A proteomic approach identifies SAFB-like transcription modulator (SLTM) as a bidirectional regulator of GLI family zinc finger transcription factors

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ABSTRACT

In Sonic hedgehog (SHH) signaling, GLI family zinc finger (GLI)-mediated diverse gene transcription outcomes are strictly regulated and are important for SHH function in both development and disease. However, how the GLI factors differentially regulate transcription in response to variable SHH activities is incompletely understood. Here, using a newly generated, tagged Gli3 knock-in mouse (Gli3TAP), we performed proteomic analyses and identified the chromatin-associated SAFB-like transcription modulator (SLTM) as a GLI-interacting protein that context-dependently regulates GLI activities. Using immunoprecipitation and -blotting, RT-qPCR, and ChIP assays, we show that SLTM interacts with all three GLI proteins and that its cellular levels are regulated by SHH. We also found that SLTM enhances GLI3 binding to chromatin and increases GLI3 repressor (GLI3R) form protein levels. In a GLI3-dependent manner, SLTM promoted the formation of a repressive chromatin environment and functioned as a GLI3 co-repressor. In the absence of GLI3 or in the presence of low GLI3 levels, SLTM co-activated GLI activator (GLIA)-mediated target gene activation and cell differentiation. Moreover, in vivo Sltm deletion generated through CRISPR/Cas9-mediated gene editing caused perinatal lethality and SHH-related abnormal ventral neural tube phenotypes. We conclude that SLTM regulates GLI factor binding to chromatin and contributes to the transcriptional outcomes of SHH signaling via a novel molecular mechanism.

Sonic hedgehog (SHH) signaling plays important roles during development and in cancer growth. Mutations that affect the SHH pathway cause severe birth defects and cancers (1-5). SHH signaling mediated through Patched (Ptc1) and Smoothened (Smo) controls target gene expression by differentially regulating activities of the GLI family of Zn-finger transcription factors (1,3,6). GLI family transcription factors carry out the diverse transcription outcomes of SHH signaling. In the absence of SHH, full-length GLI3 (GLI3FL) can be proteolyzed, and the C-terminal truncated protein GLI3R functions as the main repressor of expression of SHH target genes (7). In the presence of SHH, SHH signaling activates GLI1/2 proteins and inhibits GLI3 proteolysis. GLI1 and GLI2 are the main transcription activators that mediate SHH-induced transcription. The balance of protein levels and activities of GLI activator (GLIA) and GLI repressor (GLIR) forms determines transcription outcomes in specific cell types during specific developmental stages (8-10).
How GLI factors differentially regulate transcription in response to different SHH activities during development is under active investigation. GLI proteins share a conserved zinc finger domain containing five zinc fingers, which is responsible for binding to DNA and may also interact with co-factors (11). The three GLI family members could bind to the same consensus DNA sequences, but exerts different transcription outcomes (1,10,12,13). The N- and C-terminal regions of GLI proteins are more diverse and display repressing or activating functions, likely through interactions with different proteins. Interestingly, some proteins could interact with all three GLI proteins and exert context-dependent opposite functions in SHH signaling (14,15).

SHH regulates GLI proteins by influencing protein expressing, processing, localization, and degradation. GLI transcription activities are also regulated by post-translational modifications such as phosphorylation, acetylation, ubiquitination and sumoylation (10,12,16). SUFU is a main negative regulator of SHH signaling, which regulates GLI activities at several levels. Without the SHH signal, SUFU binds to GLI3 and the complex is recruited to primary cilia, leading to the efficient processing of GLI3FL into GLI3R (17,18). SHH activation leads to the dissociation of SUFU from Glli3FL allowing its translocation to the nucleus, where it is phosphorylated, destabilized, and converted to a transcriptional activator (GLI3A) (19-21). In addition, SUFU negatively regulates GLIA by binding to GLI1/2 and sequestering them in the cytoplasm (20-22). SUFU has also been shown to function in the nucleus to repress GLIA activities (23,24). Primary cilia play context-dependent opposite roles in regulating GLI activities. It is not only required for GLI3 processing to produce GLIR, but it also is required for GLIA dissociation from SUFU and pathway activation (14,25).

In the nucleus, the mechanism regulating GLI activities are emerging. We and others have shown that distinct transcription co-factor complexes are formed with GLIR or GLIA to produce different transcription outcomes (10,26). Chromatin remodeling BAF complexes interact with all three GLI proteins; depending on context, BAF may repress or activate basal and signaling-induced target gene transcription (15,27). In basal condition, the SHH target genes labeled by bivalent chromatin domains are repressed by an epigenetic network that involves BAF complexes and PRC2 complexes. In response to SHH stimulation, there is an exchange from GLIR to GLIA at the gene regulatory regions resulting in a switch from co-repressor complexes to co-activator complexes including BRG1, JMJD3, and MLL (26). GLI proteins have also been shown to interact with other chromatin regulators, such as HDACs and CBP (28,29). Thus, understanding of the GLI-interacting proteins will provide insights to the mechanisms underlying the GLI-mediated diverse transcription activities and SHH-related birth defects and childhood diseases.

An obstacle to identification of GLI interacting proteins has been the lack of suitable anti-GLI antibodies. To overcome this, we engineered the Gli3 locus and generated a tagged-Gli3 knock-in mouse. Using this mouse, we affinity purified GLI3-interacting nuclear proteins and identified SAFB-like transcription modulator (SLTM) as a GLI-interacting protein. We showed that SLTM interacts with all three GLI proteins. SLTM enhances GLI3 binding to chromatin and increases GLI3R protein levels. In a GLI3-dependent manner, SLTM promotes the formation of a repressive chromatin environment. In the absence of GLI3 or in the presence of low levels of GLI3, SLTM functions to co-activate GLIA-mediated target gene activation and cell differentiation. Therefore, SLTM regulates GLI factor binding to chromatin and contributes to the precise transcription outcomes of SHH signaling with a novel mechanism.

