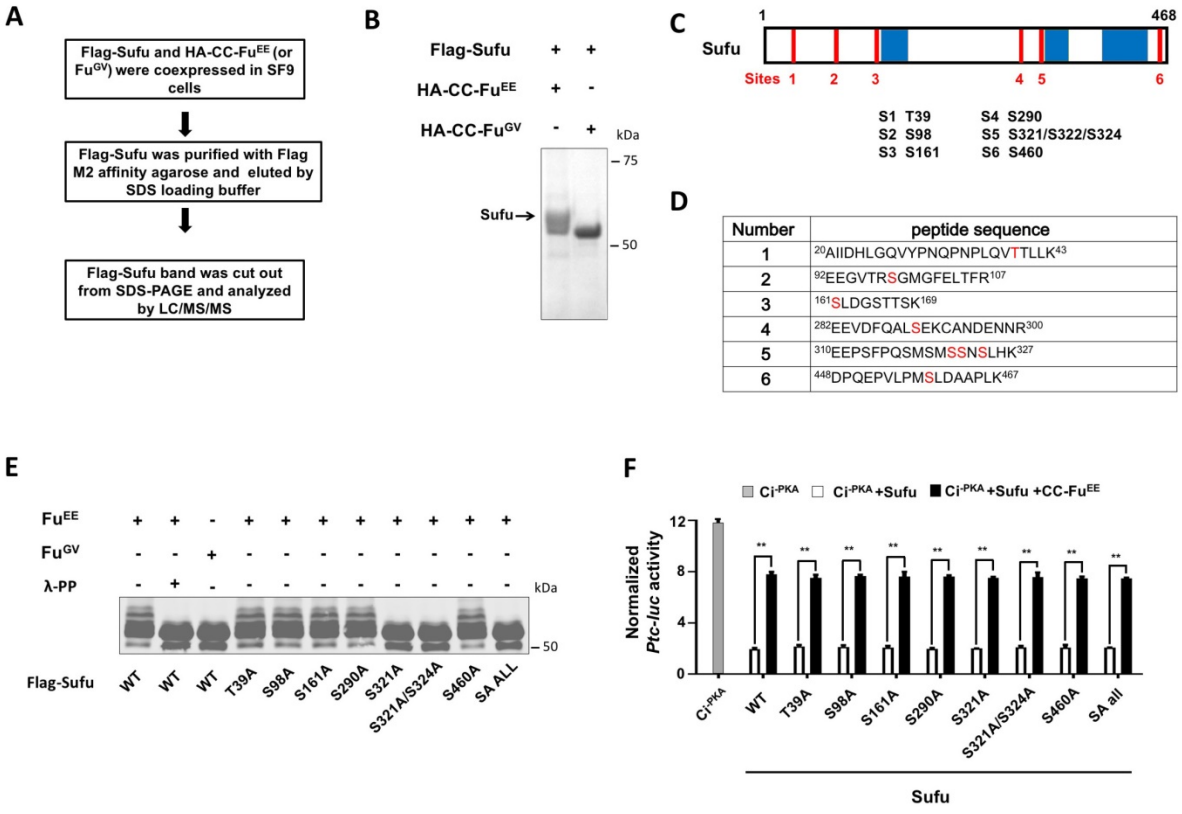

Figure S1. Mapping and characterizing Fu-mediated phosphorylation sites on Sufu. Related to Figure 1.

(A) A flow chart for expression and purification of Flag-Sufu co-expressed with CC-Fu^{EE} or CC-Fu^{GV} from Sf9 cells.

(B) Coomassie blue staining of purified Sufu protein co-expressed with CC-Fu^{EE} or CC-Fu^{GV} from Sf9 cell (p-Sufu indicates phosphorylated Sufu protein).

(C) Schematic representation of Sufu protein. Blue bars indicate the regions not covered in Mass Spec analysis. Red bars indicate the positions of potential Fu-induced Sufu phosphorylation sites (S1 to S6) identified by Mass Spec.

(D) The primary sequences of all the phosphorylated Sufu peptides yielded by trypsinization in Mass Spec analysis with the putative phosphorylation sites shown in red.

(E) Western blot analysis of wild type Sufu and indicated Sufu variants co-expressed with either Fu^{EE} or Fu^{GV} and with or without λ-protein phosphatase treatment.

(F) *ptc-luc* reporter assay in S2 cell expressing Ci^{-PKA} either alone or together with the indicated Sufu and with or without CC-Fu^{EE}. Data are mean ± SEM of normalized *ptc-luc* activity from two independent experiments (N=2). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's t test).

