Specific Requirement of Gli Transcription Factors in Hedgehog-mediated Intestinal Development*□S

He Huang[‡§1], **Jennifer L. Cotton**[‡1], **Yang Wang**[¶], **Mihir Rajurkar**[‡], **Lihua J. Zhu**[∥], **Brian C. Lewis**[∥], **and Junhao Mao**[‡2]

From the ‡Department of Cancer Biology, ∥Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, Massachusetts 01605, the §Department of Histology and Embryology, Xiangya School of Medicine, Central South University, Changsha, China 410013, and the ¶University of Massachusetts MassBiologics, Boston, Massachusetts 02126

Background: Hedgehog signaling plays important roles during intestinal development.
Results: Mesenchymal Gli2 activation, but not Gli3 removal, rescued Hedgehog pathway defects. Screen identified small leucine-rich glycoproteins (SLRPs) downstream of Hedgehog in the gut.
Conclusion: Hedgehog activity is mediated by Gli2 and involves SLRP regulation in the developing intestine.
Significance: This study uncovers novel genetic and molecular mechanisms underlying Hedgehog function in intestinal development.

Hedgehog (Hh) signaling is involved in multiple aspects of embryonic gut development, including mesenchymal growth and smooth muscle differentiation. The Gli family transcription factors is thought to collectively mediate Hh signaling in mammals. However, the function of different Gli proteins in gut development remains uncharacterized. Here, we genetically dissect the contribution of Gli transcriptional activation and de-repression in intestinal growth and patterning. We find that removal of the Gli3 repressor is dispensable for intestinal development and does not play a major role in Hh-controlled gut development. However, Gli2 activation is able to fully rescue the Smoothened (Smo)-null intestinal phenotype, suggesting that the Gli2 transcription factor is the main effector for Hh signaling in the intestine. To understand further the molecular mechanism underlying Hh/Gli function in the developing gut, we identify a subset of small leucine-rich glycoproteins (SLRPs) that may function downstream of Hh signaling in the mesenchyme. We show that osteoglycin, a SLRP, inhibits Hh-induced differentiation toward the smooth muscle lineage in C3H10T1/2 pluripotent mesenchymal cells. Taken together, our study reveals, for the first time, the distinct roles of Gli proteins in intestine development and suggests SLRPs as novel regulators of smooth muscle cell differentiation.

Hedgehog (Hh)[3] signaling has emerged as one of the critical pathways regulating the development, homeostasis, and tumorigenesis of the gastrointestinal tract (1–8). Hh ligands, including Sonic hedgehog (Shh) and Indian hedgehog (Ihh), are produced by the endodermal gut epithelium and are believed to signal to the adjacent mesenchyme to regulate tissue growth and patterning (4, 5, 9). In a recent study, we genetically removed both Shh and Ihh ligands from the early embryonic gut endoderm and found that Hh signaling is essential for gut mesenchymal development (5). Specifically, we found that Hh proteins are the primary factor responsible for maintenance and expansion of early mesenchymal progenitor populations (5). In addition, our results suggested that Hh signaling is important for later differentiation, including specification of the smooth muscle lineage from primitive mesenchyme, a prominent event during intestinal mesenchymal development (5). Despite its importance, the genetic and cellular mechanisms underlying Hh control of intestinal development remain poorly understood.

In vertebrates, Hh signaling is mediated by the Gli family of transcription factors, Gli1, Gli2, and Gli3. Hh ligands bind to its transmembrane receptor Patched1 (Ptch1), relieving inhibition of another transmembrane protein, Smoothened (Smo), which in turn activates downstream intracellular signaling events to regulate Gli activity (10–12). Of the Gli proteins, Gli2 and Gli3 are the primary signal responders, with Gli2 mainly acting as a transcriptional activator and Gli3 predominantly acting as a repressor (13, 14). Gli1, a direct transcriptional target of Hh signaling, is dispensable for mouse embryonic development (15). Therefore, the balance between the activities of Gli2/3 transcriptional activator and repressor dictates Hh responses that are both tissue-specific and developmental stage-specific. During limb and ureter development, Shh acts mainly by opposing Gli3 repression (16, 17). During neural tube and skeletal development, integrated regulation of Gli2 activation and Gli3 de-repression has both overlapping and distinct functions (18–21). Surprisingly, the Gli regulatory mechanism and the relative contribution of Gli proteins to Hh function in the intestine have yet to be examined.

In this study we demonstrate the direct genetic requirement of Hh responsiveness in the gut mesenchyme, and found that removal of Gli3 repression had no effect on Hh-mediated mes-

enchymal growth and differentiation. However, activation of Gli2 fully rescued Smo-null defects in the intestine, suggesting that Gli2, but not Gli3, is the major effector for Hh signaling during intestine development. Furthermore, our study suggests that a subset of SLRPs may act downstream of Hh signaling in the mesenchyme to regulate smooth muscle differentiation in the developing intestine.

EXPERIMENTAL PROCEDURES

Mouse Strains—$Nkx3.2^{Cre}$, Smo^{C}, $Gli3^{C}$, $R26^{SmoM2}$, and $R26^{mT/mG}$ mice have been described before (22–26). To generate the $R26^{Gli2}$ allele, the cDNA fragment encoding Gli2 fused with a C-terminal FLAG tag was inserted into the shuttling vector pBigT before being cloned into pROSA-PAS to produce the final targeting construct. ES cell targeting and blastocyst injection were performed by University of Massachusetts Medical School Transgenic Core to generate chimeric animals. $Nkx3.2^{Cre};R26^{Gli2}$, $Nkx3.2^{Cre};R26^{SmoM2}$, and $Nkx3.2^{Cre};R26^{mT/mG}$ mice were generated by crossing $Nkx3.2^{Cre}$ to $R26^{Gli2};R26^{SmoM2}$ or $R26^{mT/mG}$ mice. $Nkx3.2^{Cre};Smo^{C/C}$ and $Nkx3.2^{Cre};Gli3^{C/C}$ mice were obtained by crossing $Nkx3.2^{Cre};Smo^{C/+}$ and $Nkx3.2^{Cre};Gli3^{C/+}$ to $Smo^{C/C}$ or $Gli3^{C/C}$ mice. To generate $Nkx3.2^{Cre};Smo^{C/C};Gli3^{C/C}$ and $Nkx3.2^{Cre};Smo^{C/C};R26^{Gli2}$ embryos, $Nkx3.2^{Cre};Smo^{C/+};Gli3^{C/+}$ or $Nkx3.2^{Cre};mo^{C/+}$ mice were crossed to $Smo^{C/C};Gli3^{C/C}$ and $Smo^{C/C};R26^{Gli2}$ mice. All mouse experiments were performed according to the guidelines of IACUC at University of Massachusetts Medical School.