Results
Generation of Gli3TAP knock-in mice
To identify GLI3 interacting proteins, we engineered the mouse Gli3 locus and knocked in a tandem affinity purification (TAP) tag using homologous recombination. The TAP tag contains a protein A tag and an HA tag separated by a Tev protease cleavage site (Figure 1A). Correctly recombined embryonic stem (ES) cell clones were identified by PCR (Figure 1B) and Southern blot (Figure 1C). The
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Gli3\textsuperscript{TAP-neo} mouse line was generated through germ line transmission. The neomycin expression cassette was then removed by crossing to a Cre transgene. The resulting Gli3\textsuperscript{TAP+/−} heterozygous and Gli3\textsuperscript{TAP+/TAP} homozygous mice are normal and fertile, indicating the normal function of the protein encoded by the Gli3\textsuperscript{TAP} allele. In the Gli3\textsuperscript{TAP+/−} heterozygous telencephalon, TAP-GLI3 and wild-type GLI3 proteins were expressed and processed at comparable levels (Figure 1D). To further determine whether the TAP tag alters the function of GLI3, we crossed Gli3\textsuperscript{TAP+/TAP} mice to mice with a Gli3 null allele. Gli3\textsuperscript{TAP−} mice grow normally but have one extra first digit (Figure 1E), a phenotype similar to Gli3\textsuperscript{+/−} extra toe mice (30). Therefore, TAP-GLI3 protein likely functions similarly to wildtype GLI3 and can be used to study GLI3 protein properties.

Affinity purification of TAP-GLI3 and associated proteins

Previously, we have shown that GLI3 interacts with a chromatin remodeler BRG1 and mediates recruitment of BRG1 to the regulatory regions of SHH/GLI target genes during development (15). Using E13.5 Gli3\textsuperscript{TAP+/TAP} telencephalons, where GLI3 expression levels are high, we immunoprecipitated endogenous TAP-GLI3 with IgG beads, which could bind to the Protein A fragment in the TAP tag; endogenous BRG1 was enriched in this precipitate (Figure 2A). ChIP analyses with IgG beads of chromatin from E13.5 telencephalons also showed binding of TAP-GLI3 at the regulatory regions of SHH/GLI target genes (Figure 2B). These results further confirmed that the addition of the TAP tag did not disrupt the molecular properties of GLI3.

We then performed affinity purification of GLI3-associated complexes and identified GLI3 interacting proteins with mass spectrometry. E13.5 telencephalons were used to prepare nuclear extracts. Due to the difficulty of releasing GLI3 proteins by Tev digestion from the beads after the first step purification, we performed a one-step affinity purification using IgG Sepharose beads (Figure 2C). Western blot showed a significant enrichment of TAP-GLI3 (GLI3FL and GLI3R) in the elution fractions (Figure 2D). Wild-type telencephalons were used as a negative control. The proteins identified from the TAP-GLI3-expressing telencephalons but not the wild-type tissues were candidate GLI3 interacting proteins. Forty specific proteins were identified including many proteins with known or predicted functions in transcription regulation (Table S1). Importantly, among these hits, GLI3 was identified with the most number of recovered peptides. The known GLI3 interacting proteins KIF7 (Cos2 homolog) (31) and SUFU ranked numbers 3 and 5, respectively (Table S1, Figure 2E). The identification of GLI3 (Figure 2F) and these known GLI3 interacting proteins validates our purification approach. Notably, one of the top hits is SAFB-like transcription modulator (SLTM) with 14 peptides recovered (Figure 2E, F). SLTM is a member of the SAF-box protein family; other family members often function as transcription co-repressors or co-activators through diverse and context-dependent mechanisms (32-34). Thus it is possible that SLTM regulates GLI3 functions.

SLTM interacts with GLI proteins

To understand the interactions between SLTM and GLI proteins, we expressed both GLI3 and HA-SLTM in SHH-responsive NIH3T3 cells. Immunoprecipitation (IP) with anti-GLI3 antibodies co-purified GLI3 and SLTM (Figure 3A). To determine which region of the GLI3 protein interacts with SLTM, we performed co-IP experiments using HA-tagged GLI3 fragments and FLAG-SLTM. We observed interactions between SLTM and GLI3R, the N-terminal domain (GLI3N) and to a lesser degree the C-terminal domain (GLI3C), but not the zinc finger domain (Figure 3B). We also examined the interactions between both GLI1 and GLI2 proteins with SLTM. SLTM co-IP with all three GLI proteins from NIH3T3 cell extracts (Figure 3C).

SLTM represses SHH/GLI target gene expression

The transcriptional outcome of SHH signaling is mainly determined by the balance between GLI1A and GLI1R activities. In primary mouse embryonic fibroblast (MEF) cells and similarly NIH3T3 cells, under basal conditions, GLI3 represses the expression of target genes
SLTM is a bidirectional GLI regulator such as Gli1 and Ptc1. SHH treatment or introduction of exogenous GLI1 induces the activation of these genes. Since SAFB family proteins often function as repressors, we reasoned that SLTM likely facilitates the repression of SHH/GLI target genes. To determine the function of SLTM, we generated Sltm−/− mice using the CRISPR/Cas9 method. The second coding exon was targeted and a DNA fragment encoding GFP was inserted in frame (Figure 4A, 4B). SLTM proteins were not produced in Sltm−/− MEF cells (Figure 4C). Therefore, it is likely a null allele. GFP expression in Sltm−/+ and Sltm−/− embryos indicated that SLTM is ubiquitously expressed (Figure 4D). Sltm−/− mice die soon after birth without obvious growth defects (data not shown).

