Supplementary Materials and Methods

Generation of rhabdomyosarcoma cell lines

Ptch1$^{+/-}$ and *Hip1*$^{+/-}$ mice that developed flank masses were sacrificed and the tumors were removed within their capsules. A portion of each tumor was placed directly into Bouin's fixative and stained with Hematoxylin and Eosin following standard protocol. Another portion was flash frozen for further analysis and stored at -80. Cell lines were generated from the remaining tissue as follows. The tumor was dissected from the surrounding capsule and cut into 1-3 mm pieces. The diced tumor was placed in 0.05% trypsin solution (Invitrogen) at 37^{o} for 15 minutes, followed by 2-3 minutes of incubation in 0.25% trypsin solution. The dissociated cells were then plated using different concentrations of Matrigel in 6-well dishes according to the manufacturer's instructions (BD Biosciences). Clones were subsequently isolated and expanded on rat-tail collagen (Sigma)-coated dishes. The rhabdomyosarcoma cell lines derived from *Ptch1*$^{+/-}$ and *Hip1*$^{+/-}$ mice were designated as RMP and RMH respectively in this study. The standard growth media used was DMEM (Cellgro) supplemented with glutamine, penicillin, streptomycin and 15% fetal bovine serum (FBS) (all from Invitrogen).

Plasmids

pRK5-*Gli1* was constructed by cloning an *EcoR* V/*Xba* I restriction DNA fragment encoding the full-length mouse Gli1 protein into the pRK5 expression vector; pcDNA3-*MycGli1* was constructed by inserting oligonucleotides encoding a Myc tag (EQKLISEEDL) into the 5' end of Gli1, which was then cloned into the pcDNA3 expression vector. pcDNA3- *FLAGGli1* was constructed by inserting oligonucleotides encoding a FLAG tag (DYKDDDDK) into the 5' end of the full-length mouse *Gli1* cDNA, which was then cloned into pcDNA3. pcDNA3-*FLAGGli2* was constructed by inserting oligonucleotides encoding a FLAG tag (DYKDDDDK) into the 5' end of the full-length mouse *Gli2* cDNA, which was then cloned into pcDNA3. pcDNA3-*Gli3* was constructed by cloning a *Sac* I/*Sal* I restriction DNA fragment encompassing the full-length cDNA of human *Gli3* into pcDNA3; pcDNA-*FLAGGli3* was constructed by inserting oligonucleotides encoding a FLAG tag into the 5' end of the human *Gli3* cDNA, which was subsequently cloned into pcDNA3. N-terminal deletions of Gli1 were constructed using a PCR-based strategy to introduce an *EcoR* V site and an in-frame start codon at amino acid 176 or 243. The C-terminal Gli1 deletion was created using a similar PCR-based strategy to introduce an in-frame stop codon at amino acid position 1020. Gli1Δ404-593 was constructed by inserting *Nar* I sites at amino acids 402-3 and 592-593 using a PCR-based strategy. The region was subsequently deleted by *Nar* I digestion and ligation. Gli1Δ572-707 and Gli1Δ764-1026 were constructed by restriction digestion with *Pfl*M I and *Msc* I respectively and subsequent ligation. Sequencing was used to verify that the deletions were made correctly. Deletions were subcloned into appropriate expression plasmids. Tagged forms of the deletion mutations were constructed by cloning restriction fragments encompassing the deletion into appropriate sites of tagged full-length Gli1. Other plasmids used in this study have been previously described.

RT-PCR

RT-PCR was performed using the Invitrogen Superscript III First Strand kit according to the manufacturer's instructions. The primer pairs used to amplify resultant cDNAs were: Gli1 5' ATCACCTGTTGGGGATGCTGGAT 3'; 5' GGCGTGAATAGGACTTCCGACAG 3'. Gli2 5' GTTCCAAGGCCTACTCTCGCCTG 3'; 5' CTTGAGCAGTGGAGCACGGACAT 3'. β-actin 5' TGTTACCAACTGGGACGACA 3'; 5' CTCTCAGCTGTGGTGGTGAA 3'.

Titles and Legends to Supplementary Figures

Supplementary Figure 1. RT-PCR analysis of *Gli1* and *Gli2* expression in primary rhabdomyosarcoma tissues from *Ptch1*$^{+/-}$ and *Hip1*$^{+/-}$ mice
Total RNA was isolated from frozen primary rhabdomyosarcoma tissues and analyzed for expression of *Gli1* and *Gli2* using RT-PCR. A day 9.5 transformed mouse embryonic fibroblast cell line (tMEF) was used as control. *Gli1* expression was easily detectable in both primary tumor samples, while *Gli2* expression was weakly detectable. Lane 1: tMEF; lane 2: no RNA; lane 3: primary *Ptch1*$^{+/-}$ rhabdomyosarcoma; lane 4: primary *Hip1*$^{+/-}$ rhabdomyosarcoma.

Supplementary Figure 2. Gli1 and Gli2 inhibit myogenic differentiation of C2C12 cells
(A-D) X-gal staining of C2C12 cells after transfection with RSV-*lacZ* (which is constitutively active) and either control (A) or plasmids encoding Gli1 (B), Gli2 (C) or Gli3 (D) as indicated. Compare the elongated blue myotubes in the control cells (A) to cells expressing Gli1 (B) and Gli2 (C), the majority of which are round in shape. Gli3 had little effect on myotube formation (compare B to D).
(E) Luciferase assays of C2C12 cells to assess the activity of a Myogenin reporter, Mgn-*luc* after transfection with Gli expression plasmids as indicated. Gli1 and Gli2 substantially inhibited the activity of Mgn-*luc*.