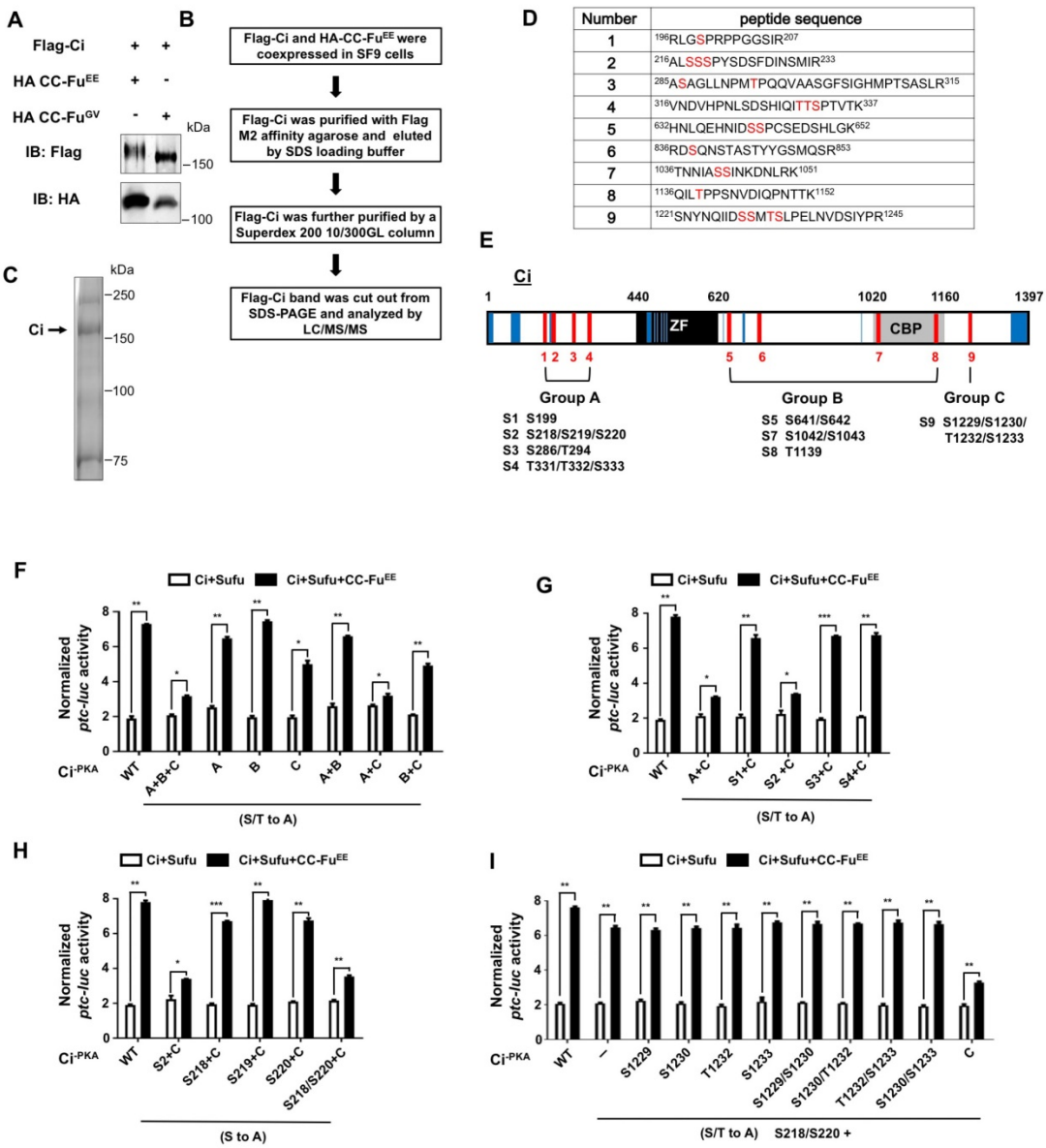

Figure S2. Mapping and characterizing Fu-mediated phosphorylation sites on Ci. Related to Figure 1.

(A) Western blot analysis of Flag-Ci protein co-expressed with CC-Fu^{EE} or CC-Fu^{GV} in Sf9 cells.

(B) A flow chart for expression and purification of Flag-Ci co-expressed with CC-Fu^{EE} from Sf9 cells.

