII; Bs, BstZ171; E, EcoRI; Sp, SpeI. B, Southern blot analysis of representative ES cell (ESC) clones using the 5′- and 3′-probes shown in A. Mut, mutant. C, diagrams of the predicted Gli2K2R mutant protein. D, Gli2K2R expression level is similar to wild type GLI2 in vivo. GLI2 proteins were first precipitated with agarose beads conjugated with double-stranded GLI-binding or control (Ctrl) oligonucleotides and then immunoblotted with GLI2 antibodies. GLI2 full-length protein (Gli2FL) and GLI2 repressor (Gli2Rep) are indicated. ZFs, zinc fingers.
expression level is lower than that of FLAG-HDAC5 (Fig. 6, middle panel). Coimmunoprecipitation analysis showed that the GLI2 antibody precipitated residual HDAC5 even when GLI2 was not overexpressed (Fig. 6, upper panel, lane 5). This could be the result from interaction between FLAG-HDAC5 and endogenous GLI2. When GLI2 and FLAG-HDAC5 were coexpressed, the significantly higher amount of FLAG-HDAC5 was coimmunoprecipitated. However, when coexpressed with Gli2K630/716R, the amount of HDAC5 coimmunoprecipitated was similar to that when HDAC5 was expressed alone (Fig. 6, upper panel, compare lane 7 with lane 9). In contrast, the amount of FLAG-HDAC4 coimmunoprecipitated was similar when it was coexpressed with either GLI2 or Gli2K630/716R (Fig. 6, upper panel, compare lane 6 with lane 8). Taken together, these results indicate that GLI2 preferentially binds HDAC5 over HDAC4 and that only GLI2 but not Gli2K630/716R can bind HDAC5. Therefore, the inhibition of GLI2 activity by sumoylation is most likely through its recruitment of HDAC proteins.

**DISCUSSION**

In the present study, we show that GLI2 is sumoylated in amino acid residues Lys-630 and Lys-716. Mutations in the two-sumoylation sites result in an increase in GLI2 activity both in cell-based reporter assay and in vivo, although to a lesser extent. The increase in Gli2K630/716R mutant activity is most likely the result from its inability to recruit HDACs.

Much is known about the molecular mechanism of GLI2 and GLI3 proteolytic processing. In contrast, little is known about how GLI2 and GLI3 are activated by HH signaling. Our previous studies showed that both GLI2 and GLI3 mutants that are not phosphorylatable at the first four PKA sites can up-regulate SHH target genes in the absence of SHH in vivo, suggesting that PKA phosphorylation is at least partially responsible for their inactivation (16, 22). In supporting this view, a recent study showed that GLI2 is activated in PKA mutant mice (23). The present study shows that the sumoylation of GLI2 protein is another type of modification to regulate GLI2 protein activity. GLI2 contains five potential sumoylation sites. This study focuses on only Lys-630 and Lys-716 because they are located in the GLI2 C-terminal region that was used to identify PIAS1 as a GLI2 interactor in the yeast two-hybrid screen. GLI2 protein with mutations in these two sites exhibits a significantly higher transcriptional activity than the wild type GLI2 in the cell-based reporter assay (Fig. 3). Similarly, the same mutant protein expressed at a physiological level also displays slightly higher activity (Fig. 5). The difference in Gli2K630/716R activity between cell culture and in vivo is likely that Gli2K630/716R mutant protein could still efficiently be sumoylated at other SUMO sites in vivo, whereas Gli2K630/716R in cell culture could not because endogenous sumoylation enzymes may become limited when Gli2K2R is overexpressed. Nevertheless, our results indicate that SUMO modification plays an inhibitory role in the GLI2 activation.

This observation is in contrast to a recent study showing that SUMO conjugation enhances the transcriptional activity of GLI proteins (18). The discrepancy between that study and ours is most likely due to the difference in the way in which the experiments were conducted. First, our study investigates the effect of mutations of two sumoylation sites on GLI2, whereas
the previous study focused on the effect of overexpression of E3 SUMO ligase Pias1 on Gli1 protein activity. Because the overexpression of Pias1 may directly and also indirectly influence Gli1 activity, the increase of Gli1 activity may partially result from an adverse effect of Pias1 overexpression on the Gli1 activity. Second, our conclusion is based on the Gli2 sumoylation mutant protein when it is either overexpressed in cultured cells or expressed in a physiological level in mouse embryos, whereas the previous study relies on only the overexpression in cell culture and the neural tube.

As described above, Gli2 and Gli3 proteins are phosphorylated by PKA and subsequently by GSK3 and CK1. The phosphorylation initiates proteolytic processing to generate Gli2 and Gli3 repressors, thus exerting an inhibitory role on Gli2 and Gli3 function (4–6). Interestingly, mutations in different PKA sites accumulatively reduce the extent of Gli2 sumoylation (Fig. 2A), suggesting that PKA phosphorylation of Gli2 is necessary for Gli2 sumoylation. It is possible that phosphorylation of Gli2 protein by PKA may enhance recruitment of sumoylation enzymes. In contrast, HH stimulation, which activates Gli2 and Gli3 proteins, can decrease Gli2 sumoylation level (Fig. 2B). Therefore, phosphorylation of Gli2 by PKA and activation of Gli2 by HH signaling are directly correlated with Gli2 sumoylation level. These observations raise the possibility that one potential mechanism by which HH signaling activates Gli2 is to inhibit Gli2 sumoylation, whereas PKA phosphorylation inhibits Gli2 activity by enhancing Gli2 sumoylation.

It is well established that sumoylation inhibits transcription factor activity through its recruitment of HDAC to the gene regulatory region. Similarly, we found that HDAC5 can interact with wild type Gli2 but not Gli2K2R mutant. This finding suggests that many other transcription factors, the mechanism by which sumoylation inhibits Gli2 activity is also through its recruitment of HDAC.

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