

FoxF1 and FoxL1 Link Hedgehog Signaling and the Control of Epithelial Proliferation in the Developing Stomach and Intestine^{*[5]}

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The hedgehog (Hh) signaling pathway is a key component of cross-talk during vertebrate gut development, involving endodermally secreted Sonic (Shh) and Indian hedgehog (Ihh) proteins that directly signal to adjacent mesoderm. Here we show that the closely linked mesenchymal forkhead transcription factors *Foxf1* and *Foxl1* are part of this signaling cascade. Analysis of conserved non-coding sequences surrounding *Foxf1* and *Foxl1* identified seven Gli binding sites, with two sites near *Foxl1* being identical among mammalian, bird, fish, and amphibian species. *In vitro* experiments indicate that Gli2 binds to these Gli sites, several of which are critical for Gli2-mediated activation of a luciferase reporter in 293 cells. In addition, we demonstrate occupancy of one of these elements by Gli proteins in the intestine *in vivo* using chromatin immunoprecipitation. Furthermore, expression of both *Foxf1* and *Foxl1* is reduced in the *Gli2/Gli3* mutant gut. These results provide compelling evidence that *Foxf1* and *Foxl1* are mediators of the Hh (endoderm) to mesoderm signaling pathway.

The Hedgehog (Hh)² signaling pathway is one of the earliest regulatory cascades activated during organogenesis of the gut tube. Shh and Ihh, expressed in the gut endoderm, bind to the receptor *Ptch1*, which is expressed exclusively in the mesoderm of the vertebrate gut (1–4). Interaction with the *Ptch1* receptor causes derepression of Smo, a transmembrane protein, which prevents post-translational processing of Gli2 and Gli3 by inhibiting phosphorylation and proteasomal degradation (5). In the absence of *Ptch1*-mediated repression of Smo, an N-terminal proteolytic fragment of Gli3, and to a lesser extent Gli2, emerges from the proteasome as a transcriptional repressor, whereas Gli1 appears to be completely degraded.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental table.

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² The abbreviations used are: Hh, hedgehog; Shh, Sonic hedgehog; Ihh, Indian hedgehog; Hox, Homeobox; E, embryonic day; GI, gastrointestinal; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-PCR; QPCR, quantitative PCR; ChIP, chromatin immunoprecipitation; CMF, Ca²⁺ and Mg²⁺-free; HBSS, Hanks' balanced salt solution; oligo, oligonucleotide.

Both *Shh* and *Ihh* are expressed in mouse definitive endoderm from E8.5 onward along the entire gut tube, except in the domain of pancreatic bud endoderm (6). In the intestine, *Shh* and *Ihh* are both required for proper specification and development of smooth muscle of the muscularis externa and neurons of the myenteric plexus (2, 4, 7). Severe embryonic inhibition of all Hh signals, through overexpression of an Hhip cDNA or via injection of a neutralizing antibody against Hh proteins, revealed that Shh and Ihh are also indispensable for villus development and later critical for patterning the crypt-villus axis and restricting epithelial proliferation via modulation of adjacent mesenchymal cells (3, 7).

Recent computational approaches have attempted to identify Gli target genes. One investigation used a local alignment algorithm termed "enhancer element locator," which evaluates the spatial order of transcription factor binding sites from two different species (8). Gli binding sites near *Foxf1*, *Foxc2*, and *Foxl1*, all located within a 50-kb region of mouse chromosome 8, scored very highly using the enhancer element locator method. In addition, expression of *Foxf1* is dependent on *Shh* in the developing oral cavity, lung, and sclerotome, and both *Foxf1* and *Foxf2* expression can be extinguished in E12.5 intestine explants when treated with the Smo inhibitor cyclopamine (9, 10). These reports suggest that *Foxf1* and *Foxf2* could be Hh target genes.

Intestinal epithelial phenotypes of *Foxf1*, *Foxf2*, and *Foxl1* mutants also suggest connections to the Hh signaling pathway. *Foxf1*^{+/-}, *Foxf2*^{-/-}, and *Foxl1*^{-/-} mice all have perturbations in the crypt-villus axis, characterized by increased proliferation and Wnt signaling, reminiscent of epithelial abnormalities caused by Hh signal ablation (3, 7, 11, 12). *Foxl1*^{-/-} mice also have delayed villus morphogenesis, with very stunted villi evident at E16.5 and E18.5, similar to the severe defects in villus development caused by Hh inhibition (7, 11). The *Foxl1* mRNA expression pattern also highly resembles that of the known Hh target genes *Ptch1*, *Gli1*, and *Bmp4* during intestine development (2, 13–15).

Our studies indicate that *Foxf1* and *Foxl1* are direct target genes of the Hh signaling pathway in the developing stomach and intestine. Their expression is dependent on *Gli2* and *Gli3* and induced by Shh-N recombinant protein. Several highly conserved Gli binding sites appear crucial for Gli-mediated binding and transcriptional activation of the *Foxf1* and *Foxl1* promoters. These findings provide a critical link toward understanding the molecular mechanisms that control epithelial pro-

liferation and organization of the developing gastrointestinal (GI) tract.

EXPERIMENTAL PROCEDURES

EMSA Analysis—Stomach and intestine primordia (E14.5) were isolated from ~36 CD1 embryos and snap-frozen in liquid N₂, and nuclear extracts were prepared as described previously (16), with some modifications. Complete (Roche Applied Science) protease inhibitor tablets were used in all buffers, supplemented with 0.1 μM phenylmethylsulfonyl fluoride and 1.0 mM sodium metavanadate. Oligonucleotides (oligos) were labeled by incubating 50 ng of double-stranded DNA with 30 μCi of a [³²P]dCTP and 2.5 units of Klenow (New England Biolabs) for 30 min at room temperature followed by removal of unincorporated nucleotides with a MicroSpin G-50 column (Amersham Biosciences). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (17); 3–4 μg of protein extract were incubated with 20 mM Hepes, pH 7.9, with 0.1 mM EDTA, 1 mM MgCl₂, 12% glycerol, 60 mM KCl, 0.5 mM dithiothreitol (added fresh), 50 ng/μl sonicated dIdC, and 125 ng/μl bovine serum albumin for 10 min to prebind. Labeled probe (25,000 cpm) was then added and incubated for 30 min at room temperature. For some assays, 1 μg of a polyclonal rabbit antibody to Gli2 (AbCam ab26056) or 1 μg of rabbit IgG (R&D Systems) was added during the prebind. Oligos used in all experiments are listed in supplemental Table 1.

