

Figure S1

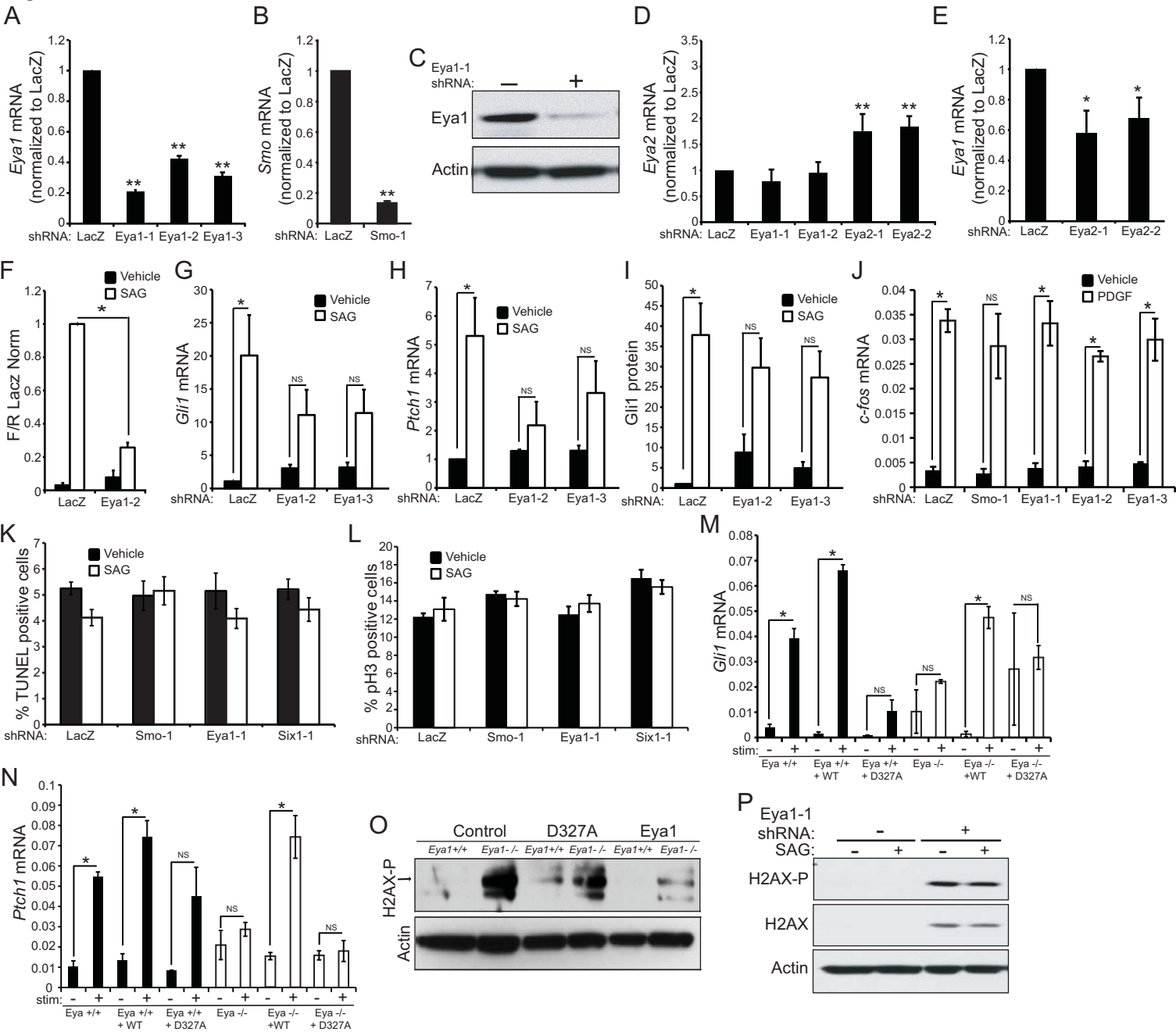


Figure S2

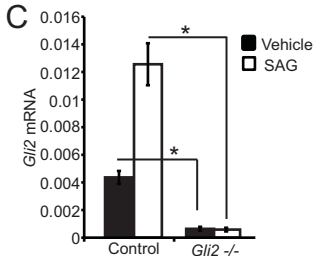
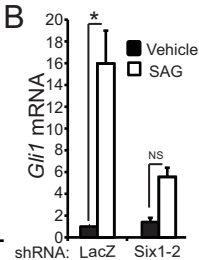
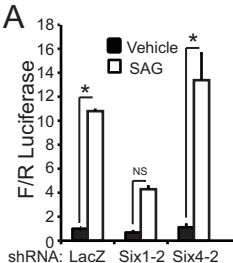