Tissue Collection and Histology—Upon euthanasia, embryonic intestine was dissected and fixed in 4% paraformaldehyde for 6 h. For paraffin sections, tissues were dehydrated, embedded in paraffin blocks, and cut at a thickness of 6 μm. For frozen sections, tissues were dehydrated in 30% sucrose and embedded in OCT, and sections were cut at a thickness of 12 μm. For RNA analysis, tissues were flash frozen in liquid nitrogen. Tissue sections (6 μm) were stained with hematoxylin and eosin (H&E) using standard reagents and protocols.

Immunohistochemistry, Immunofluorescence, and Immunoblotting—For immunohistochemistry, high temperature antigen retrieval was conducted on paraffin sections in sodium citrate buffer (pH 6.0) for 30 min. Sections were blocked in a buffer containing 5% BSA and 0.1% Triton X-100 in PBS and incubated overnight at 4 °C in primary antibodies diluted in blocking buffer. Primary antibodies used were: Ki67 (Abcam), cleaved caspase 3 (Cell Signaling), α-smooth muscle actin (SMA; Sigma), β-tubulin (Covance), β-catenin (Santa Cruz Biotechnology), and CD44 (eBioscience). Signal was detected with biotinylated secondary antibodies in the Vectastain ABC kit (Vector Laboratories).

For immunofluorescence, tissue frozen sections or C3H10T1/2 cells were washed with PBS, fixed with 4% paraformaldehyde for 5 min, incubated in blocking buffer containing 5% sheep serum, 1% FBS, and 0.1% Triton X-100 for 1 h, and incubated overnight at 4 °C with SMA antibody (1:800) diluted in blocking buffer. Secondary antibodies were Alexa Fluor- conjugated antibodies (Invitrogen) at a 1:500 dilution in blocking buffer. Slides were mounted in mounting media containing DAPI. For immunoblotting, primary antibodies were: V5 (ThermoFisher),

FLAG (Sigma), and β-actin (Sigma). HRP-conjugated secondary antibodies were obtained from Jackson Laboratories.

C3H10T1/2 Cell Culture, Lentiviral Infection, and Cell Differentiation Assay—C3H10T1/2 cells were obtained from ATCC and maintained in DMEM with 10% FBS at 37 °C in 5% CO_2. Cells were cultured at 30–50% confluence to prevent differentiation. For lentiviral infection, a C-terminally V5-tagged osteoglycin (OGN) cDNA fragment was inserted into the pGIPZ based lentiviral expression vector. The OGN lentiviral construct was transfected along with the packing plasmids into growing HEK293T cells, and viral supernatants were collected 48 h later. C3H10T1/2 cells were infected with OGN lentivirus in the presence of Polybrene and selected with puromycin for 4 days to establish the OGN-expressing cell line. For smooth muscle cell differentiation, cells were cultured in growth media for 24 h before switching to differentiation medium (DMEM with 0.5% FBS) for 12 h, followed by addition of Shh conditioned media (1:100) in culture for 36 h. Smooth muscle differentiation was assayed by immunostaining or qPCR analysis of SMA expression.

Luciferase Reporter Analysis—C3H10T1/2 cells with or without OGN overexpression were transfected with the luciferase reporter construct, GliBS-Luc (gift of Dr. H. Kondoh, Osaka University) and the *Renilla* luciferase expression vector. The cells were either treated with Shh conditioned medium or co-transfected with a Gli2 expression construct. Luciferase assays were conducted 72 h after transfection using the dual-luciferase reporter kit (Promega). Assays were conducted in triplicate.

Quantitative RT-PCR—cDNA synthesis was conducted using an Invitrogen Superscript II kit. Primers used for quantitative RT-PCR were: mouse β-actin (forward, TGACAGGATGCA-GAAGGAGA; reverse, CTGGAAGGTGGACAGTGAGG), Ptch1 (forward, AACAAAAATTCAACCAAACCTC; reverse, TGTCTTCATTCCAGTTGATGTG), HIP1 (forward, CTAT-TGGGCCTCACGACCAC; reverse, TTCCAGAAACACCCT-GGCTG), Myocd (forward, AAGGTCCATTCCAACTGCTC; reverse, CCATCTCTACTGCTGTCATCC), SMA (forward, GAACACGGCATCATCACCAAC; reverse, CTCCAGAGTC-CAGCACAATACC), Axin2 (forward, TGACTCTCCTTCCA-GATCCCA; reverse, TGCCCACACTAGGCTGACA), and Lgr5 (forward, CGGGACCTTGAAGATTTCCT; reverse, GAT-TCGGATCAGCCAGCTAC). All qPCR assays were conducted in triplicates, and standard deviation was used to calculate error bars.

Affymetrix Gene Chip Analysis—Intestinal tissues were dissected from E13.5 wild type and $Nkx3.2^{Cre};R26^{SmoM2}$ embryos. RNA was isolated, labeled, and hybridized to mouse GeneST1.0 chips (Affymetrix) according to Affymetrix protocols. Three independent biological samples were used for chip analysis. Statistical analyses were performed using R, a system for statistical computation and graphics. Genes with p value <0.05 and -fold change >1.5 were considered potential targets for further investigation.