Luciferase Assays—HEK293 cells (CRL-1573) were purchased from ATCC. Genomic fragments of *CNS1*, *Foxf1*, and *Foxl1* were obtained by PCR of mouse genomic tail DNA with PfuUltra (Stratagene). For *Foxf1* PCR, a concentration of 1 M betaine was necessary for amplification. PCR products were cloned into pSC-B (Stratagene) and subcloned into pGL3-Basic (Promega) between XhoI and NcoI (*Foxf1* promoter). Using PfuUltra (Stratagene), the *Foxl1* promoter PCR product was amplified with nested primers and then subcloned into pGL3-Basic. In 24-well plates, 1.5 × 10⁵ 293 cells were seeded in each well. About 24 h later, 200 ng of DNA (100 ng of reporter plasmid, 5 ng of pRL-SV40 (Stratagene) and 70 or 45 ng of pBlueScript II (Stratagene)) were transfected using FuGENE 6 (Roche Applied Science) at a ratio of 3:1 (FuGENE:DNA). For *Foxf1* assays, 50 ng of pCS2-Gli2* was co-transfected, whereas 25 ng was co-transfected for *Foxl1*. Gli2* lacks the 328 N-terminal amino acids of Gli2 and has been shown to be a 30 times more potent transactivator in co-transfection assays than Gli2 (18). Luciferase assays were performed as described previously (17).

Gli sites within the *Foxl1* promoter and within *CNS1* were mutated according to specifications of the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenesis of the Gli site within the *Foxf1* promoter was performed by overlap extension PCR using the PfuUltra polymerase (19).

Quantitative RT-PCR—RNA was isolated using TRIzol (Invitrogen) per the manufacturer's instructions. The Bio-Rad iScript kit was used for cDNA synthesis from 500 ng of total RNA. To prevent amplification of contaminating genomic DNA in RNA samples, 0.5 μl of RQ1 RNase-free DNase (Promega) was added to each reverse transcriptase (RT) reaction, prior to the addition of RT. Reactions were incubated for 15 min at 37 °C, 10 min at 65 °C, and then cooled on ice. Using the

Brilliant Sybr Green mix (Stratagene), QPCR amplification was performed in triplicate with 0.5 μl of cDNA per 19-μl reaction on a Stratagene MX3000 thermocycler. Amplification thresholds were normalized to those of *HPRT*.

Chromatin Immunoprecipitation Assay (ChIP)—Chromatin was prepared from the small intestine of embryos at day 14.5 of gestation (*n* = 5) as described previously (20). Chromatin was immunoprecipitated with a polyclonal goat anti-Gli1 antibody (Santa Cruz Biotechnology, antibody sc-6153). After reversal of the cross-link, the *Foxf1* promoter fragment was PCR-amplified using primers and QPCR as described above. Enrichment between input DNA and immunoprecipitated DNA was calculated relative to that of the promoter of the myelin basic protein gene, which is not expressed in the intestinal mucosa and not a target of Gli regulation.

Isolation of Gli2 and Gli3 Mutant Tissue—*Gli2*^{+/-} mice were kindly provided by Dr. Alex Joyner (Memorial Sloan-Kettering Cancer Center). *Gli3*^{+/-} (*Gli3*^{Xt-j}) mice were obtained from The Jackson Laboratory. Mice were maintained on a mixed CD1-C3H/HeJ background. Embryos were isolated from *Gli2*^{+/-}*Gli3*^{+/-} intercrosses and genotyped as described previously (21, 22). Stomach and intestine primordia from E12.5 embryos were removed and homogenized in buffer RLT (Qiagen) in conjunction with QIAshredder columns (Qiagen). RNA was then extracted using the RNeasy micro kit (Qiagen). Quantitative RT-PCR was performed as indicated above.

E14.5 Gut Mesoderm Stimulation with Shh-N—Stomach and intestine primordia (E14.5) were isolated from 12–14 CD1 embryos and placed into cold Ca²⁺ and Mg²⁺-free (CMF) HBSS (Invitrogen). Tissues were incubated 10 min at room temperature in CMF-HBSS containing 10 mM EDTA and then cut lengthwise to expose the endoderm and transferred to an Eppendorf tube containing cold CMF-HBSS. Tissue was vortexed until all endoderm was removed, as evaluated under a Leica dissecting microscope. Mesoderm was rinsed in medium (Dulbecco's modified Eagle's medium (catalog number 11995, Invitrogen), 10% fetal calf serum, + penicillin/streptomycin). Mesoderm was then incubated at 37 °C for 30 min, with periodic mixing, in 1 ml of a 50% solution of Dispase (25 caseolytic units/ml, BD Biosciences) diluted with medium. Medium (9 ml) was added and then centrifuged at 1000 rpm for 5 min at 4 °C. Tissue was washed with 5 ml of medium and then distributed between 6 wells of a 24-well plate in 0.5 ml of medium/well. After 24 h of culture, 0.5% bovine serum albumin or mouse recombinant Shh-N (catalog number 461-SH, R&D Systems) was added to a final concentration of 2 μg/ml. After an additional 72 h of culture, RNA was extracted using TRIzol (Invitrogen) and quantitative RT-PCR was performed as described above.

RESULTS

Activation of Hh target genes is mediated by Gli factors binding to the consensus sequence GACCACCCA (23, 24). To identify conserved sequences within a 150-kb region surrounding *Foxf1* and *Foxl1*, we compared mammalian and non-mammalian species using the VISTA browser (25, 26), which provides multispecies global alignment. Using this tool, we discovered two sites near *Foxf1* and five sites near *Foxl1* that resembled or