Sltm deletion in MEF cells led to a de-repression of SHH target genes under basal conditions. SHH target gene Gli1 and Ptc1 were present at higher levels in Sltm−/− MEFs than in wild-type control cells (Figure 4E). SLTM is not a general repressor since Wnt target genes Axin2 and C-Myc were not de-repressed in Sltm−/− MEFs (Figure 4E). Furthermore, overexpression of SLTM in NIH3T3 cells significantly repressed the SHH-induced target gene expression (Figure 4F). Overexpression of SLTM also inhibited endogenous Gli1 expression induced by exogenous GLI1 activator (Figure 4G). Therefore, SLTM is required for repression of SHH/GLI target genes such as Gli1 and Ptc1, whereas excess SLTM represses SHH-induced expression of these genes.

**SLTM enhances GLI3 binding to chromatin and is regulated by SHH**

SHH signaling activation inhibits GLI3 processing and leads to the degradation of GLI3 proteins and departure of GLI3R from the chromatin and nucleus. To determine how SLTM regulates GLI3 repressor activities, we examined the SLTM and GLI3 protein levels in NIH3T3 cells that were treated with SHH and overexpressed increasing amounts of SLTM. SHH treatment led to decreased endogenous SLTM levels as well as lower GLI3R levels (Figure 5A). Overexpression of SLTM in the presence of SHH increased GLI3 protein levels and repressed SHH-induced Gli1 expression in an SLTM dose-dependent manner (Figure 5A). These results suggest that SLTM protein level is regulated by SHH and it positively regulates GLI3 protein levels.

In NIH3T3 cells that express exogenous SLTM, SLTM was localized in the nucleus and was enriched in the chromatin fraction (Figure 5B). Interestingly, overexpression of SLTM significantly increased the level of GLI3R in the chromatin fraction relative to overexpressing control proteins, but levels of the full length GLI3 were similar in control cells and cells that overexpressed SLTM (Figure 5B). The increase of GLI3R in the chromatin fraction in the presence of excess SLTM suggests that GLI3R binding to the regulatory regions of its target genes was increased. ChIP analyses indicated that SLTM binds to the regulatory regions of SHH target genes such as Gli1 and that the binding was attenuated upon SHH treatment (Figure 5C). Overexpression of SLTM increased GLI3 binding to the Gli1 regulatory region (Figure 5D). In contrast, in Sltm−/− MEFs binding of GLI3 to the Gli1 regulatory region was significantly reduced compared to levels in control cells (Figure 5E). These results suggest that SLTM enhances GLI3 retention in the chromatin fraction.

**SLTM facilitates the formation of a repressive chromatin environment at the Gli1 locus**

Previously, we showed that GLI3-mediated repression of SHH target genes is associated with a repressive chromatin environment (15,26). Under basal conditions, SHH target genes are marked by a bivalent domain containing a repressive H3K27me3 and an active H3K4me3 mark. H3K27 methyltransferase complex PRC2 maintains the H3K27me3 mark repressing the expression of the SHH target genes. SHH activation induces an epigenetic switch. GLI3R is displaced from SHH target gene promoters, and GLIA binds and recruits H3K27me3 demethylase Jmjd3 to remove H3K27me3 marks and activate target gene expression.

In a recent proteomic study, SLTM was shown to interact with PRC2 (35); therefore, we examined whether an excess of SLTM would alter H3K27me3 levels in SHH target gene
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regulatory regions. In NIH3T3 cells, overexpression of SLTM significantly increased the H3K27me3 levels at the Gli1 locus (Figure 6A). Consistently, overexpression of SLTM also increased local PRC2 levels as shown by the increase in levels of the PRC2 subunit SUZ12 in the Gli1 regulatory regions (Figure 6B). Interestingly, we also observed another repressive histone mark, H3K9me2, in the regulatory region, and SLTM overexpression led to an increase in this mark (Figure 6C). Histone H3 ChIP-qPCR was used as a control; similar levels of nucleosome occupancy were observed in control cells and in cells in which SLTM was overexpressed (Figure 6D). Thus, SLTM may repress SHH target genes by both enhancing GLI3 binding and facilitating the generation of a repressive chromatin environment.

**SLTM activates SHH/GLI target genes in the absence of GLI3**

Our experiments indicate that SLTM facilitates the binding of GLI3R to specific SHH target gene regulatory regions and helps to generate and maintain a repressive chromatin environment. Since GLI3R is the major repressor in SHH signaling, we examined whether the repressive function of SLTM on SHH signaling is GLI3 dependent. Surprisingly, in Gli3<sup>-/-</sup> MEFs, instead of repressing target gene expression as it did in the wild-type cells (Figure 4F), excess SLTM enhanced the expression of SHH/GLI target genes in response to SHH stimulation. SHH-induced Gli1 and Ptc1 expression were both higher upon SLTM overexpression in Gli3<sup>-/-</sup> MEFs (Figure 7A).