RESULTS

Genetic Removal of Smo from the Developing Intestinal Mesenchyme—Our previous analysis of mouse embryos lacking both Shh and Ihh from early gut endoderm provided strong evidence that endoderm-derived Hh proteins control adjacent mesenchymal development (5). The majority of evidence from
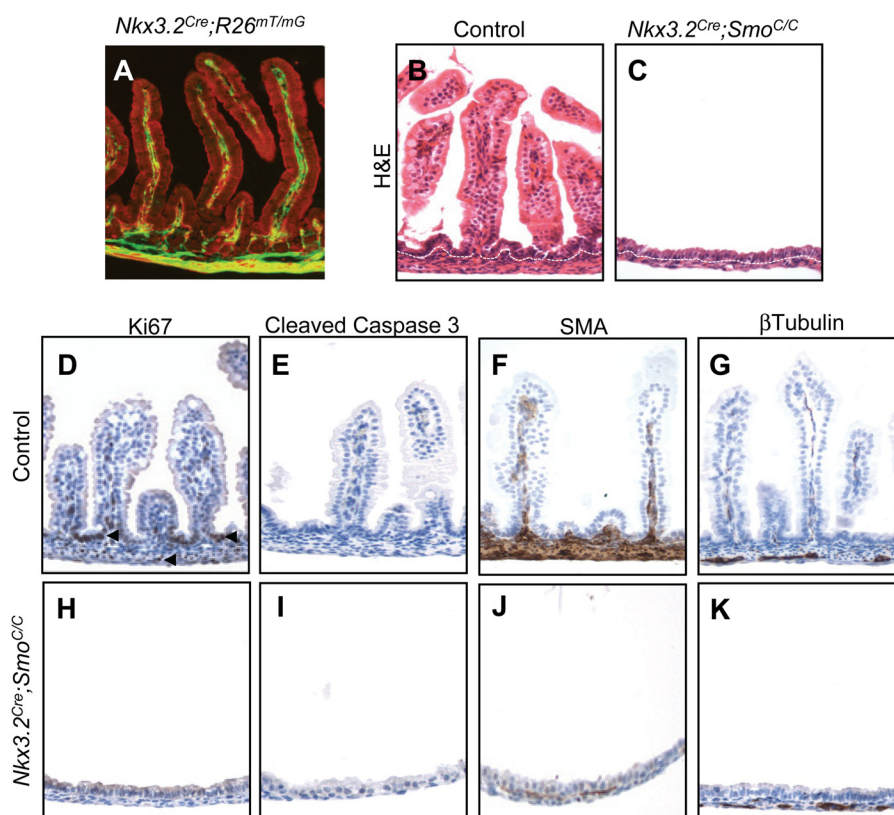
FIGURE 1. **Mesenchymal removal of Smo in the developing intestine.** *A, Nkx3.2^{Cre}*-mediated mesenchymal recombination in the intestine. Fluorescence image of the *Nkx3.2^{Cre};R26^{mT/mG}* intestine shows widespread Cre-mediated recombination and GFP expression exclusively in the intestinal mesenchymal compartment. *B* and *C*, H&E staining of the intestine of control and *Nkx3.2^{Cre};Smo^{C/C}* embryos at E18.5. *White dashed lines* delineate the boundary of gut epithelium and mesenchyme. *D–K*, defects in mesenchymal growth and patterning in *Nkx3.2^{Cre};Smo^{C/C}* embryos at E18.5. Shown is immunohistochemical staining of Ki67 (*D* and *H*), cleaved caspase 3 (*E* and *I*), SMA (*F* and *J*), and *β*-tubulin (*G* and *K*) in the intestine of control and *Nkx3.2^{Cre};Smo^{C/C}* mutant embryos.

the literature also supports a paracrine mode of Hh signaling from the epithelium to the mesenchyme (4, 6, 7, 9); however, direct genetic evidence for such a requirement within the gut mesenchyme is still lacking. Furthermore, the extremely small size of the intestine in the endodermal Shh/Ihh mutants restricted our previous analysis mainly to fetal stomach mesenchyme (5). Therefore, we set out to examine further the role of Hh signaling in intestinal development and determine whether cell-autonomous Hh reception is genetically required in the mesenchyme. We performed targeted deletion of Smo from the intestinal mesenchymal compartment. Smo, a cell surface transmembrane protein, is essential for all Hh signal transduction in recipient cells (27). We crossed a conditional *Smo* allele, *Smo^C* (23), to a recently established *Nkx3.2^{Cre}* line (22), which initiates Cre expression in the gut mesenchyme at E9.5. When *Nkx3.2^{Cre}* was crossed to a dual-fluorescent *Rosa26* reporter allele, *R26^{mT/mG}* (26), widespread Cre-mediated recombination was observed exclusively in the intestinal mesenchyme of *Nkx3.2^{Cre};R26^{mT/mG}* mice (Fig. 1A). *Nkx3.2^{Cre};Smo^{C/C}* mutant embryos survived gestation but died immediately after birth, therefore allowing us to analyze the late stage development of embryonic intestine. Intestinal mesenchymal proliferation and expansion in the *Nkx3.2^{Cre};Smo^{C/C}* embryos were dramatically affected (Fig. 1, *B* and *C*), producing a phenotype similar to that seen in the Shh/Ihh double mutant (5). At E18.5, significantly fewer mesenchymal cells remained between the thin layers of mesentery and intestinal epithelium (Fig. 1, *B* and *C*) in the Smo

mutant embryos. In agreement with our previous report for a primary mitogenic role of Hh signaling on mesenchymal populations (5), mesenchymal proliferation was absent, although apoptosis was not increased in the Smo-mutated intestinal mesenchyme, as assayed by Ki67 and cleaved caspase 3 staining, respectively (Fig. 1, *D*, *E*, *H*, and *I*).

Smo deletion also dramatically affected mesenchymal differentiation, including smooth muscle differentiation. Smooth muscle cells, detected by the expression of SMA, are specified from local intestinal mesenchyme (28). Earlier studies suggested that Hh signaling may have an inhibitory effect on this process (29, 30). However, our analysis of fetal intestine harboring deletion of endodermal Shh/Ihh suggests that Hh signaling is required for smooth muscle specification in the developing gut (5). Consistent with this, we found that in E18.5 *Nkx3.2^{Cre};Smo^{C/C}* embryos, the number of SMA-expressing or Desmin-expressing intestinal smooth muscle cells was drastically decreased (Fig. 1, *F* and *J*, and data not shown). Further, the *β*-tubulin-expressing enteric neuron population was also decreased in the Smo mutant embryos (Fig. 1, *G* and *K*). Together, these findings demonstrate the genetic requirement of Hh responsiveness in the mesenchyme to maintain growth and promote differentiation in the fetal intestine.