Hedgehog Regulation of FoxF1 and FoxL1

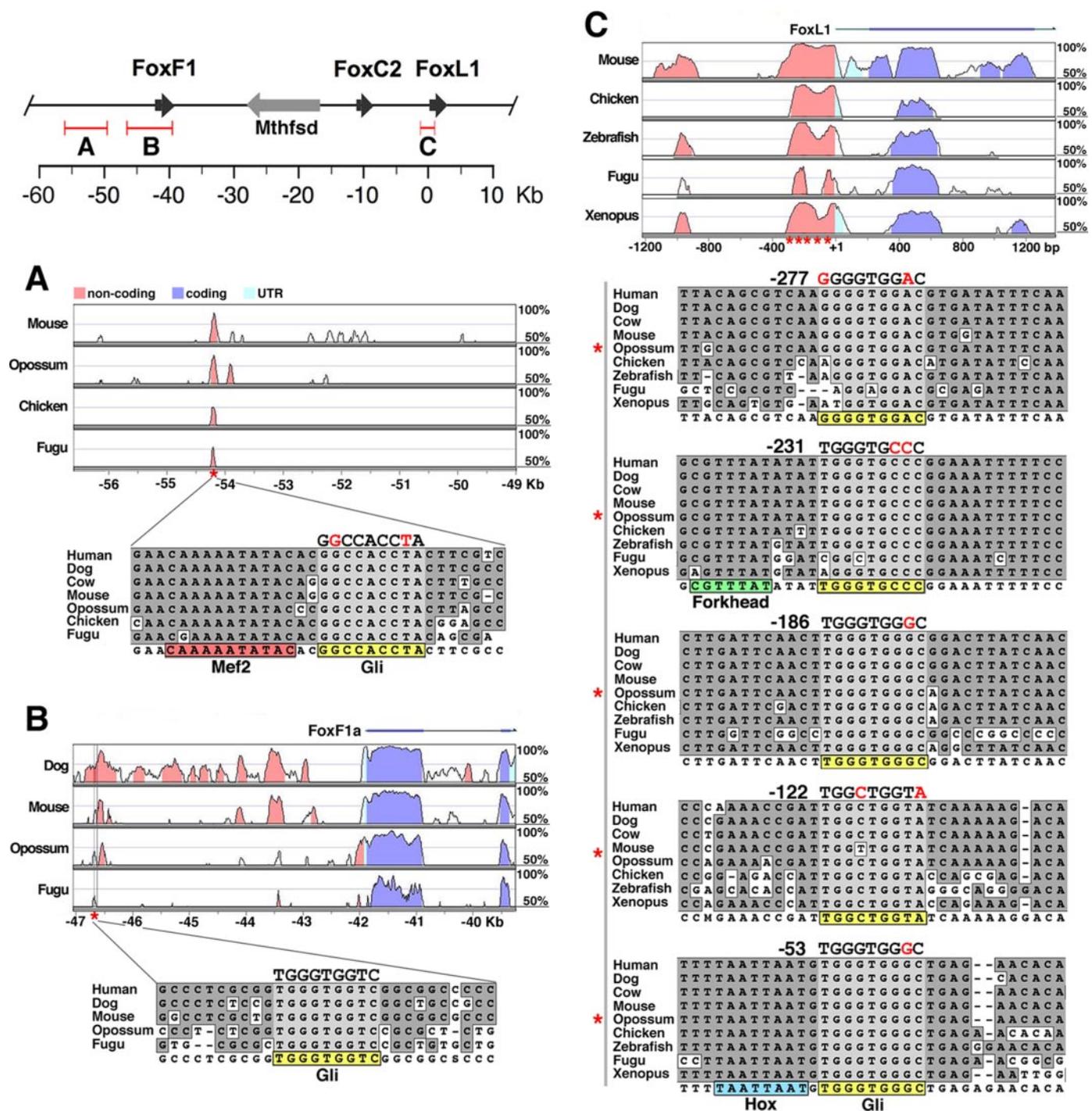


FIGURE 1. Identification of phylogenetically conserved Gli binding sites. *Foxf1*, *Foxc2*, and *Foxl1*, along with *Mthfsd*, are located within a 50-kb region of mouse chromosome 8 (top left). Conserved regions identified by VISTA analysis were analyzed for the Gli binding consensus (A, B, and C). About 7.5 kb upstream of the *Foxf1* gene, a small, ~100-bp conserved region (CNS1) contains two forkhead sites (not pictured) and one Gli site adjacent to a Mef2-like consensus (A). One additional Gli site was identified near *Foxf1*, ~3.4 kb upstream (B). Five conserved Gli sites were found in the proximal promoter region of *Foxl1*, at the indicated positions (C). Gli sites are highlighted in yellow, and the consensus binding sites are noted above with mismatches colored red. Physical genomic distances are relative to the *Foxl1* gene.

matched the Gli binding consensus (Fig. 1). One Gli site near *Foxf1* was within a small (~100 bp) conserved region (CNS1), which also contained two forkhead sites (AYAAACA) and one site similar to the consensus of Mef2 transcription factors, known to be involved in striated and smooth muscle development (27) (Fig. 1A). The two Gli sites nearest to *Foxl1* were within a region of the proximal promoter that exhibited even

greater sequence conservation than the coding region of *Foxl1* (Fig. 1C). Gli binding sites often appeared within close proximity to forkhead binding sites, with four out of seven Gli sites located within 16 bp of a conserved forkhead site (data not shown). The Gli site nearest *Foxl1*, at -53, was also immediately adjacent to a highly conserved Homeobox (Hox) consensus site, TAAT (Fig. 1C).