To determine whether this effect of SLTM on SHH target gene expression was due to the absence of GLI3, we reintroduced GLI3 into Gli3<sup>+/+</sup> MEFs. Lentivirus expressed modest levels of GLI3R diminished the activator effect of SLTM on target gene expression (Figure 7B), suggesting that the absence of GLI3 switched SLTM from a repressor to an activator. Since SLTM also interacts with GLI activators, albeit with a lower affinity (Figure 3C), it is possible that in the absence of GLI3 or in the presence of low levels of GLI3R, SLTM could facilitate the binding of GLI2 to chromatin. Indeed, although excess SLTM did not enhance GLI2 binding to the Gli1 regulatory regions in wild-type MEFs, in Gli3<sup>-/-</sup> cells, SLTM significantly increased GLI2 binding to the regulatory regions (Figure 7C). These results suggest that the relative levels or activities of GLIA and GLIR determine whether SLTM functions as an activator or a repressor of SHH target genes.

Since SLTM positively regulated GLI3R levels, we examined GLI3 levels in Sltm<sup>-/-</sup> MEFs. In Sltm<sup>-/-</sup> MEFs, levels of GLI3R were lower than in wild-type cells and were similar to the low GLI3 levels observed in wild-type cells upon SHH stimulation (Figure 7D). Interestingly, in Sltm<sup>-/-</sup> MEFs, SHH-induced Gli1 activation was impaired (Figure 7E), suggesting that SLTM functions as a co-activator for GLIA. The low GLI3R levels in SHH-stimulated cells may be responsible for switching SLTM from a repressor to an activator. The repressor function of SLTM observed in unstimulated NIH3T3 and MEF cells and in stimulated NIH3T3 cells in which SLTM is overexpressed depends on the presence of high GLI3 levels. Thus, in the absence of GLI3 or when GLI3 levels are low, SLTM functions as an activator of SHH target genes.

**SLTM deletion leads to abnormal expression of ventral neural tube markers**

To determine whether SLTM regulates SHH-dependent developmental processes, we examined Sltm<sup>-/-</sup>embryos for expression of ventral neural tube markers. As a morphogen, SHH is important for neural tube patterning and neural progenitor specification. In developing neural tube, SHH/GLIA are required for the specification of the most ventral neural progenitors (8,9). GLI3 levels are low in the ventral neural tube. In E10.5 Sltm<sup>-/-</sup> neural tubes, V3 interneuron progenitor marker NKX2.2 levels were significantly reduced and OLIG2-expressing motor neuron progenitor regions were expanded (Figure 8). This phenotype was seen in other mutant embryos that were defective in SHH signaling (36,37). Together with our observations in Sltm<sup>-/-</sup> MEF and NIH3T3 cells, this result indicates that SLTM is important for GLI-dependent target gene expression and ventral neural tube progenitor specification.
SLTM enhances SHH-induced osteoblast differentiation

To determine whether SLTM functions in other SHH-dependent differentiation processes, we investigated the effect of excess SLTM on SHH-induced osteoblast differentiation of the mesenchymal stem cell-like C3H10T1/2 cells. SHH induces the differentiation of C3H10T1/2 cells into osteoblasts in a GLIA-dependent manner (38,39). Alp is a direct target gene of GLI1/2 that is activated during differentiation (40). SHH treatment induced Alp expression in C3H10T1/2 cells as shown by staining for alkaline phosphatase activities in plated cells (Figure 9A). Overexpression of SLTM further enhanced SHH-induced Alp expression relative to levels in cells transfected with a control vector (Figure 9B) and also significantly enhanced SHH-induced expression of Gli1 and Ptc1 (Figure 9B). These results further confirmed that SLTM is a GLI regulator.

Discussion

In this study, using a newly generated Gli3TAP knock-in mouse and a proteomic approach, we found that SLTM interacts with GLI proteins to regulate SHH signaling bidirectionally. SLTM facilitates the binding of GLI3R to chromatin and enhances the repressor function of GLI3R. In the absence of GLI3R or when levels of GLI3R were low, SLTM increases the binding of GLIA to regulatory regions of SHH target genes and enhances GLIA-mediated gene activation and cell differentiation.

The Gli3TAP mouse we generated proved to be a useful tool to study GLI3 function. We first showed that addition of the TAP tag did not significantly alter GLI3 activities. TAP-GLI3 protein was expressed and processed in a similar fashion as the wild-type GLI3. The Gli3TAP mice grow normally but have one extra first digit, a phenotype similar to Gli3+/− extra toe mice (30). The TAP tag allowed us to study the properties of endogenous GLI3. A similar tag knock-in mouse was generated to study GLI3 function (41). However, we believe that this study represents the first purification of endogenous GLI3 interacting complexes, which indicates the value of this mouse.

Using immunoprecipitation via the TAP tag, we identified SLTM as a novel GLI regulator. Our data indicate that SLTM interacts with GLI proteins in the nucleus and regulates the local and global binding of GLI proteins to chromatin. The exchange of the GLIR for GLIA transcription factors is a critical step in production of SHH signaling outcomes. However, it was unknown how the binding of GLI proteins to target genes is regulated. It has been shown that nuclear GLI proteins are unstable after dissociation from SUFU (16,21). Here, we demonstrated that SLTM plays an important role in regulating the balance between different GLI protein activities by differentially affecting their binding to chromatin. We showed that SLTM increased both GLI3R protein levels and GLI3 binding to chromatin. Since SLTM is a chromatin-associated protein that binds to GLI regulatory regions, we favor the scenario that SLTM stabilizes GLI3 proteins by interacting and retaining it on the chromatin. However, it is also possible that SLTM stabilizes GLI3, whereas increased GLI3 levels passively increased GLI3 binding to DNA. In the absence of GLI3, SLTM enhances GLIA activities, possibly through similar mechanisms.