Hh signaling mediates the reciprocal interaction between intestinal epithelium and mesenchyme (2, 6, 7, 31), although its exact role in regulation of epithelial differentiation is not fully understood. In the *Villin-HIP1* and *Villin^{Cre};Ihh^{C/C}* mutant
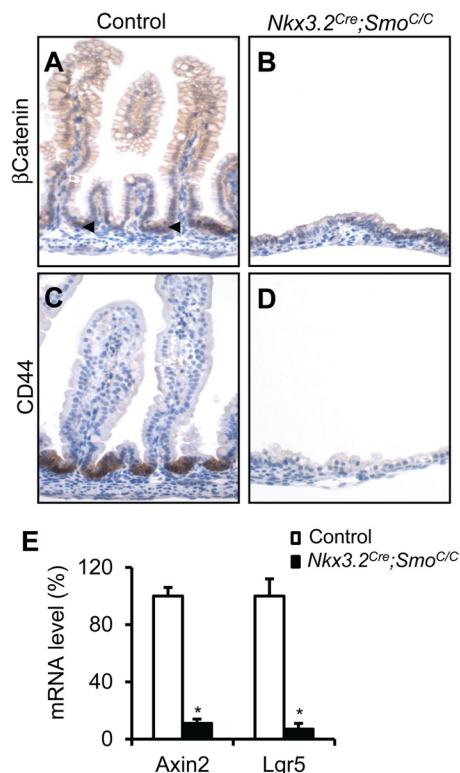
FIGURE 2. **Epithelial Wnt activity in embryonic intestine with mesenchymal Smo deletion.** *A–D*, immunohistochemical staining of β-catenin and CD44 in the intestine of control and $Nkx3.2^{Cre};Smo^{C/C}$ embryos at E18.5. *E*, qPCR analysis of mRNA levels of Axin2 and Lgr5 in control and $Nkx3.2^{Cre};Smo^{C/C}$ intestine. *, $p < 0.01$; *error bars* indicate mean ± S.D.

mice, partial inhibition of Hh signaling leads to Wnt pathway activation and intestinal epithelial overproliferation in newborn animals (2, 31). Therefore, we further analyzed the intestinal epithelial development in $Nkx3.2^{Cre};Smo^{C/C}$ mutant embryos. We found that both epithelial proliferation and differentiation were severely affected by mesenchymal Smo deletion. The intestinal epithelium of mutant embryos at E18.5 was not proliferative (Fig. 1, *D* and *H*), as assayed by Ki67 staining, and lacked differentiation of the Goblet and enteroendocrine cell lineages (data not shown). Smo mesenchymal deletion also blocked epithelial activation of Wnt/β-catenin signaling, measured by β-catenin nuclear staining and the expression of the Wnt target gene *CD44* (Fig. 2, *A–D*), as well as qPCR analysis of the Wnt target genes, *Axin2* and *Lgr5* (Fig. 2*E*). Although the perinatal death of mutant embryos prevented postnatal analysis, these results highlight the importance of Hh signaling in mediating the interactions between the epithelial and mesenchymal compartments, and underline the fact that these two tissue layers are highly interdependent during intestinal development.

Gli3 Is Dispensable for Intestinal Mesenchymal Development—Removal of Gli3 transcriptional repression plays a key role in mediating Hh signaling in different developmental contexts (14, 16, 17, 19). Although a modest glandular hyperplasia has been reported in the Gli3 mutated embryonic stomach (32), the role of Gli3 in intestinal development and contribution to Hh function remain unknown. We generated mice in which Gli3 was deleted in the intestinal mesenchyme using the $Nkx3.2^{Cre}$ and $Gli3^{C}$ allele (24). $Nkx3.2^{Cre};Gli3^{C/C}$ mice were born at the

expected Mendelian frequency and did not display a gross phenotype compared with the littermate controls (data not shown). At the embryonic stage, $Nkx3.2^{Cre};Gli3^{C/C}$ mutant embryos exhibited normal fetal intestinal epithelial and mesenchymal development (Fig. 3, *B* and *G*, and 4*B*). This suggests that Gli3 is not required for intestinal development.

Despite the lack of phenotype after Gli3 deletion in mouse ureter mesenchyme, Gli3 removal completely rescues Smo mutant phenotype (17). Therefore, we further investigated whether Gli3 de-repression contributes to intestinal development in the context of Hh pathway inactivation. We generated embryos with both Smo and Gli3 deficiency in the intestinal mesenchyme. Surprisingly, we found that elimination of Gli3 in the $Nkx3.2^{Cre};Smo^{C/C}$ background failed to restore the Smo-null defects in the intestine (Fig. 4*D*), suggesting that removal of Gli3 repression does not play a significant role in mediating Hh signaling in the developing intestinal mesenchyme.

Gli2 Activation Rescues Intestinal Development in the Smo-null Embryos—Previous studies report that both Gli2 and Gli3 are expressed in the developing gut mesenchyme (4). Given that Gli3 removal failed to restore intestinal development in the Smo-null embryos, we hypothesized that Gli2-mediated transcriptional activation is the predominant mediator of Hh signaling in the gut. To test this idea, we generated a *Rosa26* knock-in allele of Gli2, $R26^{Gli2}$, which allowed Cre-dependent expression of a FLAG-tagged form of Gli2 from the ubiquitously expressed *Rosa26* locus (Fig. 3, *E* and *F*).

We generated $Nkx3.2^{Cre};R26^{Gli2}$ embryos by crossing $Nkx3.2^{Cre}$ to the $R26^{Gli2}$ mice. Interestingly, we found that similar to Gli3 removal, Gli2 activation alone by $R26^{Gli2}$ did not significantly affect embryonic intestinal development (Figs. 3 and 4). At E18.5, intestinal mesenchymal and epithelial development appeared phenotypically normal in the $Nkx3.2^{Cre};R26^{Gli2}$ mutant embryos (Fig. 4, *A* and *C*). This is drastically different from the phenotype we observed in the $Nkx3.2^{Cre};R26^{SmoM2}$ mutant embryos (Ref. 5 and Fig. 3*D*). $R26^{SmoM2}$ is a *Rosa26* conditional knock-in allele of SmoM2, a constitutively active form of Smo (25). $Nkx3.2^{Cre};R26^{SmoM2}$ mutant embryos died at approximately E14.5 (5). SmoM2 activation dramatically expanded the intestinal mesenchymal compartment and induced the expression of the smooth muscle lineage markers, myocardin (Myocd) and SMA at E13.5, as assayed by immunostaining and qPCR (Fig. 3, *D* and *H*). We further examined Hh pathway activity in Gli3-deleted, Gli2- or SmoM2-expressing embryonic intestines and found that expression of the Hh target genes, such as *HIP1* and *Ptch1*, was markedly increased in the $Nkx3.2^{Cre};R26^{SmoM2}$ mutant intestine at E13.5, but was largely unaffected in the $Nkx3.2^{Cre};R26^{Gli2}$ or $Nkx3.2^{Cre};Gli3^{C/C}$ embryos (Fig. 3*H*), which likely accounts for the lack of Hh gain-of-function phenotype in Gli3 or $R26^{Gli2}$ mutant embryos. However, it is interesting to note that in transiently transfected NIH3T3 cells, Gli2 induced stronger downstream transcriptional activation than SmoM2 (data not shown), suggesting that additional posttranslational regulatory mechanisms may modulate Gli2 activity or stability *in vivo*.