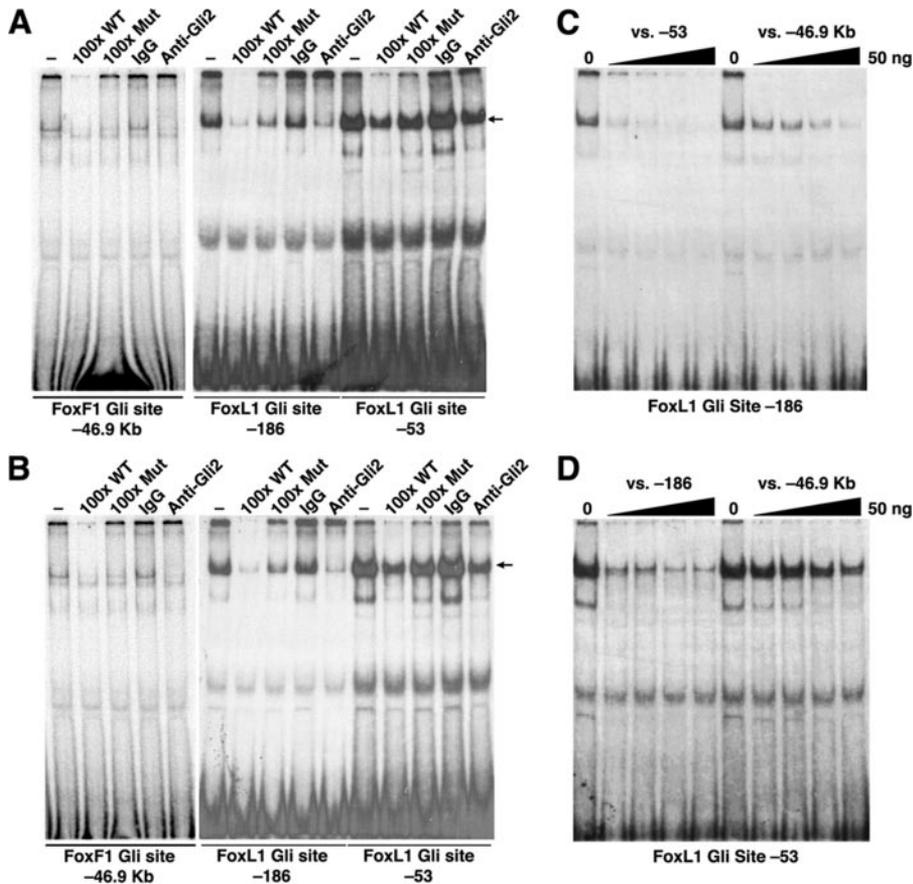


FIGURE 2. Gli2 from E14.5 gut extracts binds to Gli consensus sequences near *Foxf1* and *Foxl1*. Nuclear extracts from E14.5 stomach (A) and intestine (B) bind Gli sites from the *Foxf1* promoter (–46.9 kb) and from the *Foxl1* promoter (–186 and –53). Binding is diminished by competition with an unlabeled oligo encompassing a known Gli binding site from the *Ptch1* promoter (100x WT) but is not effectively competed by a mutant oligo (100x Mut). Anti-Gli2 disrupts binding with all three oligos, whereas rabbit IgG does not. Using E14.5 intestine extracts, oligos from each site were competed against increasing amounts of unlabeled oligos (0, 5, 10, 25, and 50 ng) to determine relative binding affinity (C and D). The –186 site was effectively competed with 10–25 ng of cold –53 oligo, whereas the –46.9-kb oligo did not completely quench binding, even at 50 ng (C). Similar results were seen with the –53 site, although this site appears to have an upper band not effectively competed by either cold competitor (D). This may represent association of other transcription factors, such as Hox proteins, binding independently of Gli.

To assess the Gli binding potential of these conserved sequences, we performed EMSAs on sites with the closest match to the Gli consensus. Gli sites near *Foxl1* at –186 and –53, with respect to the start of the *Foxl1* mRNA at +1, exhibited strong binding, which was diminished upon competition with an unlabeled oligo encompassing a Gli site from the *Ptch1* promoter (28) (Fig. 2, A and B). Similarly, an antibody against Gli2 reduced the level of the shifted signal of the –186 and –53 oligos, indicating a disruption of Gli2 binding (Fig. 2, A and B). The same held true in assays performed with the Gli site nearest to *Foxf1* (*Foxf1* Gli site, –46.9 kb), except that the intensity of the shifted complex was much less (Fig. 2, A and B). To compare the relative binding affinity of these Gli sites, competitions with unlabeled oligos were performed (Fig. 2, C and D). These indicated that sequences at –53 and –186 had higher affinity than the Gli site near *Foxf1* at –46.9 kb. These experiments also revealed that the –53 oligo produced two closely migrating complexes, and the top band in this doublet was partially resistant to competition with the –186 oligo (Fig. 2D).

This upper band could represent binding of a Hox factor to the highly conserved Hox site immediately adjacent to the Gli site.

Next, we tested the Gli sites within *Foxf1* and *Foxl1* promoter fragments for their ability to activate a luciferase reporter in HEK293 cells. Individual Gli sites were mutated, and promoter activity was assayed in the context of a constitutively active Gli2 protein that contains an N-terminal deletion of the repressor domain (Gli2 Δ N or Gli2*) (18). The 3.6-kb *Foxf1* promoter exhibited very high level reporter activity in 293 cells but was still stimulated ~2-fold when co-transfected with a Gli2*-expressing plasmid (Fig. 3A). Mutation of the Gli site significantly reduced Gli2*-mediated enhancement but did not affect the basal level of promoter activity (Fig. 3A). The conserved non-coding region (CNS1) near *Foxf1*, about 7.5 kb distal to the Gli site near *Foxf1*, was assayed for its effects on reporter activity in the context of the 3.6-kb *Foxf1* promoter. CNS1 did not affect *Foxf1* promoter activity, and likewise, when the Gli site within CNS1 was mutated, reporter activity was unchanged (Fig. 3A). This may not be so surprising considering that a transgenic mouse with a LacZ cDNA driven by a *Foxf1* promoter lacking the CNS1 region largely recapitulates the expression pattern of *Foxf1* in the developing and adult gut (29).

In the context of a 1.0-kb *Foxl1* promoter fragment, we observed a 4-fold induction of reporter activity upon co-transfection of the Gli2*-expressing plasmid (Fig. 3B). Gli sites at –53 and –186 were assessed first because of their high conservation and ability to bind Gli2 in EMSAs. The Gli site mutation at –186 caused substantial attenuation of Gli2*-mediated induction, whereas mutation of the –53 site had somewhat lesser, although significant, effects. Mutation of both sites decreased Gli2*-mediated induction to less than 1.6-fold, indicating that these two sites provide the large majority of the Gli2 responsiveness of the *Foxl1* promoter in this assay (Fig. 3B). However, a small induction was still observed in the –53/–186 double mutant, suggesting that some Gli2 responsiveness may remain. Additional mutations at the –277, –231, and –122 Gli sites, in which all sites except –231 are mutated or in which all five sites are mutated, completely eliminated any significant induction by Gli2* (Fig. 3B). Among all five Gli sites, the –186 site appears to have preeminence because of its comparatively