As an important developmental signal, SHH signaling and transcription outcomes are precisely regulated. Interestingly, SHH signaling pathway components often play bidirectional roles, functioning both as activators and repressors of the pathway in a context-dependent manner. For example, the primary cilium is required for both GLI3R processes under basal conditions and for GLI1/2 activation in response to SHH (14,42), and the chromatin remodeling factor BRG1 is required for both repressing basal expression and for signaling-induced target gene expression (15,27). Similarly, our data indicate that SLTM functions as a co-repressor of GLI3R under basal conditions and in the absence of GLI3 SLTM functions as a co-activator of GLIA. Therefore, SLTM provides another level of precision in SHH signaling regulation.

The seemingly bidirectional function of SLTM in modulating GLIR and GLIA activities is regulated by SHH activities and GLI3R protein levels. SLTM facilitates GLI3 binding to target genes in cells under basal condition. In
response to SHH, SLTM protein levels decrease, which likely contributes to the departure of GLI3R from the target sites and reduction of GLI3R levels. In SHH-stimulated cells in which SLTM was overexpressed, SLTM functioned as a co-repressor, possibly by increasing the amount of GLI3R bound to chromatin. In the absence of GLI3 (e.g., in Gli3-/- cells) or when GLI3 levels were relatively low (e.g., in SHH-stimulated cells or in Sltm-/- cells), SLTM enhanced GLI1 binding and functioned as a co-activator of SHH target gene expression. In the absence of GLI3 (e.g., in Gli3-/- cells) or when GLI3 levels were relatively low (e.g., in SHH-stimulated cells or in Sltm-/- cells), SLTM enhanced GLI1 binding and functioned as a co-activator of SHH target gene expression. Our data suggest that SLTM prefers to bind to GLI3R rather than GLI1 in unstimulated NIH3T3/MEF cells (Figure 3): the differential effects of SLTM on SHH target gene expression are likely due to this difference in binding affinity and to the relative amount of GLI1 and GLI3R in the cells. The SAFB proteins mainly serve as co-repressors. We found that SLTM overexpression led to a repressive chromatin environment in regulatory regions of SHH target genes. It is possible that SLTM recruits other co-repressors such as PRC2 as previously reported proteomic analysis demonstrated an interaction between PRC2 and SLTM (35). It is also possible that the increase of GLI3R binding to the enhancer region facilitated by SLTM indirectly results in a repressive chromatin environment. The fact that SLTM functions as a GLI1 co-activator suggests, however, that SLTM-mediated effects on GL1 protein binding to chromatin plays a major role in determining the transcriptional outcomes.

SLTM is a member of the SAFB family proteins. Although it is less similar to the other two family members SAFB1 and SAFB2 than they are to each other (32,33), it is not clear whether SAFB1 and SAFB2 compensate for the absence of SLTM in vivo. Sltm-/- MEF cells display defects in SHH target gene expression under basal and SHH-induced conditions. Sltm-/- embryos have defects in ventral neural tube, indicating its function in GLI1 mediated progenitor specification. However, Sltm-/- mice did not display defects previously observed in Gli3-/- embryos (30). It is possible that other co-repressors such as SAFB family members may have redundant functions in vivo. It is also possible that Sltm deletion led to impaired function of both GLIR and GLIA, which may rescue certain of the gross defects caused by GLIR deletion. These rescuing phenotypes were previously observed in Shh/Gli3 and Smo/Gli3 double knock-outs (43-45). The positive function of SLTM in SHH-induced C3H10T1/2 osteoblast differentiation further suggests a physiological role of SLTM in SHH-dependent developmental process.

In summary, we generated a Gli3TAP knock-in mouse and used it to analyze GLI3 function. We performed a proteomic analysis of endogenous GLI3 complexes and identified SLTM as a novel regulator of GLI activities and SHH signaling outcomes. We uncovered a bidirectional function of SLTM: This protein can act as an activator or a repressor depending on GLI3R levels. The function of SLTM in regulating GLI protein binding to chromatin was previously unknown, and this study revealed another layer of precise regulation of SHH signaling.

**Experimental procedures**

**Generation of Gli3TAP knock-in mice**

Gli3TAP knock-in mice were generated using homologous recombination. A BAC (RP22-256H21) containing the 5’ part of the Gli3 gene (129S6/SvEvTac strain) was used to construct the knock-in template plasmid. The recombinaseering method was used to retrieve a 16.6-kb fragment flanking exon 2 (4.7 kb upstream and 11.6 kb downstream). A fragment encoding a TAP tag including both Protein A and HA tags was inserted immediately after the start codon of the Gli3 gene. A Neo cassette flanked by two LoxP sites was inserted 0.3 kb downstream of the 3’ end of exon 2. The construct was transfected into SM-1 ES cells (129SvEv origin). Clones were digested with SacI and screened using Southern blot using an outside probe (Figure 1C). The upstream recombination was confirmed by PCR using a primer in the TAP tag and an outside primer (Figure 1B). A clone containing the desired sequence was injected into C57/Bl6 blastocysts which were used to generate chimeric mice. These mice were bred to C57/Bl6 mice to generate heterozygotes. To remove the Neo cassette, Gli3TAP-neo heterozygotes were crossed to Nestin-Cre transgenic mice (46), which express Cre in the germ cells. Gli3TAP mice were
bred to homozygotes, which display no obvious defects and are fertile. *Gli3*+/− mice (30) were kindly provided by Dr. J. Reiter (UCSF) and bred as a *Gli3*+/− intercross. All mice are maintained on a mixed genetic background at UT Southwestern Medical Center Animal Facility. All experiments in this study were approved by the IACUC at UT Southwestern Medical Center.