(C) Coomassie blue staining of purified Flag-Ci protein from Sf9 cell co-expressed with CC-Fu^{EE}.

(D) The primary sequences of all the phosphorylated Ci peptides yielded by trypsinization in Mass Spec analysis with the putative phosphorylation sites shown in red.

(E) Schematic representation of Ci protein. “ZF” and “CBP” indicate Zinc-Finger DNA binding

domain and dCBP binding domain, respectively. Blue bars indicate the regions not covered by Mass Spec analysis. Red bars indicate the positions of potential Fu-induced Ci phosphorylation sites (S1 to S9) identified by the Mass Spec analysis.

(F- I) *ptc-luc* reporter assays in S2 cell expressing wild type Ci^{-PKA} or the indicated Ci^{-PKA} variants together with Sufu or with Sufu + CC-Fu^{EE}. Data are mean ± SEM of normalized *ptc-luc* activity from two independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's t test).

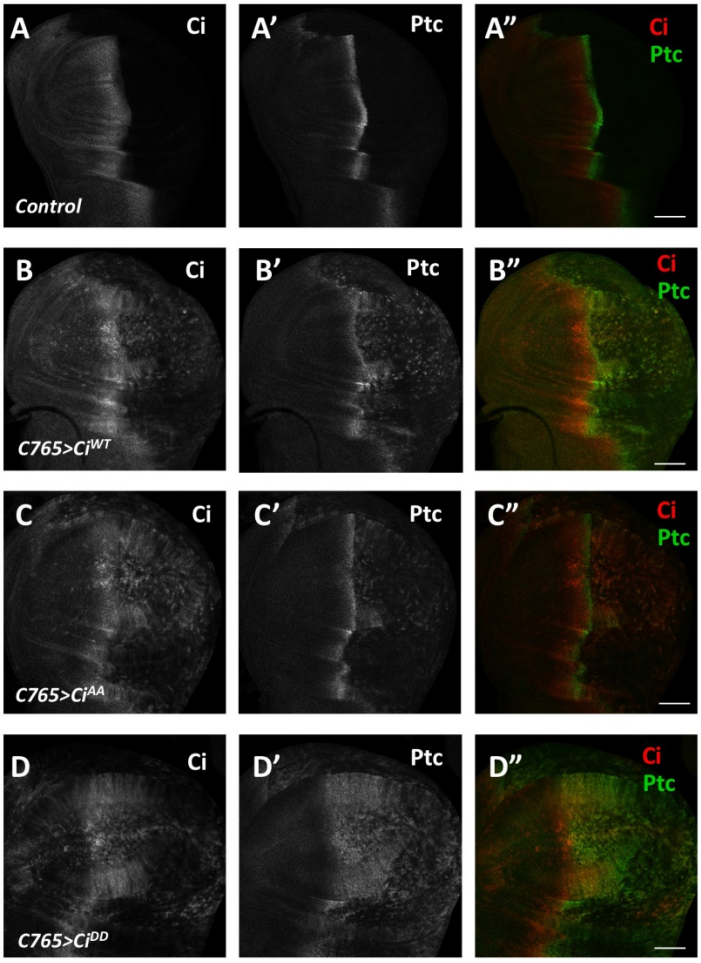

Figure S3. Phosphorylation of C-I and C-II regulates Ci activity *in vivo*. Related to Figure 2.

(A-D'") Control (A-A") wing disc or wing discs from late third instar larvae expressing *UAS-Myc-Ci^{WT}* (B-B"), *UAS-Myc-Ci^{AA}* (C-C"), or *UAS-Myc-Ci^{DD}* (D-D") under the control of *C765* Gal4 driver were immunostained to the expression of Ci (red) and Ptc (green). Larvae were grown at 25 ^{0}C. Scale bars are 50 μM.