Hedgehog Regulation of FoxF1 and FoxL1

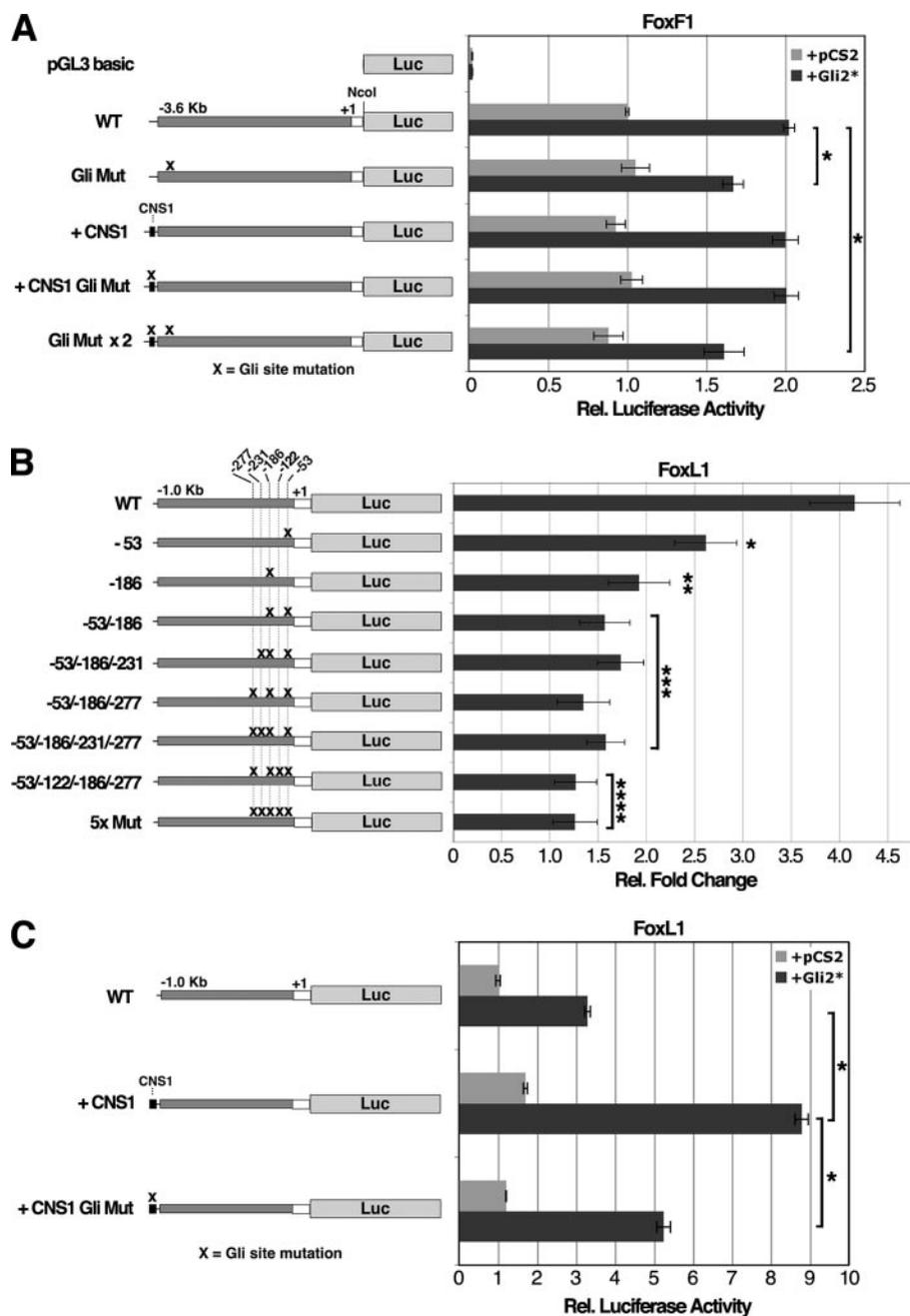


FIGURE 3. Gli sites near *Foxf1* and *Foxl1* are necessary for Gli2-mediated transcriptional activation. A 3.6-kb promoter fragment of *Foxf1*, plus a 239-bp 5' untranslated region, were assayed for luciferase (*Luc*) activity in 293 cells (A). The *Foxf1* promoter was assayed in the context of the CNS1 element and with the indicated Gli site mutations (*Gli Mut*) (A). Data represent the average of two experiments, each performed in triplicate. Cells were harvested 24 h after transfection. *WT*, wild type. *Rel.*, relative. As in A, a 1.0-kb promoter fragment of *Foxl1*, plus a 190-bp 5' untranslated region, were similarly assayed in B, except that -fold changes represent induction of reporter activity by Gli2* when compared with empty vector. Asterisks indicate a significant decrease in Gli2*-mediated induction (B). Data represent the average of four experiments, each done in triplicate (B). C, *Foxl1* promoter activity in the context of the CNS1 element, with or without a mutation in the Gli site. Data are representative of three experiments, each done in triplicate (C). pGL3-Basic basal luciferase activity, not shown (B and C), is greater than the basal activity of the *Foxl1* promoter, suggesting that the *Foxl1* promoter may have repressive *cis*-regulatory elements. Data represent the mean \pm S.E. For A and C, (*) indicates p value < 0.001 ; for B, (*) indicates p value < 0.05 , (**) indicates p value < 0.01 , (***) indicates p value < 0.005 , and (****) indicates p value < 0.001 according to Student's *t* test.

high conservation, strong Gli binding affinity, and key importance in Gli2*-mediated induction in this assay. Although the CNS1 element did not enhance the activity of the *Foxf1* promoter, there was a pronounced stimulatory effect on *Foxl1* regulatory sequences, more than doubling the Gli2*-mediated

induction of this promoter (Fig. 3C). Mutation of the Gli site within CNS1 significantly diminished the effect of CNS1 (Fig. 3C). These data suggest that CNS1 may act as a distal enhancer for the *Foxl1* promoter.

To determine whether Gli proteins occupy any of the potential binding sites identified above *in vivo*, we performed chromatin immunoprecipitation assays with an antibody specific to Gli1 on chromatin isolated from fetal intestine (see "Experimental Procedures"). As shown in Fig. 4, the Gli binding site in the promoter of the *Foxf1* gene, located at position -46.9 kb with respect to the transcriptional start site of *Foxl1*, is highly enriched in the immunoprecipitated DNA when compared with input DNA, indicating strong binding to this site by Gli1 *in vivo*. To our knowledge, this is the first description of Gli1 binding to *in vivo* targets in the GI tract using chromatin immunoprecipitation.

The *in vitro* and *in vivo* data described above indicate that Gli transcription factors directly activate the *Foxf1* and *Foxl1* genes, predicting that genetic inactivation of *Gli2* and/or *Gli3* would cause reductions of *Foxf1* and *Foxl1* mRNA levels *in vivo*. *Gli1* is not expressed in the absence of *Gli2* and *Gli3* and thus cannot compensate for their inactivation (30). Therefore, we evaluated *Foxf1* and *Foxl1* mRNA levels in E12.5 *Gli2* and *Gli3* null stomach and intestine (midgut) primordia (Fig. 5, B and C). In the stomach primordium of *Gli2*^{-/-}*Gli3*^{-/-} double mutants, *Foxl1* expression is significantly decreased, whereas *Foxf1* expression is not altered (Fig. 5B). The stomach primordium of *Gli2*^{+/-}*Gli3*^{-/-} embryos exhibited a significant increase in expression of *Foxf1* and a small increase of *Foxl1* mRNA. This may reflect strong repressive effects of Gli3 that normally function to limit expression of *Foxf1* (and perhaps *Foxl1*) in the stomach. However, this did not hold true in the intestine, where *Foxf1* and *Foxl1* expression was lower when both alleles of either *Gli2* or *Gli3* are inactivated, although *Foxl1* mRNA levels were not significantly reduced in