**Generation of Sltm-null allele with the CRISPR/Cas9 method**

RNA encoding Cas9 and guide RNA targeting exon 2 of *Sltm* were injected into pronuclei. The repair plasmid DNA containing the GFP coding sequence and polyA signal was co-injected to generate the *Sltm*-null allele with GFP inserted in frame in exon 2. The region including exon 2 and flanking sequence was sequenced in mice obtained. One progeny was obtained with GFP inserted correctly. The *Sltm*-null allele was genotyped by PCR using GFP1F (GCATGGACGAGCTGTACAAG) and Sltm1R (GTATCCCCATACCTAAACTTC) primers and the wild-type allele was genotyped using Sltm2F (CCCCTTTCTGTGTAGCATACATAATTCT) and Sltm2R (CCCCAGAATCAATAAAGAAGACTTT) primers. *Sltm*+/− mice were crossed to generate *Sltm*−/− mice; the *Sltm*−/− mice die shortly after birth without obvious growth defects.

**Affinity purification of TAP-GLI3 and mass spectrometry analysis**

Telencephalons from E13.5 wild-type or *Gli3*TAP/TAP embryos (30 embryos per sample) were homogenized in Buffer A (25 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, with protease inhibitor freshly added). The nuclei were washed once with Buffer A and then lysed with Purification Buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40). The TAP tag was designed for tandem affinity purification with IgG followed by anti-HA antibodies. Due to the difficulty eluting GLI3 proteins by Tev digestion from several types of beads after the first purification step, we used a one-step affinity purification with IgG Sepharose 6 Fast Flow beads (GE Healthcare). Purification Buffer was used to wash the beads and the same buffer with 1% SDS was used for elution. The eluted GLI3 complexes were run on SDS-PAGE gels for a short distance. The total proteins were isolated and subjected for mass spectrophotometry analyses. Proteins from the gel slice were digested, extracted and analyzed by LC/MS/MS using an Orbitrap Elite mass spectrometer (UTSW Proteomic Core Facility). Peptide identification was performed using the X!Tandem (47) and open MS search algorithm (OMSSA) search engines (48) against the mouse protein database from Uniprot. The precursor mass tolerance was 20 ppm and the fragment mass tolerance was 0.5 Da. The false discovery rate (FDR) was set to 0.01. Forty proteins that were only identified from the TAP-GLI3 samples but not from the control samples were listed in Table S1.

**Generation of expression plasmids**

The SLTM expression plasmid was obtained from Dr. Michael Norman (University of Bristol) (32). The *Sltm* coding region was cloned into the pSin-EF2 lentiviral vector to add the HA-Tag. GLI expression vectors and GLI3 fragments were described previously (15). GLI3N, GLI3ZnF and GLI3C encode 1-425 aa, 426-633 aa and 633-1580 aa of GLI3, respectively.

**Cell culture and lentiviral infection**

Primary *Sltm*−/− and control MEF cells were cultured from E13.5 to E15.5 embryos. Briefly, embryo trunks were dissected, trypsinized, and dissociated to single cells. MEF cells were cultured in DMEM media with 10% fetal bovine serum. Immortalized *Gli3*−/− MEF cells were provided by Dr. Wade Bushman (University of Wisconsin) (49). SHH conditioned medium was prepared as previously described (50). For SHH treatment, MEF cells or NIH3T3 cells were cultured in SHH-containing low-serum media for 24 hours. Lentiviruses were prepared according to a previously described procedure (51). PolyJet (Signagen) was used for plasmid transfection of cultured cells. Attached MEF cells were infected at an MOI of 5 for 24 hours with 8 µg/ml polybrene.

**Cell fractionation**
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Cell fractionation was performed as described in (52). Briefly, NIH3T3 cells expressing exogenous SLTM were pelleted and resuspended in 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT. The supernatant was the cytoplasmic fraction and the pellet was the nuclei. The nuclei were resuspended in 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT. The pelleted chromatin fraction was resuspended in Laemmli buffer and sonicated prior to analysis.

**Immunoblotting**

For immunoblotting, cells or ground tissues were lysed in RIPA buffer (50 mM Tris, pH 8, 250 mM NaCl, 0.05% SDS, 0.5% DOC, 1% NP-40). Histone fractions were prepared with acid extraction (0.2 N HCl). Cell lysates or histone fractions were separated on SDS-PAGE gels. Antibodies used were against GLI1 (Cell Signaling), GLI3 (Roche), SLTM (Bethyl Laboratory), GAPDH (Sigma), GFP (Clontech), HA (HA-7, Sigma), and histone H3 (ab1791, Abcam). HRP-conjugated secondary antibodies were purchased from Jackson Immunology.

**Immunohistology**

Timed mouse pregnancies were determined by plugging date as day 0.5. Immunostaining were performed on paraffin sections. Antibodies used were against OLIG2 (Chemicon) and NKX2.2 (Developmental Studies Hybridoma Bank, University of Iowa). The images were visualized using an Olympus BX50 microscope. OLIG2 and NKX2.2 positive cells were counted from comparable sections of wildtype and Sltm-mutant neural tubes at the mid-trunk region. Averages of 15 sections from three embryos in each group (5 sections/embryo) were calculated and compared.

**Co-immunoprecipitation experiments**

Cells expressing tagged SLTM and tagged GLI proteins were lysed in Buffer A. Nuclear extracts were prepared in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) with rotation at 4 °C for 1 hour. After centrifugation, antibodies against one tag were added to pre-cleared nuclear extracts and incubated at 4 °C overnight. Samples were incubated with protein A beads (GE Healthcare) for 1 hour; beads were washed with RIPA buffer four times. Precipitated proteins were eluted by boiling in 2X Sample Buffer before SDS-PAGE and western blot analysis.