Figure S3

A



B



Figure S4

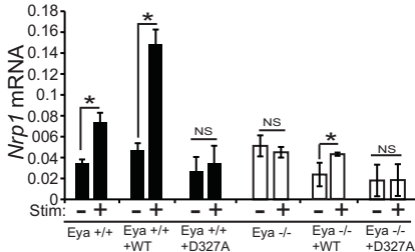


Figure S5

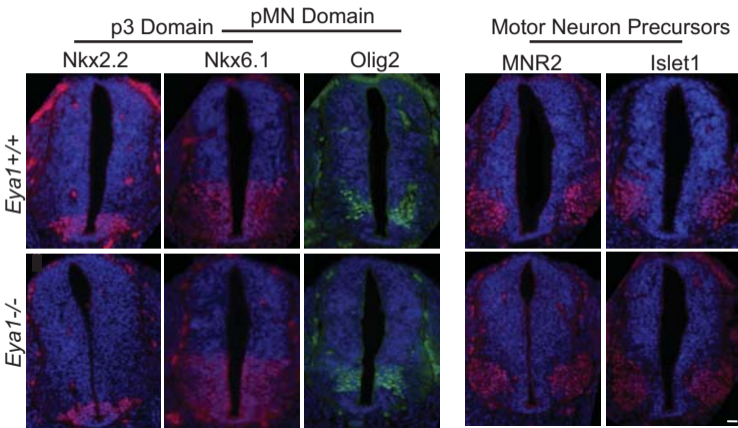


Table 3: Screen hits previously identified as Hh regulators

Symbol	Gene Name	RefSeq	NCBI Gene ID	Re-Screen Hit	Previously Identified
Acp1	acid phosphatase 1, soluble	NM_021330	11431	Acp1	Hilman et al., 2011
Dusp13	dual specificity phosphatase 13	NM_013849	27389		Hilman et al., 2011
Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_019411	19052	Ppp2ca	Nybakken et al., 2005
Ppp2r3a	protein phosphatase 2, regulatory subunit B", alpha	XM_135153	235542		Nybakken et al., 2005
Ppp2r5a	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	NM_144880	226849		Nybakken et al., 2005
Ppp2r5b	protein phosphatase 2, regulatory subunit B (B56), beta isoform	NM_198168	225849	Ppp2r5b	Nybakken et al., 2005
Pten	phosphatase and tensin homolog	NM_008960	19211		Hilman et al., 2011
Ptp4a3	protein tyrosine phosphatase 4a3	NM_008975	19245		Hilman et al., 2011
Ptpn2	protein tyrosine phosphatase, receptor type, N polypeptide 2	NM_011215	19276		Hilman et al., 2011