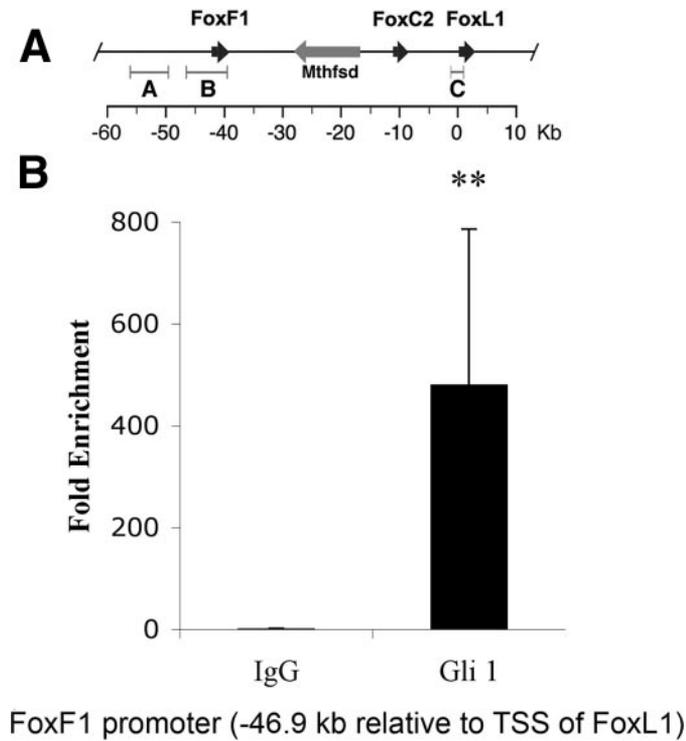


FIGURE 4. Gli1 occupies its site in the Foxf1 promoter in intestinal chromatin. *A*, schematic representation of the Foxf1/Foxl1 locus. Physical genomic distances are relative to the Foxl1 gene. The binding site assayed here is located in *region B*. *B*, quantification of real-time PCR analysis of the enrichment of the Foxf1 promoter element at position -46.9 relative to the transcriptional start site (TSS) of Foxl1. QPCR was performed on five input samples and five ChIP samples, and the enrichment was calculated relative to a non-target gene (myelin basic protein). (***) p value <0.01 according to Student's t test. Data represent the mean \pm S.E. *C*, representative agarose gel of the ChIP experiment showing the presence of the Foxf1 promoter fragment in the Gli1 ChIP but not the IgG control ChIP.

Gli2^{+/-}*Gli3*^{-/-} embryos (Fig. 5C). In terms of *Foxf1* and *Foxl1* regulation, expression of these genes appears more dependent on levels of both Gli2 and Gli3 in the developing intestine than in the stomach. Reduction of *Foxf1* and *Foxl1* mRNA levels was not secondary to a deficiency of mesodermal tissue caused by *Gli2* and/or *Gli3* mutations because QPCR did not reveal significant reductions of the mRNA for vimentin, an intermediate filament broadly expressed in mesenchymal cells (data not shown). As expected, expression of *Gli1* was dramatically reduced in *Gli2*^{-/-}*Gli3*^{-/-} embryos (Fig. 5B).

Next we investigated whether activation of the Hh pathway was sufficient to induce the expression of these genes in a relevant tissue. Gut mesoderm from E14.5 embryos was isolated and treated with a recombinant mouse N-terminal fragment of Shh (Shh-N) and assayed for induction of *Foxf1* and *Foxl1* mRNA. *Foxl1* was significantly induced in both stomach and intestine mesoderm, whereas *Foxf1* was significantly induced only in the intestine (Fig. 6A). In this assay, the Hh signaling

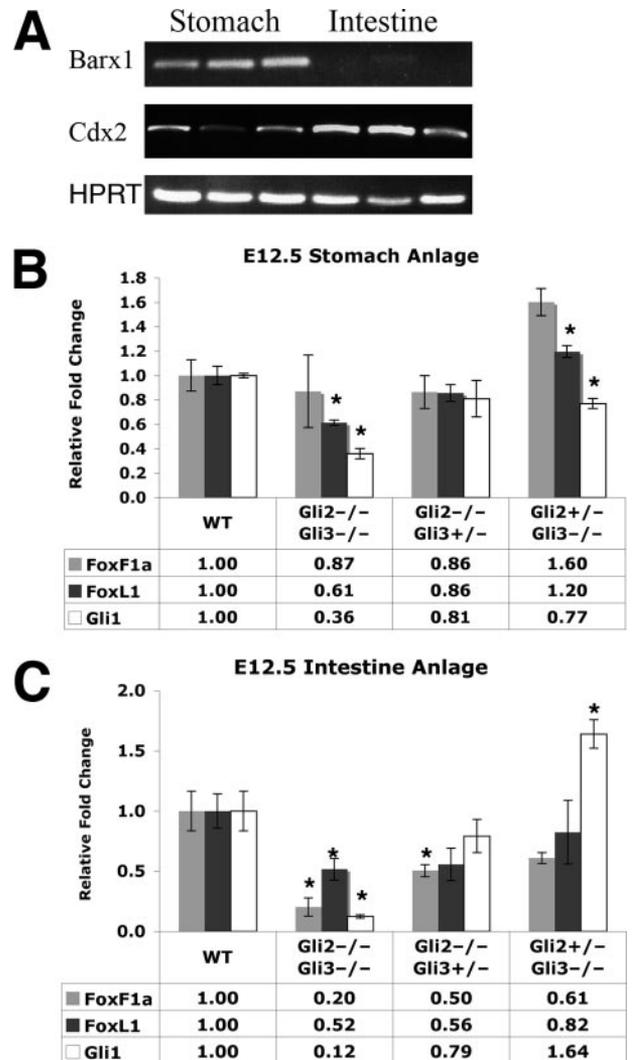


FIGURE 5. Foxf1 and Foxl1 expression are dependent on Gli2 and Gli3, in vivo. *A*, prospective stomach and intestine were dissected from day 12.5 days postcoital embryos. RT-PCR for Barx1 confirms that our stomach anlage is expressing this gastric marker. Cdx2 expression is strong in the intestine, as expected. Some Cdx2 expression is also present in the stomach fraction, indicating that these samples also contained some prospective duodenal tissue. *B* and *C*, Foxf1, Foxl1, and Gli1 mRNA levels in E12.5 stomach (*B*) and intestine (*C*) anlage in *Gli2* and *Gli3* compound mutants, as assessed by quantitative RT-PCR. Tissues from 3–5 embryos were assayed for each genotype. Wild type (WT) levels are set equal to 1.0. (*) p value <0.05 according to Student's independent t test. Data represent the mean \pm S.E.

pathway is clearly activated, as indicated by robust induction of the Gli2/Gli3 target gene *Gli1* (Fig. 6B). Collectively, these data demonstrate that *Foxf1* and *Foxl1* are dependent on *Gli2* and *Gli3* and induced upon Hh pathway activation in the mesoderm of developing stomach and intestine.