**RT-PCR and q-PCR**

RNA from cells or ground tissues was extracted with TRIZOL (Invitrogen). cDNAs were synthesized by reverse transcription with a blend of oligo(dT) and random primers using Iscript (Bio-Rad), followed by PCR or quantitative PCR analysis. A Bio-Rad real-time PCR system (C1000 Thermal Cycler) was used for quantitative PCR. Levels of GAPDH mRNA were used to normalize input RNA. Graphics shown are representative of experiments performed in triplicate. The experiments were repeated for at least three times. Standard errors were calculated according to a previously described method (15). Primers for ChIP-q-PCR and RT-PCR are:

Gli1ChIP_P5F
CGTAACTGAGCTTTCCCATGT
Gli1ChIP_P5R
CCTTCATGTCCATAGGTCGC
ChIP-Ptch1F
GAAGCCACAGAAAACCCTGTC
Ptch1R GCCGCAAGCCTTCTCTAGG
Gli1F GGTCTCGGGGTCTCAAACTGC
Gli1R CGGCTGACTGTGTAAGCAGAG
Chromatin immunoprecipitation

ChIP experiments were performed as described previously (15). Dounced tissue or dissociated cells were crosslinked with PFA or double crosslinked with DSG (Pierce) and sonicated into fragments of 200-500 bp. Antibodies used were against HA (Abcam), FLAG (Sigma), GLI3 (Roche), H3K27me3 (EMD Millipore), SUZ12 (Cell Signaling), H3K9Me2 (EMD Millipore), and histone H3.
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Precipitated DNA was purified and subjected to real-time PCR.

**Alkaline phosphatase (ALP) activity assay**

ALP activity was determined as described previously (39). The C3H10T1/2 cells were washed with PBS, fixed with 3.7% formaldehyde, and stained with a mixture of 330 µg/ml nitro blue tetrazolium, 165 µg/ml bromochloroindoyl phosphate, 100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris (pH 9.5).

**Statistical analysis**

At least 3 independent experiments were performed with each experiment analyzed in triplicate. Graphics shown are representative experiments. Data are expressed as means ± s.d.. Statistical analysis was performed by either analysis of variance with ANOVA post hoc t-test for multiple comparisons or a two-tailed unpaired Student’s t-test. A p value of <0.05 was considered significant.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

Z. Z., X. Z., B. K., and J.W. designed and performed experiments and analyzed results. J.W. wrote the manuscript with the help from all authors.
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**Footnotes**

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Abbreviations used are: SHH, Sonic Hedgehog; SLTM, SAFB-like transcription modulator; GLIA, GLI activator; GLIR, GLI repressor; TAP, tandem affinity purification; qPCR, quantitative polymerase chain reaction; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; MEF, mouse embryonic fibroblast; H3K27me3, histone H3 lysine 27 trimethylation; CRISPR, clustered regularly interspaced short palindromic repeats; ZnF, zinc finger.
Figure 1. Generation of Gli3\textsuperscript{TAP} knock-in mice. A. Structures of the Gli3 allele, the Gli3\textsuperscript{TAP} knock-in construct, the Gli3\textsuperscript{TAP-neo} allele and the final Gli3\textsuperscript{TAP} allele. B. Genotyping of correctly recombined Gli3\textsuperscript{TAP} allele in ES cells using primers P1 and P2 indicated in panel A. C. The correctly engineered allele was also confirmed by Southern blot using an external probe on the 3’ side. D. TAP-GLI3 expression analyzed in E13.5 Gli3\textsuperscript{TAP/+} telencephalons using western blot with anti-HA or anti-GLI3 antibodies. E. Digit phenotypes of Gli3\textsuperscript{TAP/TAP} homozygous mice, Gli3\textsuperscript{+/-} extra toe mice, and Gli3\textsuperscript{TAP/-} mice.
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Figure 2. Affinity purification of TAP-GLI3 and associated proteins. A. Immunoprecipitation of TAP-GLI3 from E13.5 Glil3TAP/TAP telencephalons with IgG also pulled down BRG1. Wild-type telencephalons were used as a negative control. B. ChIP-qPCR analyses of TAP-GLI3 binding to SHH/GLI target gene regulatory regions in E13.5 Glil3TAP/TAP knock-in telencephalons using IgG (black bars). Wild-type telencephalons were used as a negative control (gray bars). (n=3) C. Procedures of affinity purification of TAP-GLI3 and interacting proteins. D. Western blot analysis of TAP-GLI3 proteins in different fractions during purification shows the enrichment of TAP-GLI3 after affinity purification. E1: elution fraction 1; E2: elution fraction 2. E. Top protein hits identified by mass spectrometry in TAP-GLI3 affinity purification. F. Representative MS/MS fragment ion spectrum with peak assignments for GLI3 and SLTM.
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Figure 3. SLTM interacts with GLI proteins. A. Extracts of NIH3T3 cells expressing HA-SLTM and GLI3 were immunoprecipitated with anti-GLI3 antibodies and western blotted with antibodies against GLI3 and SLTM. HA-SLTM co-immunoprecipitated with GLI3. B. SLTM interacts with GLI3R and the N-terminal (GLI3N) domain, but not the zinc finger (ZnF) region of GLI3. NIH3T3 cells expressing FLAG-SLTM and HA-tagged GLI3 fragments were immunoprecipitated with anti-HA antibody and analyzed by western blot with antibodies against FLAG or HA. * HA tagged GLI3 fragments. C. SLTM interacts with GLI1 and GLI2 as shown by expression of indicated tagged proteins in NIH3T3 cells followed by immunoprecipitation and western blot. * HA tagged GLI proteins. Shown are representative results from at least 3 independent experiments.
Figure 4. SLTM represses SHH/GLI target genes expression. A. Sltm null allele was generated using CRISPR-Cas9. The genomic structures of wild-type and mutant Sltm alleles are shown. A GFP gene was inserted into the Sltm null allele. B. PCR was used to compare genotypes of wild-type (+/+) and Sltm null (-/-) embryos in the region of the Sltm allele. C. As shown by western blot of extracts from wild-type and Sltm null MEFs, SLTM was not expressed in Sltm null MEFs and GFP was. D. Ubiquitous expression of GFP driven by the Sltm promoter in E13.5 Sltm null embryos. E. Basal expression of SHH target genes Gli1 and Pcth1 in Sltm null MEFs (black bars, n=3) and wild-type (gray bars, n=3) as indicated by RT-qPCR. **:
p<0.01. F. Overexpression of SLTM but not the empty vector control in SHH-treated NIH3T3 cells repressed SHH-induced expression of Gli1 and Ptc1 as measured by RT-qPCR. **: p<0.01. G. Overexpression of SLTM in NIH3T3 cells inhibited Gli1 expression induced by exogenous Gli1 as measured by RT-qPCR. **: p<0.01. RT-qPCR graphics in F and G are representative of at least 3 experiments performed in triplicate (n=3). Significance was determined by Student’s t-test.
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Figure 5. SLTM enhances GLI3 binding to chromatin. A. Levels of SLTM and GLI were evaluated by western blot in NIH3T3 cells that overexpressed different amounts of SLTM; cells were stimulated with SHH or left unstimulated. B. NIH3T3 cells overexpressing SLTM or transfected with a control vector were fractionated. SLTM, GLI3FL, and GLIR in cytoplasmic fraction and in chromatin were detected by western blot. C. SLTM binding to the Gli1 regulatory region in control (gray) and SLTM-expressing (black) NIH3T3 cells was measured by ChIP-qPCR. D. Binding of GLI3 to the Gli1 regulatory region in control (gray) and SLTM-expressing (black) NIH3T3 cells was measured by ChIP-qPCR. E. ChIP-qPCR analyses of GLI3 binding to the Gli1 regulatory region in wild-type or Sltm^-/- MEFs. ChIP-qPCR graphics in C to E are representative of at least 3 experiments performed in triplicate (n=3). Significance was determined by Student’s t-test. **: p<0.01.
Figure 6. SLTM facilitates the formation of a repressive chromatin environment at the Gli1 locus. ChIP-qPCR analyses were performed on extracts of control (gray) and SLTM-expressing (black) NIH3T3 cells using antibodies to (A) H3K27me3, (B) SUZ12, (C) H3K9me2, and (D) histone H3 in the Gli1 locus. Histone H3 occupancy was used as a control. ChIP-qPCR graphics are representative of at least 3 experiments performed in triplicate (n=3). Significance was determined by Student’s t-test. **: p<0.01.
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Figure 7. SLTM activates SHH/GLI target genes in the absence of GLI3R.  