Table 4: Primary screen hits rescreened

Symbol	Gene Name	RefSeq	NCBI Gene ID	Re-Screen Hit
Acp1	acid phosphatase 1, soluble	NM_021330	11431	Acp1
Acp12	acid phosphatase-like 2	NM_153420	235534	
Akp-ps1	alkaline phosphatase pseudogene 1	XM_136795	208256	
Alpi	alkaline phosphatase, intestinal	XM_129951	76768	Alpi
Alpl	alkaline phosphatase, liver/bone/kidney	NM_007431	11647	
Arid1a	AT rich interactive domain 1A	NM_033566	93760	Arid1a
Arpp21	cyclic AMP-regulated phosphoprotein, 21	NM_033264	74100	
<i>Atp6v0e</i>	ATPase, H ⁺ transporting, lysosomal V0 subunit E	NM_025272	11974	<i>Atp6v0e</i>
BC005764	cDNA sequence aka Prg2; Lppr3; KIAA4076	NM_181681	216152	
Cant1	calcium activated nucleotidase 1	NM_029502	76025	Cant1
<i>Dupd1</i>	dual specificity phosphatase and pro isomerase domain containing 1	XM_487320	435391	<i>Dupd1</i>
Dusp11	dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	NM_028099	72102	
Dusp19	dual specificity phosphatase 19	NM_024438	68082	
Dusp21	dual specificity phosphatase 21	XM_135794	73547	
Dusp26	dual specificity phosphatase 26 (putative)	NM_025869	66959	Dusp26
Dusp28	dual specificity phosphatase 28	NM_175118	67446	Dusp28
Dusp4	dual specificity phosphatase 4	NM_176933	319520	
Ebf2	early B-cell factor 2	NM_010095	13592	
<i>Enoph1</i>	enolase-phosphatase 1	NM_026421	67870	
Entpd4	ectonucleoside triphosphate diphosphohydrolase 4	NM_02617	67464	
Epb4.1l4a	erythrocyte protein band 4.1-like 4a	NM_013512	13824	
Eya1	eyes absent 1	NM_010164	14048	Eya1
Eya2	eyes absent 2	NM_010165	14049	Eya2
Fam48a	family with sequence similarity 48, member A	NM_019995	56790	
Fbp1	fructose biphosphatase 1	NM_019395	14121	
G6pc2	glucose-6-phosphatase, catalytic, 2	NM_021331	14378	
Gfi1b	growth factor independent 1B	NM_008114	14582	<i>Gfi1b</i>
Gm5601	Gm5601 predicted pseudogene 5601	XM_485994	434233	
Impa1	inositol (myo)-1(or 4)-monophosphatase 1	NM_018864	55980	<i>Impa1</i>
Impa2	inositol (myo)-1(or 4)-monophosphatase 2	NM_053261	114663	
Inpp5d	inositol polyphosphate-5-phosphatase D	NM_010566	16331	<i>Inpp5d</i>
LOC381574	LOC381574 similar to Protein phosphatase 2, regulatory subunit B (B56), alpha isoform NOTE THIS RECORD WAS DISCONTINUED	XM_485481	381574	
Mtm1	X-linked myotubular myopathy gene 1	NM_019926	17772	
Mtmr4	myotubularin related protein 4	NM_133215	170749	
<i>Mtmr6</i>	myotubularin related protein 6	NM_144843	219135	
Nudt6	nudix (nucleoside diphosphate linked moiety X)-type motif 6	NM_153561	229228	Nudt6
<i>Olf1199</i>	olfactory receptor 1199	NM_146458	258450	
Olf140	olfactory receptor 140	NM_020515	57272	
Olf1506	olfactory receptor 1506	NM_146265	257665	Olf1506
Phlpp1	PH domain and leucine rich repeat protein phosphatase 1	XM_129968	98432	
Phlpp2	PH domain and leucine rich repeat protein phosphatase 2	XM_146511	244650	
Pou2f2	POU domain, class 2, transcription factor 2	NM_011138	18987	
Ppapdc1a	phosphatidic acid phosphatase type 2 domain containing 1A	XM_355946	381925	
Ppm1k	protein phosphatase 1K (PP2C domain containing)	NM_175523	243382	

Ppp1cb	protein phosphatase 1, catalytic subunit, beta isoform	NM_172707	19046	Ppp1cb
<i>Ppp2ca</i>	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	NM_019411	19052	<i>Ppp2ca</i>
Ppp2r3a	protein phosphatase 2, regulatory subunit B", alpha	XM_135153	235542	
Ppp2r5a	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	NM_144880	226849	
<i>Ppp2r5b</i>	protein phosphatase 2, regulatory subunit B (B56), beta isoform	NM_198168	225849	<i>Ppp2r5b</i>
Ptp4a2	protein tyrosine phosphatase 4a2	NM_008974	19244	
Ptp4a3	protein tyrosine phosphatase 4a3	NM_008975	19245	
Ptpn22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	NM_008979	19260	Ptpn22
Ptprg	protein tyrosine phosphatase, receptor type, G	NM_008981	19270	Ptprg
Ptprq	protein tyrosine phosphatase, receptor type, Q	XM_137234	237523	
<i>Ptpru</i>	protein tyrosine phosphatase, receptor type, U	NM_011214	19273	<i>Ptpru</i>
<i>Ptprv</i>	protein tyrosine phosphatase, receptor type, V	NM_007955	13924	<i>Ptprv</i>
R3hdm2	R3H domain containing 2	NM_027900	71750	R3hdm2

Supplemental Figure Legends

Figure S1 (related to Figure 1) - shRNA screen for regulators of Shh signaling.