DISCUSSION

In summary, this study provides clear evidence that *Foxf1* and *Foxl1* are target genes of the Hh signaling pathway via Gli transcription factors. Although a total absence of Gli factors (in *Gli2*^{-/-}*Gli3*^{-/-} double mutants) does not completely extinguish *Foxf1* and *Foxl1* expression in the developing gut, this is not surprising. The remaining expression of *Foxf1* and *Foxl1* in *Gli2*^{-/-}*Gli3*^{-/-} gut tissue is likely due to other signals and transcription factors acting on their respective promoters.

Hedgehog Regulation of FoxF1 and FoxL1

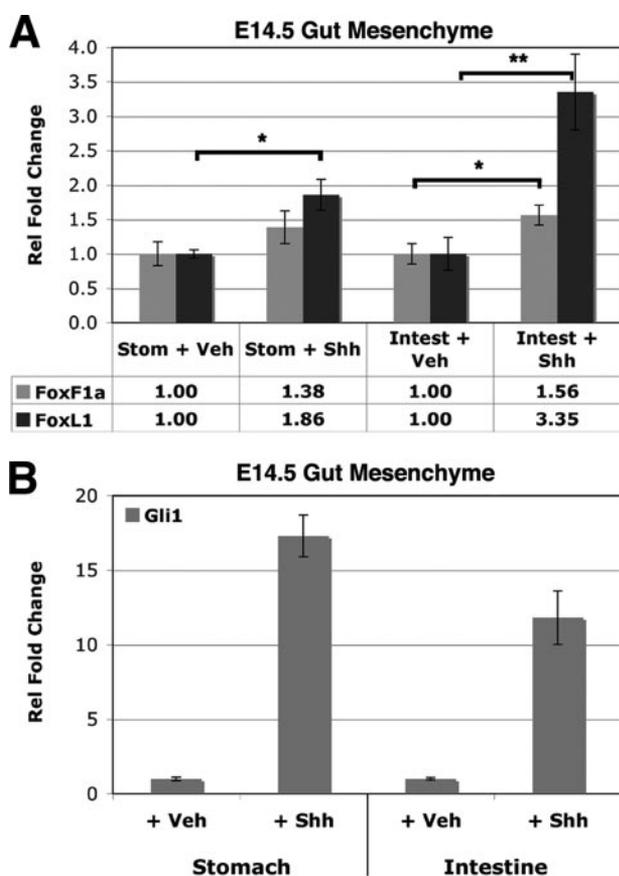


FIGURE 6. Foxf1 and Foxl1 are induced in gut mesenchyme upon administration of Shh. E14.5 mesenchyme from stomach (*Stom*) and intestine (*Intest*) were cultured with 0.5% bovine serum albumin (vehicle (*Veh*), set equal to 1.0) or recombinant Shh-N for 72 h, and *Foxf1* and *Foxl1* mRNA levels were assessed by quantitative RT-PCR (A). *Rel*, relative. *Gli1* induction by Shh-N is also shown (B). Data are representative of two experiments, each performed in triplicate. (*) p value <0.05 or <0.01 (**) according to Student's independent t test. Data represent the mean \pm S.E.

Indeed, as shown in Fig. 1, conserved Hox sites, forkhead sites, and other binding sites are located in these promoters and could provide Hh-independent positive influences on *Foxf1* and *Foxl1* expression. In the developing mouse, expression of *Foxf1* in the lateral mesoderm and yolk sac is absent in *Shh*^{-/-}*Ihh*^{-/-} and *Smo*^{-/-} embryos but maintained in the allantois and primitive streak (31), indicating that Hh-independent signals maintain some level of *Foxf1* expression in certain tissues.

Although complete inactivation of Gli activity in *Gli2*^{-/-}*Gli3*^{-/-} embryos did not entirely eliminate expression of *Foxf1* and *Foxl1* in the stomach and intestine, an 80% reduction of *Foxf1* and 50% reduction of *Foxl1* in the intestine could account for a portion of the comprehensive defects seen upon Hh inhibition in the developing gut. In addition to *Foxf1* and *Foxl1*, there are likely to be multiple other Hh target genes activated via Gli transcription factors in the developing gut mesoderm. These target genes may individually execute small roles in larger developmental processes. One remarkable example of this phenomenon involves the platelet-derived growth factor (PDGF) signaling pathway, in which each of 12 identified platelet-derived growth factor target genes individually contributes small phenotypic aspects of the much more serious phenotypes

of the *Pdgfra* and *Pdgfrb* mutants (32). Indeed, genetic inactivation of *Foxl1* results in a developmental phenotype of the gut considerably less severe than that caused by potent inhibition of the Hh signal (7, 11).

Foxf1 and *Foxl1* dependence on the Hh signal also differs between different tissues. It is interesting that both *Foxf1* and *Foxl1* appear less responsive to Shh-N stimulation in stomach mesoderm and somewhat less dependent on *Gli2* and *Gli3* in the developing stomach when compared with similar experiments with intestinal tissue. This could reflect a difference of *Gli2* and *Gli3* expression levels in the stomach, the degree to which the Hh pathway is activated there, and/or differential dependence on other transcription factors expressed in the stomach.

Where possible, primary tissue was used to evaluate the connection between the Hh signal and *Foxf1* and *Foxl1* in this study. Luciferase assays of *Foxf1* and *Foxl1* Gli-dependent promoter activity were the only instances where primary tissue could not be used due to technical limitations. However, it is compelling that Shh induction of *Foxf1* and *Foxl1* mRNAs in E14.5 intestinal mesenchyme closely mirrors the induction seen by Gli2* in HEK293 cells; note a 3.4-fold induction of *Foxl1* in E14.5 mesenchyme (*versus* a 4-fold induction in HEK293 cells) and a 1.6-fold induction of *Foxf1* in E14.5 mesenchyme (*versus* a 2-fold induction in HEK293 cells) in Figs. 3 and 6. This similarity may suggest that testing these promoter fragments in HEK293 cells represents an appropriate model for assaying Gli-mediated activation of these mesenchymally expressed forkhead genes.