To examine whether Gli2 activation contributes to Hh function in the intestine, we generated $Nkx3.2^{Cre};Smo^{C/C};R26^{Gli2}$ embryos and found that Gli2 expression from the *Rosa26* locus
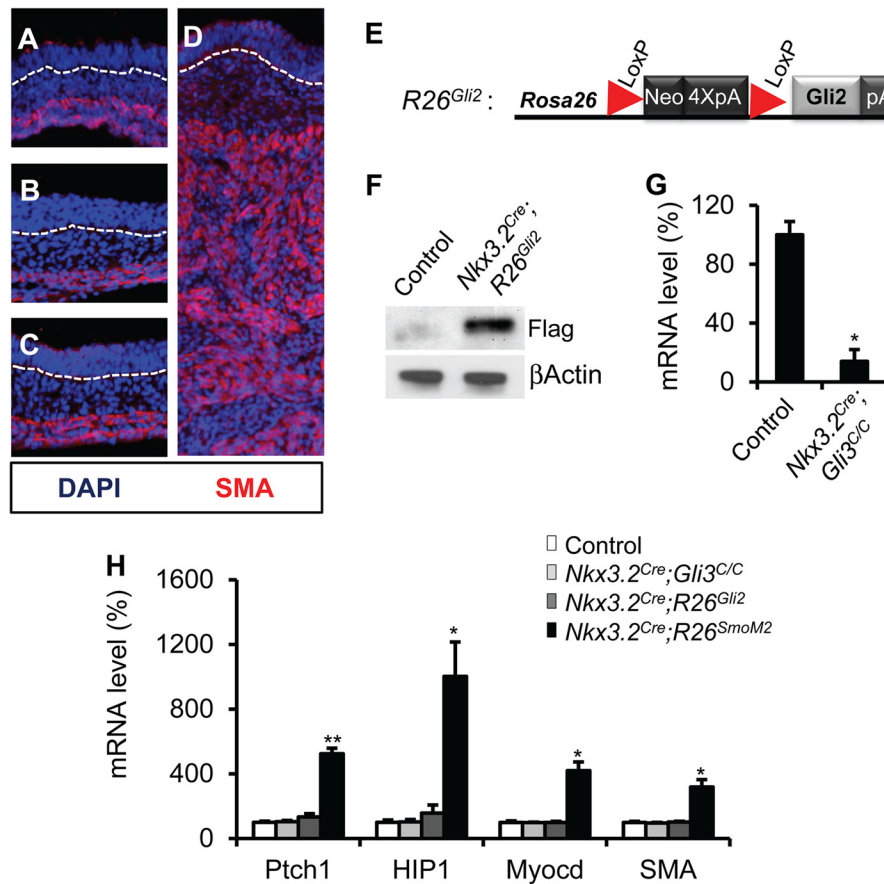
FIGURE 3. **Removal of Gli3 and ectopic expression of Gli2 in the intestinal mesenchyme.** *A–D,* immunofluorescence staining of SMA in the developing intestine of control (*A*), *Nkx3.2^{Cre};Gli3$^{C/C}$* (*B*), *Nkx3.2^{Cre};R26^{Gli2}* (*C*), and *Nkx3.2^{Cre};R26^{SmoM2}* (*D*) embryos at E13.5. *White dashed lines* delineate the boundary of epithelium and mesenchyme. Note the normal thickness and SMA expression pattern in the *Nkx3.2^{Cre};Gli3$^{C/C}$* and *Nkx3.2^{Cre};R26^{Gli2}* embryos compared with control embryos. *E,* schematic representation of the *Rosa26* conditional *R26^{Gli2}* allele. *F,* immunoblot analysis of Gli2-FLAG protein expression in control and *Nkx3.2^{Cre};R26^{Gli2}* embryos. *G,* qPCR analysis of Gli3 mRNA levels in control and *Nkx3.2^{Cre};Gli3$^{C/C}$* gut. *H,* relative mRNA levels of Ptch1, HIP1, Myocd and SMA in control, *Nkx3.2^{Cre};Gli3$^{C/C}$*, *Nkx3.2^{Cre};R26^{Gli2}*, and *Nkx3.2^{Cre};R26^{SmoM2}* intestine at E13.5. *, $p < 0.05$; **, $p < 0.01$; *error bars* indicate mean ± S.D.
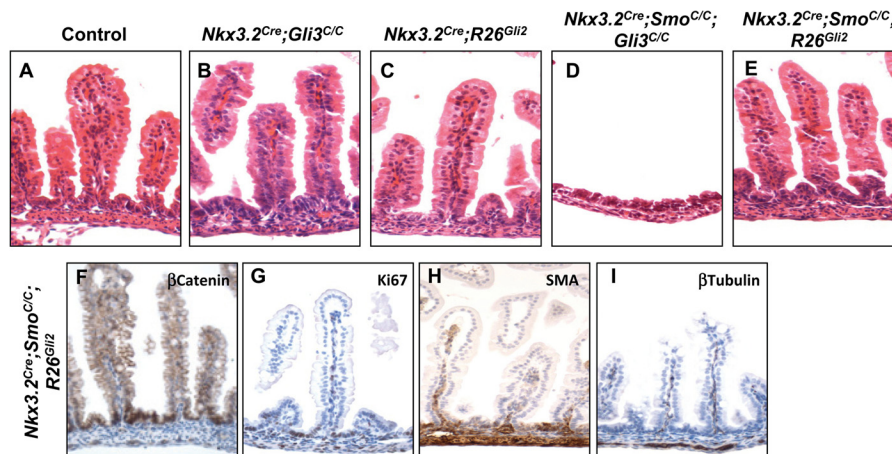

FIGURE 4. **Gli2 activation, but not Gli3 de-repression, mediates Hh function in the developing gut.** *A–E,* histology of the control, *Nkx3.2^{Cre};Gli3$^{C/C}$*, *Nkx3.2^{Cre};R26^{Gli2}*, *Nkx3.2^{Cre};Smo$^{C/C}$;Gli3$^{C/C}$*, and *Nkx3.2^{Cre};Smo$^{C/C}$;R26^{Gli2}* intestine at E18.5. *F–I,* epithelial and mesenchymal development in the *Nkx3.2^{Cre};Smo$^{C/C}$;R26^{Gli2}* intestine at E18.5. Immunohistochemical staining of β-catenin (*F*), Ki67 (*G*), SMA (*H*), and β-tubulin (*I*) in the *Nkx3.2^{Cre};R26^{Gli2}* embryos is shown.

completely rescued the Smo-null intestinal phenotypes. Histological analyses of E18.5 embryos revealed that intestinal development in the *Nkx3.2^{Cre};Smo$^{C/C}$;R26^{Gli2}* embryos is normal (Fig. 4*E*). We observed the rescue of mesenchymal growth, smooth muscle development, and intestinal epithelial differentiation (Fig. 4, *F–I*). Together, these results strongly suggest that

Gli2, but not Gli3, is the major effector for Hh signaling during embryonic intestinal development.