A. SLTM activates expression of SHH/GLI target genes in Gli3-/- cells. RT-qPCR was used to analyze expression of Gli1 and Ptc1 in Gli3-/- MEFs transfected with a control or SLTM expressing vector in the absence or presence of SHH. B. Gli3-/- cells were transfected with empty vector (black) or with a lentiviral construct for expression of GLI3R (gray) and the effects of SHH induction in the presence and absence of SLTM were evaluated. The presence of GLI3 diminished the activator functions of SLTM upon SHH treatment of cells. Lower panel shows the exogenous GLI3R expression in Gli3-/- cells in each condition evaluated with a western blot. C. ChIP-qPCR analysis of GLI2 binding to the Gli1 regulatory region in wild-type or Gli3-/- cells expressing control (gray) or SLTM (black) indicates that GLI2 binding is enhanced by SLTM in the absence of GLI3. D. Western blot was used to evaluate levels of GLI3FL and GLI3R in wild-type and Sltn-/- MEFs in the presence and absence of SHH stimulation. E. GLI1 expression was evaluated in Sltn-/- MEFs and wild-type cells in the presence of SHH with RT-qPCR (graph) and western blot. qPCR graphics in all panels are representative of at least 3 experiments performed in triplicate (n=3). Significance was determined by Student’s t-test or ANOVA post hoc t-test. **: p<0.01.
**Figure 8. Abnormal expression of ventral neural tube markers in Sltm<sup>−/−</sup> embryos.** Cross sections of the mid-trunk neural tube regions of E10.5 wild-type and Sltm<sup>−/−</sup> embryos were stained with antibodies against ventral neural progenitor markers NKX2.2 (red) and OLIG2 (green). Representative overlay and single channel pictures are shown. Reduction of NKX2.2 expressing cell numbers and the consequent expansion of OLIG2 expressing regions were quantified (The numbers are averages of positive cells in 15 sections from 3 pairs of wild-type and mutant embryos). Significance was determined by Student’s t-test. ****: p<0.01.
Figure 9. SLTM enhances SHH-induced C3H10T1/2 cell differentiation into osteoblasts.  

**A.** Representative images of C3H10T1/2 cells treated with or without SHH for 7 days and with or without overexpression of SLTM. Cells were stained to reveal Alkaline phosphatase activity indicative of differentiation.  

**B.** RT-qPCR was used to analyze SHH target gene expression in control and SLTM-expressing C3H10T1/3 cells treated with or without SHH for 7 days. RT-qPCR graphics are representative of at least 3 experiments performed in triplicate (n=3). Significance was determined by ANOVA post hoc t-test. **: p<0.01.
A proteomic approach identifies SAFB-like transcription modulator (SLTM) as a bidirectional regulator of GLI family zinc finger transcription factors
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