When fetal intestinal mesenchyme was cultured in the presence of Shh, *Foxl1* was more responsive than *Foxf1*. In contrast, when we analyzed gene expression in the intestine anlage of *Gli2*^{-/-}*Gli3*^{-/-} embryos, *Foxf1* was the more affected gene, whereas in the stomach, *Foxl1* is more sensitive to *Gli2*/*Gli3* ablation. At present, we do not know what explains these slight discrepancies. It is possible that during the relatively long culture with Shh (72 h), there is a partial fate change in the cultured mesenchyme. Alternatively, differences could be due to alternate signaling molecules affecting gene expression in the culture experiment or non-physiological dosage of Shh. Nevertheless, our genetic data strongly support the notion that both *Foxf1* and *Foxl1* are regulated by hedgehog signaling and Gli factors.

To evaluate the *in vivo* binding of Gli factors to the *Foxf1* and *Foxl1* promoters, we developed ChIP from embryonic gut tissue for Gli1, which to our knowledge has not been reported previously. We were thus able to confirm strong Gli binding to the promoter of the *Foxf1* gene *in vivo*. To truly dissect the *in vivo* relevance of the Hh responsiveness of a target gene, it would be necessary to mutate all Gli binding sites within endogenous regulatory elements. This would be a costly and extremely complicated undertaking for linked genes such as *Foxl1* or *Foxf1*, which have multiple Gli binding sites that could additionally regulate the expression of neighboring loci. The surmounting evidence provided here (phylogenetic conservation of Gli sites, confirmed binding by EMSA, promoter responsiveness to Gli2 in luciferase assays, occupancy of target sites by Gli *in vivo*, genetic dependence on *Gli2* and *Gli3*, and

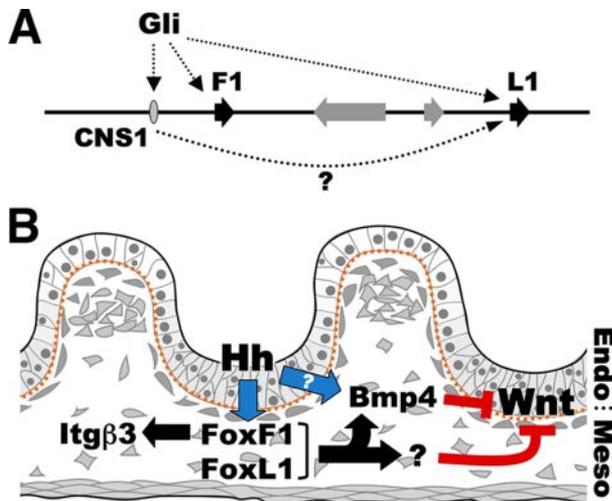


FIGURE 7. Reciprocal signaling in the gastrointestinal tract. *A*, summary of Gli-mediated regulation of *Foxf1* and *Foxl1* chromosomal region, and possible enhancer function of CNS1, which may impact *Foxl1* activity from a distance. *B*, model of Hh regulatory cascade in E14–E15 developing gut, indicating possible roles for *Foxf1* and *Foxl1*. *Endo*, endoderm; *Meso*, mesoderm.

Shh induction in primary tissue explants) strongly indicates that *Foxf1* and *Foxl1* are target genes of the Hh pathway.

Because *Foxf1* and *Foxl1* appear to be controlled in parallel by the same signaling pathway (the Hh signal) and are both expressed in the same tissue (the developing mesoderm of the gut), it is compelling to speculate whether, and to what extent, *Foxf1* and *Foxl1* are functionally or biochemically redundant. *Foxl1* and *Foxf2* mutants both exhibit reduced expression levels of *Bmp4* in the gut (10, 11), and recent characterizations indicate that *Foxf1* mutant embryos have a severe depletion of *Bmp4* expression in the lateral and extraembryonic mesoderm (31). If *Foxf1* is involved in *Bmp4* expression in the developing intestine, then the effects of *Foxf1* and *Foxl1* could be routed, in part, through *Bmp4*, a known repressor of epithelial proliferation (33–35), perhaps via direct effects on the Wnt signaling pathway (Fig. 7B). *Foxf1* was recently described as having a critical role in mediating fibroblast migration, and the integrin *Itgb3* was identified as a direct target gene (36). *Foxf1* deficiency in the gut could thus cause defects in mesodermal migration during villus development. This could affect the distribution of mesenchymal cells along the crypt-villus axis, and in turn, affect epithelial organization. Still, there are few known target genes of *Foxf1* and *Foxl1*, and no described targets that are candidate repressors of the Wnt signaling pathway, except that *Foxl1* inactivation results in higher expression of the Wnt-enhancing proteoglycan, Syndecan-1 (12). Elucidation of the *Foxl1* *in vivo* DNA binding consensus will aid bioinformatic identification of *Foxl1* target genes and enable the evaluation of whether *Foxf1* and *Foxl1* bind to the same *cis*-regulatory elements and cooperatively or redundantly regulate identical target genes.

Although mutations in either *Foxl1* or *Foxf1* affect mesenchymal signaling, their combined role in GI development cannot be easily addressed by genetic means due to their genomic proximity. Construction of a double mutant is further hampered by the presence of at least two genes, *Foxc2* and *Mthfsd*, between *Foxf1* and *Foxl1*. Nevertheless, our analysis of the regulatory circuits operative in the developing GI tract provides

firm evidence placing *Foxf1* and *Foxl1* at a central node in the reciprocal signaling mediating organogenesis of the mammalian GI tract (Fig. 7B).

Although multiple genes are involved in the morphological processes of gut development, the mutation of single genes can have profound effects on the homeostasis of the adult gastrointestinal epithelium. Mutations of the Wnt signaling pathway represent the most salient causes of sporadic and familial colorectal cancer in humans. However, very few non-cell-autonomous regulators of intestinal epithelial proliferation and/or Wnt signaling have been described. One non-autonomous regulator, *Foxl1*, is a modifier of the mouse *Apc^{Min}* mutation, acting as a suppressor of tumors formed from unchecked Wnt signaling, probably through the regulation of secreted proteins (37). Together, *Foxl1* with *Foxf1* could provide indispensable control of proliferation and homeostasis of the adult intestinal epithelium. As more mesenchymal regulators of epithelial proliferation are discovered in mice and humans, genetic risk factors associated with colorectal cancer could include an array of mutations relevant to this indirect tumor suppression, thus expanding the power of genetic testing and pharmacological intervention for preventative and therapeutic measures.

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Hedgehog Regulation of FoxF1 and FoxL1

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