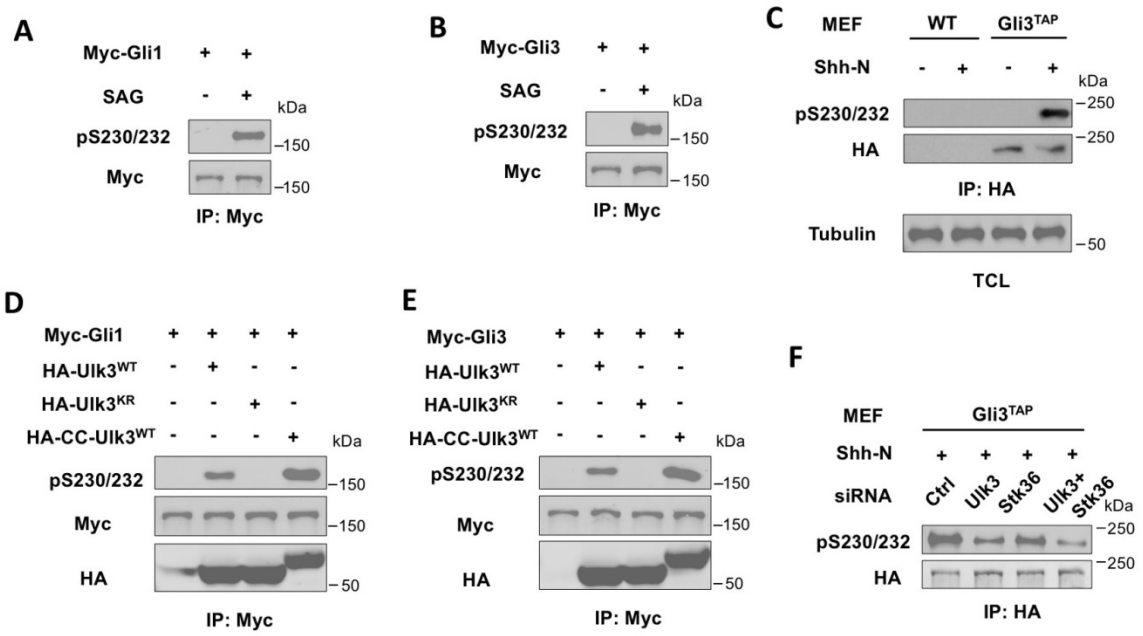

Figure S4. Shh and ULK3/STK36 regulate phosphorylation of Gli1 and Gli3 at the N-terminal conserved sites. Related to Figures 6 and 7

(A-B) Western blot analysis of phosphorylation of Myc-Gli1 (A) and Myc-Gli3 (B) expressed in NIH3T3 cells in the presence or absence of SAG using the pS230/232 phospho-specific antibody.

(C) Western blot analysis of endogenous Gli3 phosphorylation from wild type or $Gli3^{TAP}$-MEF cells in the presence or absence of Shh-N using following immunoprecipitation with HA antibody to pull down $Gli3^{TAP}$.

(D-F) Western blot analysis of phosphorylation of Myc-Gli1 (A) and Myc-Gli3 (B) expressed in NIH3T3 cells in the absence or presence of the indicated ULK3 constructs using the pS230/232 phospho-specific antibody.

(F) Western blot analysis of phosphorylation of endogenous Gli3 from $Gli3^{TAP}$-MEF cells in the presence of absence of SAG.

(F) Western blot analysis of endogenous Gli3 phosphorylation from $Gli3^{TAP}$-MEF cells treated with Shh-N and the indicated siRNAs.

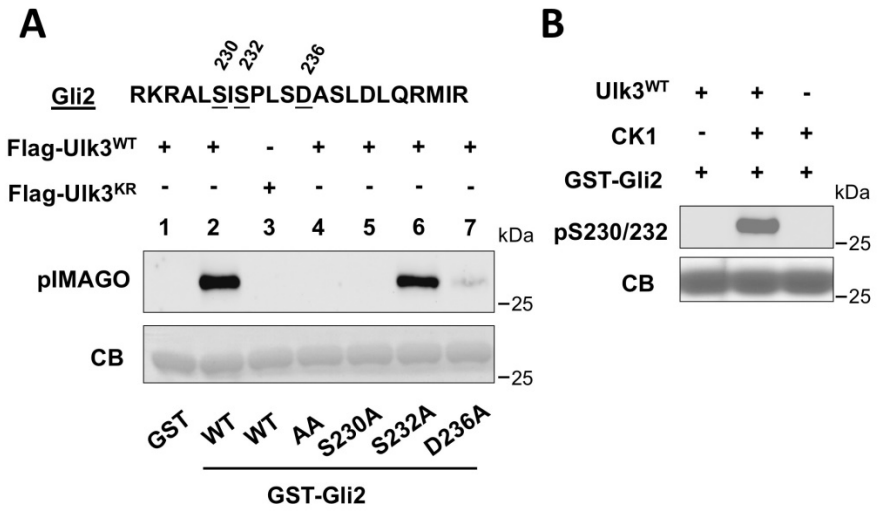

Figure S5. Phosphorylation of Gli2 on S230 by ULK3 primes CK1 phosphorylation of S232. Related to Figure 7.