Hh-Gli Regulates Expression of the Small Leucine-rich Glycoproteins—We performed expression profiling using Affymatrix chips in the wild-type and Hh gain-of-function (*Nkx3.2^{Cre};R26^{SmoM2}*) E13.5 mutant gut to investigate the Hh-
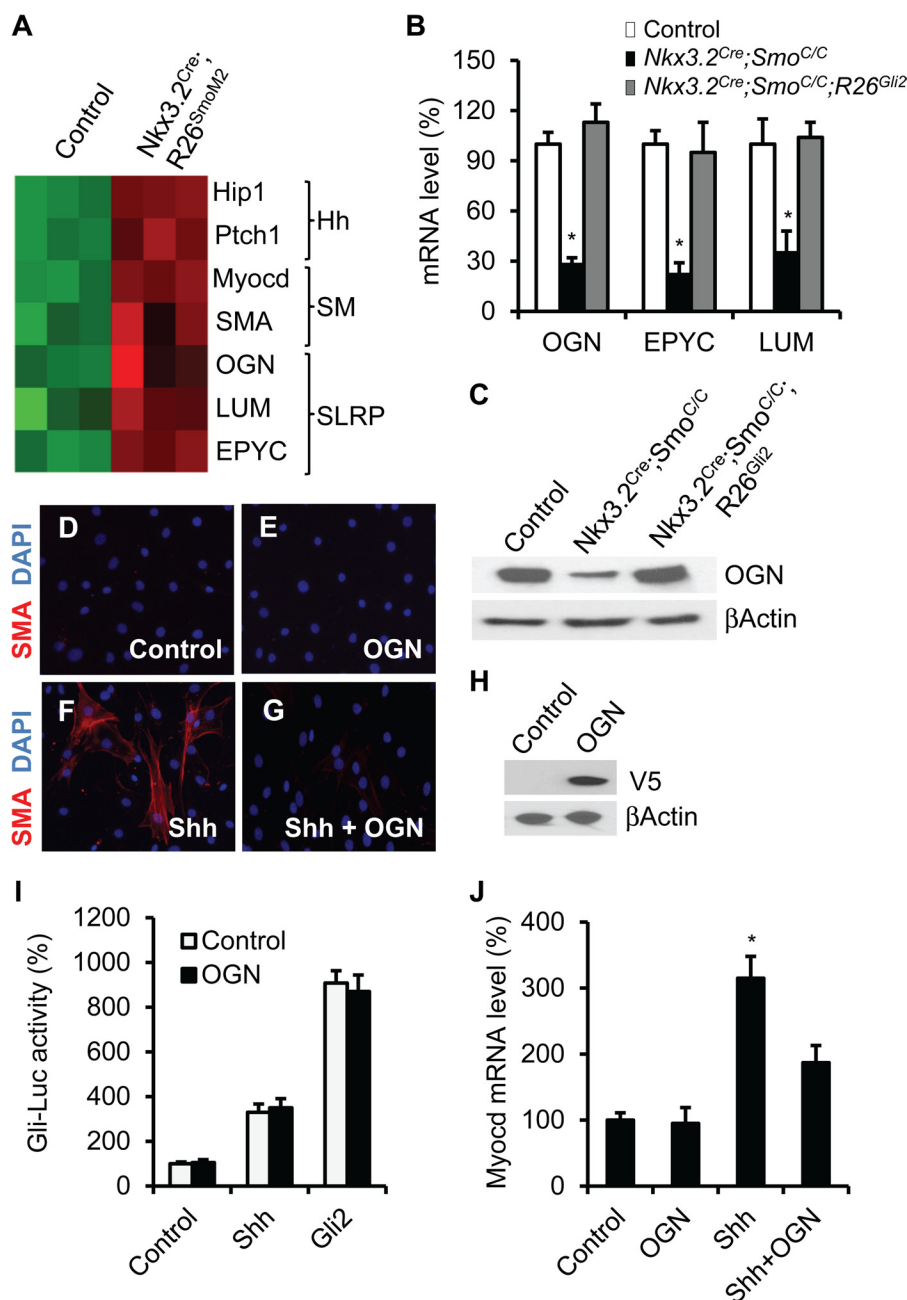
FIGURE 5. **SLRPs regulate smooth muscle cell differentiation.** *A*, heat map illustrating mRNA expression of selected genes in control and SmoM2-expressing developing gut. *SM*, smooth muscle differentiation. *B*, qPCR analysis of mRNA expression of OGN, EPYC, and LUM in control, $Nkx3.2^{Cre};Smo^{C/C}$, and $Nkx3.2^{Cre};$ $Smo^{C/C};R26^{Gli2}$ intestine at E18.5. *C*, immunoblot analysis of OGN expression in control, $Nkx3.2^{Cre};Smo^{C/C}$, and $Nkx3.2^{Cre};Smo^{C/C};R26^{Gli2}$ intestine. *D–G*, immunofluorescence staining of SMA in the control and OGN-overexpressing C3H10T1/2 cells with or without Shh treatment. *H*, immunoblot analysis of expression of V5-tagged OGN proteins in C3H10T1/2 cells. *I*, Gli-luciferase reporter activity in the control and OGN-expressing C3H10T1/2 cells with Shh treatment or Gli2 overexpression. *J*, qPCR analysis of Myocd mRNA level in the control and OGN-overexpressing C3H10T1/2 cells with or without Shh treatment. *, $p < 0.05$, *error bars* indicate mean ± S.D.

Gli downstream mechanism in developing gut. Our analysis identified 150 significantly up-regulated genes in $Nkx3.2^{Cre};$ $R26^{SmoM2}$ embryos (supplemental Table S1), including *Hip1*, *Ptch1*, *Ptch2*, and *Gli1*, which are known Hh target genes involved in the feedback regulation of pathway signal transduction (Fig. 5*A* and supplemental Table S1). Consistent with the idea that the Hh pathway positively regulates smooth muscle cell differentiation, we found that the genes associated with smooth muscle lineage specification, such as *Myocd* and *SMA*, were up-regulated as well (Fig. 5*A* and supplemental Table S1).