(A) *In vitro* kinase assay using immunopurified Flag-ULK3^{WT}or Flag-ULK3^{KR} and the indicated GST-Gli2 fusion proteins. Amino acid sequence of the Gli2 fragment included in the GST-Gli2 fusion protein is shown on the top. Phosphorylation was detected by the pIMAGO kit. GST-fusion proteins were visualized by coomassie blue gel staining (CB).

(B) *In vitro* kinase assay using immunopurified Flag-ULK3^{WT} and/or recombinant CK1 δ as the kinases and GST-Gli2 fusion protein as the substrate. Phosphorylation was detected by western blot with the pS230/232 antibody.

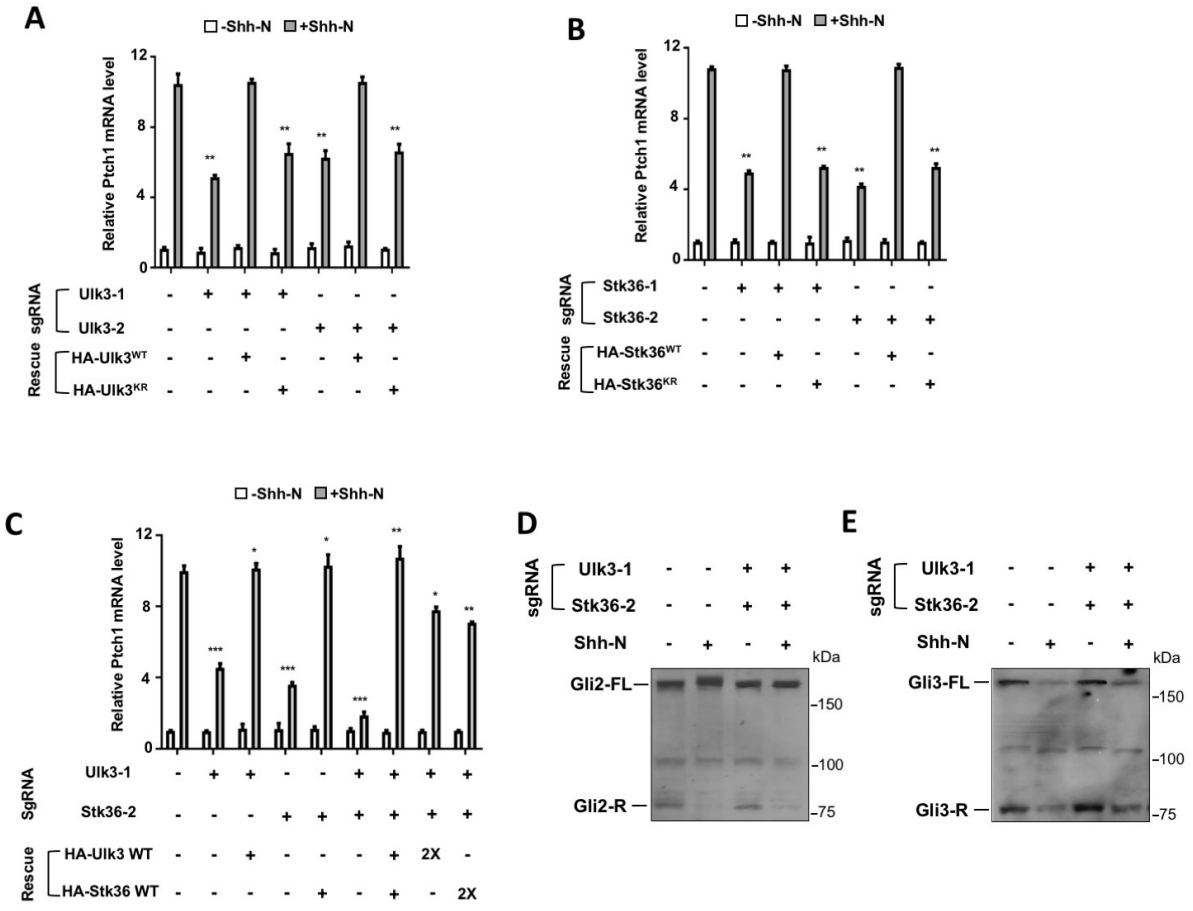

Figure S6. Shh promotes Gli2 activation through ULK3 and STK36. Related to Figure 7.

(A-C) Relative *Ptch1* mRNA levels measured by RT-qPCR in clonal NIH3T3 cells transduced with lentiviruses expressing Cas9 and the indicated sgRNAs with or without the indicated rescue constructs in the presence or absence of Shh-N. Data are mean ± SEM of normalized *ptc-luc* activity from two independent experiments. **p < 0.01 and ***p < 0.001 (Student's t test). (D-E) Western blot analysis of Gli2 and Gli3 proteolysis in clonal NIH3T3 cells transduced with lentiviruses expressing Cas9 and the indicated sgRNAs in the presence or absence of Shh-N.

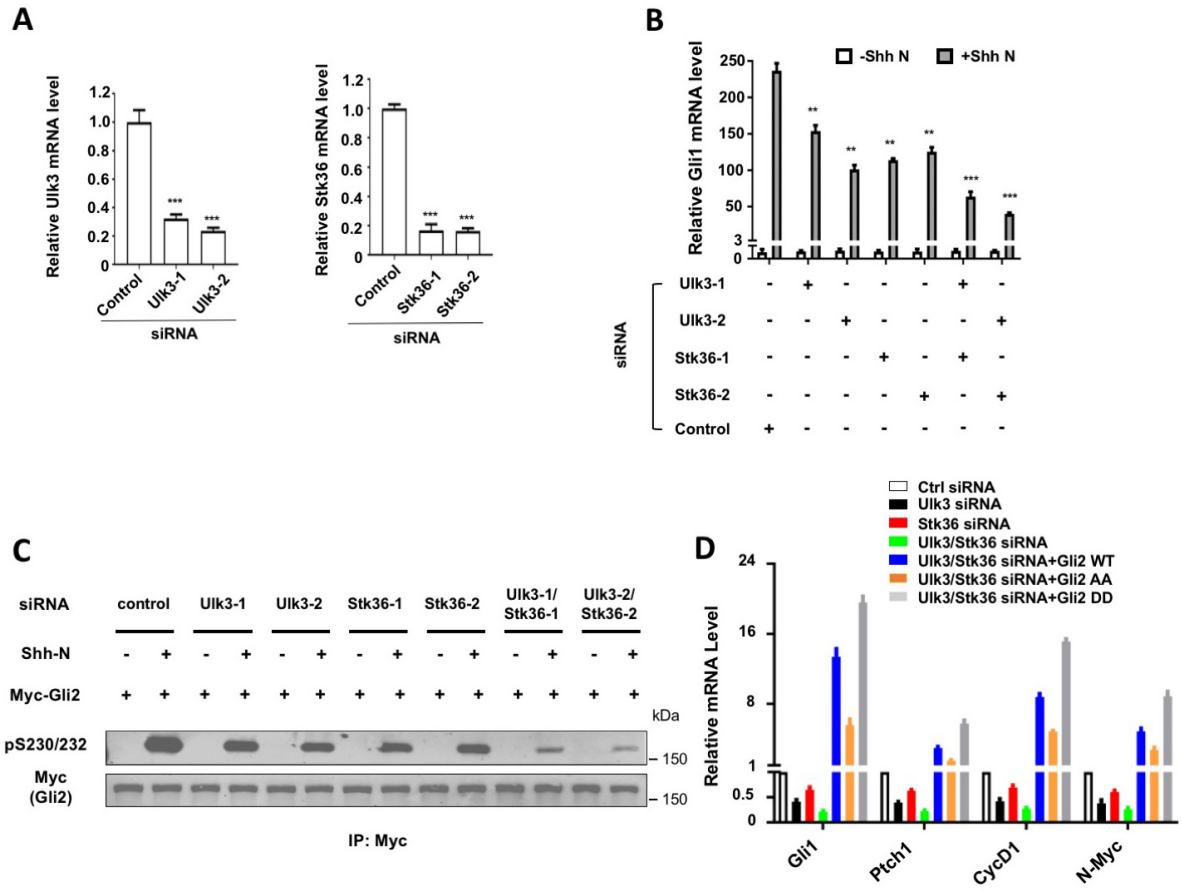

Figure S7. Knockdown of Ulk3 and Stk36 affected Shh-induced Gli2 phosphorylation and activation. Related to Figure 7.

(A) Relative Ulk3 or Stk36 mRNA levels measured by RT-qPCR of NIH3T3 cells treated with siRNAs targeting Ulk3 or Stk36. Data are mean ± SEM from two independent experiments. ***p < 0.001 (Student's t test).