Interestingly, we found that several SLRP glycoproteins, including epiphycan (EPYC), osteoglycin (OGN), and lumican (LUM), were also among the top genes up-regulated in the mutant gut (Fig. 5*A* and supplemental Table S1). EPYC, OGN, and LUM belong to the SLRP family of proteins, which are characterized by the presence of the N-terminal cysteine-rich clusters (33). SLRPs have been reported to regulate cell growth and differentiation (33), although it is not known whether they are involved in intestinal development. To further characterize the potential Hh regulation of SLRP, we performed qPCR and

immunoblot analyses of the expression of OGN, EPYC, and LUM in the intestine of wild type, Smo-deficient (*Nkx3.2^Cre; Smo^C/C*) and Gli2-rescue (*Nkx3.2^Cre;Smo^C/C;R26^Gli2*) embryos. Consistent with our transcriptional profiling data, we found that mRNA levels of OGN, EPYC, and LUM, and protein expression of OGN, were down-regulated in the Smo-null embryos, but were restored by Gli2 expression (Fig. 5, *B* and *C*). Together, these data suggest that these SLRPs may act downstream of Hh signaling in the developing intestinal mesenchyme.

OGN Regulates Smooth Muscle Cell Differentiation of C3H10T1/2 Cells in Vitro—To examine the involvement of SLRPs in smooth muscle differentiation, we utilized the *in vitro* C3H10T1/2 cell differentiation model. C3H10T1/2 is a mouse embryonic pluripotent mesenchymal cell line that differentiates into SMA-positive smooth muscle cells in response to treatment with Hh proteins and has been used as a surrogate system to study the Hh-induced differentiation program (8).

To further examine the effect of SLRPs on C3H10T1/2 cell differentiation, we used lentiviral infection to generate a C3H10T1/2 cell line that stably expresses C-terminally V5-tagged OGN proteins (Fig. 5*H*). We found that OGN expression alone was not sufficient to induce cell differentiation toward smooth muscle lineage (Fig. 5, *D* and *E*) and did not affect Gli-dependent luciferase reporter activity in the C3H10T1/2 cells with or without Shh treatment or Gli2 expression (Fig. 5*I*), suggesting that the proteoglycan is unlikely directly involved in Hh signal transduction. However, we found that OGN ectopic expression was able to inhibit Shh-mediated promotion of smooth muscle cell differentiation. Shh-induced induction of smooth muscle cell markers SMA and Myocd was decreased in OGN-expressing C3H10T1/2 cells, as assayed by immune-fluorescence staining or mRNA qPCR (Fig. 5, *F*, *G*, and *J*). Together, these data suggest that SLRPs are novel regulators of intestinal mesenchymal development and may function downstream of Hh signaling in a negative feedback fashion to modulate smooth muscle cell differentiation.

DISCUSSION

The current study investigated the genetic function of the Gli transcriptional effectors of Hh signaling in intestinal development and identified a novel function of SLRPs in smooth muscle cell differentiation. Recent studies from our laboratory and others established that Shh and Ihh proteins produced by the endodermal epithelium play a key role in intestinal development by controlling mesenchymal growth and patterning (1, 4, 5, 8, 9). However, the mechanisms through which Hh ligands carry out their diverse activities in the intestine are not well understood, specifically which Gli effectors mediate Hh function. Here, we show the first genetic analysis for the role of different Gli proteins during intestinal development and present strong evidence that the major Hh function in the intestinal mesenchyme is achieved through the Gli2 transcriptional activator.

Removal of Gli3-mediated transcriptional repression plays many critical roles in embryonic development (13, 14, 16, 19, 20). In the developing ureter, Hh signaling functions in a similar fashion as it does in the gastrointestinal system. Hh ligands are secreted by the epithelium and signal to the adjacent ureter mesenchyme (17, 30), and a recent report showed that removal of Gli3 repression is able to fully rescue the Smo-null mesenchymal phenotypes (17). Therefore, it is somewhat surprising that our genetic analysis revealed that Gli3 activity is dispensable for Hh function in the intestine, although both Gli2 and Gli3 are expressed in the mesenchyme (4). Our results highlight the context-dependent manner of Gli regulation in Hh-controlled developmental processes.

The molecular mechanism underlying gut mesenchymal development remains poorly characterized. Our transcriptional profiling and functional analysis suggest that a subset of SLRPs may act downstream of Hh signaling and is involved in regulating smooth muscle cell differentiation. Hh signaling has been reported to regulate expression of certain extracellular matrix proteins, such as collagens (34), but it is the first time that Hh signaling is linked to SLRPs. The SLRP family consists of 17 member proteins that are largely characterized by the presence of N-terminal cysteine-rich clusters (33), and the exact role of SLRPs *in vivo* is poorly understood. It has been reported that they may regulate cell proliferation and differentiation in certain developmental contexts (33), and genetic mutations in decorin (DCN) and LUM have been implicated in inherited ocular disorders such as congenital stromal dystrophy and high myopia in the cornea (35, 36). Our study suggests that SLRPs are novel regulators for smooth muscle differentiation. In Hh-mediated intestinal development, it appears that OGN acts in a negative feedback manner to modulate smooth muscle cell differentiation. Several SLRPs, including DCN and biglycan, have been reported to regulate different signaling pathways, such as insulin growth factor, epidermal growth factor, and transforming growth factor/bone morphogenetic protein (33). It is interesting to speculate that SLRPs may function to integrate critical signaling pathways in the embryonic gut mesenchyme. Clearly, future genetic studies are needed to establish the function of SLRPs in intestinal development.

REFERENCES

1. van den Brink, G. R. (2007) Hedgehog signaling in development and homeostasis of the gastrointestinal tract. *Physiol. Rev.* **87,** 1343–1375
2. Kosinski, C., Stange, D. E., Xu, C., Chan, A. S., Ho, C., Yuen, S. T., Mifflin, R. C., Powell, D. W., Clevers, H., Leung, S. Y., and Chen, X. (2010) Indian hedgehog regulates intestinal stem cell fate through epithelial-mesenchymal interactions during development. *Gastroenterology* **139,** 893–903
3. Yauch, R. L., Gould, S. E., Scales, S. J., Tang, T., Tian, H., Ahn, C. P., Marshall, D., Fu, L., Januario, T., Kallop, D., Nannini-Pepe, M., Kotkow, K., Marsters, J. C., Rubin, L. L., and de Sauvage, F. J. (2008) A paracrine requirement for hedgehog signalling in cancer. *Nature* **455,** 406–410
4. Kolterud, A., Grosse, A. S., Zacharias, W. J., Walton, K. D., Kretovich, K. E., Madison, B. B., Waghray, M., Ferris, J. E., Hu, C., Merchant, J. L., Dlugosz, A. A., Kottmann, A. H., and Gumucio, D. L. (2009) Paracrine hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. *Gastroenterology* **137,** 618–628
5. Mao, J., Kim, B. M., Rajurkar, M., Shivdasani, R. A., and McMahon, A. P. (2010) Hedgehog signaling controls mesenchymal growth in the developing mammalian digestive tract. *Development* **137,** 1721–1729
6. Zacharias, W. J., Li, X., Madison, B. B., Kretovich, K., Kao, J. Y., Merchant, J. L., and Gumucio, D. L. (2010) Hedgehog is an anti-inflammatory epi-

thelial signal for the intestinal lamina propria. *Gastroenterology* **138,** 2368–2377

7. van Dop, W. A., Heijmans, J., Büller, N. V., Snoek, S. A., Rosekrans, S. L., Wassenberg, E. A., van den Bergh Weerman, M. A., Lanske, B., Clarke, A. R., Winton, D. J., Wijgerde, M., Offerhaus, G. J., Hommes, D. W., Hardwick, J. C., de Jonge, W. J., Biemond, I., and van den Brink, G. R. (2010) Loss of Indian Hedgehog activates multiple aspects of a wound healing response in the mouse intestine. *Gastroenterology* **139,** 1665–1676

8. Zacharias, W. J., Madison, B. B., Kretovich, K. E., Walton, K. D., Richards, N., Udager, A. M., Li, X., and Gumucio, D. L. (2011) Hedgehog signaling controls homeostasis of adult intestinal smooth muscle. *Dev. Biol.* **355,** 152–162

9. Ramalho-Santos, M., Melton, D. A., and McMahon, A. P. (2000) Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127,** 2763–2772

10. McMahon, A. P., Ingham, P. W., and Tabin, C. J. (2003) Developmental roles and clinical significance of hedgehog signaling. *Curr. Top. Dev. Biol.* **53,** 1–114

11. Lum, L., and Beachy, P. A. (2004) The Hedgehog response network: sensors, switches, and routers. *Science* **304,** 1755–1759

12. Hooper, J. E., and Scott, M. P. (2005) Communicating with Hedgehogs. *Nat. Rev. Mol. Cell Biol.* **6,** 306–317

13. Ruiz i Altaba, A. (2011) Hedgehog signaling and the Gli code in stem cells, cancer, and metastases. *Sci. Signal* **4,** pt9

14. Hui, C. C., and Angers, S. (2011) Gli proteins in development and disease. *Annu. Rev. Cell Dev. Biol.* **27,** 23.21–23.25

15. Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D., and Joyner, A. L. (2002) Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* **129,** 4753–4761

16. Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F., and Chiang, C. (2002) Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418,** 979–983

17. Cain, J. E., Islam, E., Haxho, F., Blake, J., and Rosenblum, N. D. (2011) GLI3 repressor controls functional development of the mouse ureter. *J. Clin. Invest.* **121,** 1199–1206

18. Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., and Hui, C. C. (1998) Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125,** 2533–2543

19. Hilton, M. J., Tu, X., Cook, J., Hu, H., and Long, F. (2005) Ihh controls cartilage development by antagonizing Gli3, but requires additional effectors to regulate osteoblast and vascular development. *Development* **132,** 4339–4351

20. Litingtung, Y., and Chiang, C. (2000) Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3,** 979–985

21. Joeng, K. S., and Long, F. (2009) The Gli2 transcriptional activator is a crucial effector for Ihh signaling in osteoblast development and cartilage vascularization. *Development* **136,** 4177–4185

22. Verzi, M. P., Stanfel, M. N., Moses, K. A., Kim, B. M., Zhang, Y., Schwartz, R. J., Shivdasani, R. A., and Zimmer, W. E. (2009) Role of the homeodomain transcription factor Bapx1 in mouse distal stomach development. *Gastroenterology* **136,** 1701–1710

23. Long, F., Chung, U. I., Ohba, S., McMahon, J., Kronenberg, H. M., and McMahon, A. P. (2004) Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development* **131,** 1309–1318

24. Blaess, S., Stephen, D., and Joyner, A. L. (2008) Gli3 coordinates three-dimensional patterning and growth of the tectum and cerebellum by integrating Shh and Fgf8 signaling. *Development* **135,** 2093–2103

25. Mao, J., Ligon, K. L., Rakhlin, E. Y., Thayer, S. P., Bronson, R. T., Rowitch, D., and McMahon, A. P. (2006) A novel somatic mouse model to survey tumorigenic potential applied to the Hedgehog pathway. *Cancer Res.* **66,** 10171–10178

26. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007) A global double-fluorescent Cre reporter mouse. *Genesis* **45,** 593–605

27. Zhang, X. M., Ramalho-Santos, M., and McMahon, A. P. (2001) Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell* **105,** 781–792

28. McHugh, K. M. (1995) Molecular analysis of smooth muscle development in the mouse. *Dev. Dyn.* **204,** 278–290

29. Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S., and Fukuda, K. (2000) The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* **127,** 1971–1980

30. Yu, J., Carroll, T. J., and McMahon, A. P. (2002) Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129,** 5301–5312

31. Madison, B. B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X. T., and Gumucio, D. L. (2005) Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* **132,** 279–289

32. Kim, J. H., Huang, Z., and Mo, R. (2005) Gli3 -null mice display glandular overgrowth of the developing stomach. *Dev. Dyn.* **234,** 984–991

33. Schaefer, L., and Iozzo, R. V. (2008) Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J. Biol. Chem.* **283,** 21305–21309

34. Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B. R., Miura, N., and Carlsson, P. (2006) Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development* **133,** 833–843

35. Bredrup, C., Knappskog, P. M., Majewski, J., Rødahl, E., and Boman, H. (2005) Congenital stromal dystrophy of the cornea caused by a mutation in the decorin gene. *Invest. Ophthalmol. Vis. Sci.* **46,** 420–426

36. Majava, M., Bishop, P. N., Hägg, P., Scott, P. G., Rice, A., Inglehearn, C., Hammond, C. J., Spector, T. D., Ala-Kokko, L., and Männikkö, M. (2007) Novel mutations in the small leucine-rich repeat protein/proteoglycan (SLRP) genes in high myopia. *Hum. Mutat.* **28,** 336–344