(B) Relative Gli1 mRNA levels (measured by RT-qPCR) in NIH3T3 treated with the indicated siRNAs in the presence or absence of Shh-N. Data are mean ± SEM from two independent experiments. **p < 0.01 and ***p < 0.001 (Student's t test).

(C) Western blot analysis of Gli2 phosphorylation in NIH3T3 cells transduced with lentivirus expressing low level of Myc-Gli2 and treated with the indicated siRNAs in the presence or absence of Shh-N.

(D) Relative mRNA levels (measured by RT-qPCR) of the indicated Shh target genes in in vitro cultured medulloblastoma cells derived from SmoM2 mice, treated the indicated siRNAs, and transduced with or without lentiviruses expressing the indicated Myc-Gli2 constructs. Data are mean ± SEM from two independent experiments.

Supplemental table 1. List of Ci^{-PKA} variants with different combinations of phosphroylation sites mutated. Related to Figure 1 and Figure S2.

Ci^{-PKA} construct	S/T residues mutated to A in the indicated constructs
A	S199, S218/S219/S220, S286/T294, T331/T332/S333
B	S641/S642, S1042/S1043, T1139
C	S1229/S1230/T1232/S1233
A+B	S199, S218/S219/S220, S286/T294, T331/T332/S333, S641/S642, S1042/S1043, T1139
A+C	S199, S218/S219/S220, S286/T294, T331/T332/S333, S1229/S1230/T1232/S1233
B+C	S641/S642, S1042/S1043, T1139, S1229/S1230/T1232/S1233
A+B+C	S199, S218/S219/S220, S286/T294, T331/T332/S333, S641/S642, S1042/S1043, T1139, S1229/S1230/T1232/S1233
S1+C	S199, S1229/S1230/T1232/S1233
S2+C	S218/S219/S220, S1229/S1230/T1232/S1233
S3+C	S286/T294, S1229/S1230/T1232/S1233
S4+C	T331/T332/S333, S1229/S1230/T1232/S1233
AA	S218/S220, S1229/S1230/T1232/S1233
SA-I	S218/S220
SA-II	S1229/S1230/T1232/S1233
Ci^{-PKA} construct	**S/T residues mutated to D/E in the indicated constructs**
DD	S218/S220, S1229/S1230/T1232/S1233
SD-I	S218/S220
SD-II	S1229/S1230/T1232/S1233

Supplemental table 2. List of oligonucleotides used in this sutdy

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Smo dsRNA DNA Template Primer (F) GAATTAATACGACTCACTATAGGGAGAGTCGCACA TTTGTTGCTTCAG	This Paper	N/A
Smo dsRNA DNA Template Primer (R) GAATTAATACGACTCACTATAGGGAGACCGCTTAT AAAAATCATTAAA	This Paper	N/A
Fu dsRNA DNA Template Primer (F) GAATTAATACGACTCACTATAGGGAGATGTTGAGC ATCTTGAGACC	This Paper	N/A
Fu dsRNA DNA Template Primer (R) GAATTAATACGACTCACTATAGGGAGAGACCCAAG CGAACAGAGAAG	This Paper	N/A
Mouse Ulk3 siRNA GUGUACAAGGCCUACGCCA [dT][dT]	Millipore Sigma	Cat# SASI_Mm02_0 0444409
Mouse Ulk3 siRNA CUGAGAAGGUGGCCCGUGU[dT][dT]	Millipore Sigma	Cat# SASI_Mm02_0 0444410
Mouse Stk36 siRNA GGUAUACUGGCUUCAGAAA[dT][dT]	Millipore Sigma	Cat# SASI_Mm02_0 0345637
Mouse Stk36 siRNA GCCUUAUGUGCUUUGCUGU[dT][dT]	Millipore Sigma	Cat# SASI_Mm01_0 0167751
Mouse Ulk3 sgRNA GCGGGATGGCTGGGCCCAGC	This Paper	N/A
Mouse Ulk3 sgRNA GGTGTACAAGGCCTACGCCA	This Paper	N/A
Mouse Stk36 sgRNA GGCGCTCAGAGAAAGAGCTG	This Paper	N/A
Mouse Stk36 sgRNA GTCGAGCATATGCACAATGT	This Paper	N/A
Mouse Gli2 shRNA GTACCGGTATGTTTACCCGCTCCTATTTCTCGAGA AATAGGAGCGGGTAAACATATTTTTTG	Millipore Sigma	Cat# TRCN0000219 066
Mouse Kapβ2 shRNA CCGGAGATTCAGTGGGACATCATTTCTCGAGAAA TGATGTCCCACTGAATCTTTTTTG	Millipore Sigma	Cat# TRCN0000295 586
Mouse GAPDH RT-PCR primer GTGGTGAAGCAGGCATCTGA	Millipore Sigma	N/A
Mouse GAPDH RT-PCR primer GCCATGTAGGCCATGAGGTC	Millipore Sigma	N/A
Mouse Gli1 RT-PCR primer GTGCACGTTTGAAGGCTGTC	Millipore Sigma	N/A
Mouse Gli1 RT-PCR primer GAGTGGGTCCGATTCTGGTG	Millipore Sigma	N/A

Mouse Stk36 RT-PCR primer CGCATCCTACACCGAGATATGA	Millipore Sigma	N/A
Mouse Stk36 RT-PCR primer AAATCCAAAGTCACAGAGCTTGA	Millipore Sigma	N/A
Mouse Ulk3 RT-PCR primer ACGAAACATCTCTCACTTG	Millipore Sigma	N/A
Mouse Ulk3 RT-PCR primer TGCTGGGCAAAGCCAAAGTC	Millipore Sigma	N/A
Mouse Patched1 RT-PCR primer GAAGCCACAGAAAACCCTGTC	Millipore Sigma	N/A
Mouse Patched1 RT-PCR primer GCCGCAAGCCTTCTCTAGG	Millipore Sigma	N/A
Mouse CyclinD1 RT-PCR primer AGACCTGTGCGCCCTCCGTA	Millipore Sigma	N/A
Mouse CyclinD1 RT-PCR primer CAGCTGCAGGCGGCTCTTCT	Millipore Sigma	N/A
Mouse N-Myc RT-PCR primer GTCTTCCCCTTCCCGGTGAAC	Millipore Sigma	N/A
Mouse N-Myc RT-PCR primer CAAGGTATCCTCTCCGGAGGTGC	Millipore Sigma	N/A

Supplemental table 3. List of recomibnant DNA/Plasmids used in this sutdy

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pUAST 6XMyc Ci$^{-PKA_WT/AA/DD/SD-I/SD-II}$	This Paper	N/A
pUAST 6XMyc Ci$^{WT/AA/DD}$	This Paper	N/A
pUAST 3XFlag Ci^{-PKA}	This Paper	N/A
pUAST CFP Ci$^{WT/-PKA}$	This Paper	N/A
pUAST Flag Sufu	This Paper	N/A
pUAST HA Sufu	This Paper	N/A
pUAST Sufu YFP	This Paper	N/A
pUAST HA Fu$^{WT/EE/GV/KR}$	Shi et al., 2011	N/A
pUAST HA CC-Fu$^{WT/EE}$	Shi et al., 2011	N/A
pUAST Flag MATH	Zhang et al., 2006	N/A
pUAST Myc Smo	This Paper	N/A
pUAST HA Fu$^{INS\ WT/KR}$	This Paper	N/A
pGEX 4T-1	GE Healthcare	Cat#28954549
pFastBac1	Thermo Fisher Scientific	Cat#10359016
pcDNA3.1(+) 3XHA mUlk3 $^{WT/KR}$	This Paper	N/A
pcDNA3.1(+) 3XHA CC-mUlk3	This Paper	N/A
pcDNA3.1(+) 6XMyc mGli2$^{WT/AA}$	This Paper	N/A
pcDNA3.1(+) 6XMyc mGli1	This Paper	N/A
pcNDA3.1(+) 6XMyc mGli3	This Paper	N/A
FUXW 6XMyc mGli2$^{WT/AA/DD}$	This Paper	N/A
FUXW 3XHA mUlk3$^{WT/KR}$	This Paper	N/A
FUXW 3XHA mStk36$^{WT/KR}$	This Paper	N/A
psPAX2	Didier Trono Lab	Addgene Plasmid #12260
pMD2.G	Didier Trono Lab	Addgene Plasmid #12259
LentiCas9-Blast	Sanjana et al., 2014	Addgene Plasmid #52962
Lentiguide-Puro	Sanjana et al., 2014	Addgene Plasmid #52963
pGL-2 *ptc*-Luc	Chen et al., 1999	N/A
Renilla Luciferase Pol III	Nybakken et al., 2005	N/A
pδ51LucII 8XGli	Sasaki et al., 1997	N/A
pRL SV40 Renilla	Promega	Cat#E2231