(A) shRNAs targeting *Eya1* (*Eya1*-1, *Eya1*-2, *Eya1*-3) successfully knock-down *Eya1* (N=9-10, **P<0.01, Error bars = SEM). (B) An shRNA targeting *Smo* successfully knocks down *Smo* (N=5, **P<0.01, Error bars = SEM). (C) shRNA targeting *Eya1* (*Eya1*-1) reduces levels of *Eya1* when co-transfected with an *Eya1* encoding plasmid in 293T cells. (D) shRNAs targeting *Eya1* or *Eya2* do not knock-down *Eya2* mRNA (N=3-5, **P<0.01, Error bars = SEM). (E) shRNAs targeting *Eya2* (*Eya2*-1, *Eya2*-2) instead reduce *Eya1* mRNA levels (N=3, *P<0.05, Error bars = SEM). (F) An shRNA targeting *Eya1* (*Eya1*-2) blocks SAG-mediated induction of Firefly/*Renilla* luciferase (N=3, *P<0.05, Error bars = SEM). shRNAs targeting *Eya1* (*Eya1*-2, *Eya1*-3) block induction of both *Gli1* (G) and *Ptch1* (H) mRNA (N=5, *P<0.05, Error bars = SEM, NS= not significant, P>0.05). (I) shRNAs targeting *Eya1* (*Eya1*-2, *Eya1*-3) decrease SAG-induction of Gli1 protein (N=4, *P<0.05, Error bars = SEM, NS= not significant, P>0.05). (J) *c-fos* gene expression is induced by PDGF stimulation. shRNAs targeting *Eya1* do not block induction of *c-fos* mRNA (N=3, *P<0.05, Error bars = SEM, NS = not significant, P>0.05). (K) shRNAs to *Smo*, *Eya1* or *Six1* do not affect apoptosis (TUNEL-positive cells) in ShhLightII cells. (L) shRNAs to *Smo*, *Eya1* or *Six1* do not affect proliferation (pH3-positive cells) in ShhLightII cells. (M) *Gli1* mRNA or (N) *Ptch1* mRNA in control or SAG-stimulated *Eya1*^{+/+} and *Eya1*^{-/-} MEFs were measured, following reintroduction of wild type or phosphatase dead (D327A) *Eya1* (N=3, *P<0.05, Error bars = SEM, NS = not significant, P>0.05). (O) Western blot of phosphorylated H2AX in *Eya1*^{+/+} and *Eya1*^{-/-} MEFs. Actin = loading control. (P) *Eya1* knock-down increases both phosphorylated H2AX and total H2AX protein in ShhLightII cell lysate as compared to cells treated with control shRNA targeting *RFP*. H2AX dephosphorylation and total protein levels (Actin) are not modulated by SAG stimulation.

Figure S2 (related to Figure 2) - Six1 promotes Shh pathway activation. (A) An shRNA targeting *Six1* (*Six1-2*), but not *Six4* (*Six4-2*), blocks induction of Firefly/*Renilla* luciferase; no puromycin selection in these experiments, (N=3, *P<0.05, Error bars = SEM, NS = not significant, P>0.05). (B) An shRNA targeting *Six1* blocks induction of *Gli1* mRNA (N=3, *P<0.05, Error bars = SEM, NS = not significant, P>0.05). (C) *Gli2*^{-/-} MEFs show an absence of *Gli2* mRNA (N=3, *P<0.05, Error bars = SEM).

Figure S3 (related to Figure 3) - (A) ShhLightII cells expressing shRNA targeting *Sufu* (*Sufu* Knock-down cells) display decreased *Sufu* protein levels (B) ShhLightII transfected with a transposon expressing *Gli2-V5* (*Gli2*-ShhLightII cells) show *Gli2-V5* expression.

Figure S4 (related to Figure 4) - *Nrp1* mRNA induction in *Eya1*^{+/+} and *Eya1*^{-/-} MEFs expressing WT and phosphatase dead (D327A) (N=3, *P<0.05, Error bars = SEM, NS = not significant, P>0.05). Phosphatase activity is needed for *Nrp1* induction.

Figure S5. Ventral cell types of the neural tube appear properly patterned in *Eya1*^{-/-} mice at E10.5. Ventral spinal cord progenitor domains, pMN and p3, generate somatic motor neurons and V3 interneurons respectively. Cells in the p3 region are *Nkx2.2*- and *Nkx6.1*-positive (both shown in red). Cells in the pMN region are *Nkx6.1*- and *Olig2*-positive (*Olig2* shown in green). Motor neuron identity is specified in *MNR2*-positive domain and motor neuron precursors are *Islet1*-positive (both shown in red). No changes were observed in *Eya1*^{-/-} neural tubes. Scale bar = 50 μ m.

Table S1 (related to Figure 1) - Phosphatome Screen-Shh Signaling. This table lists the individual genes tested in primary screen for phosphatases involved in Shh signaling. Gene symbol, gene name and identifier and the target sequence are shown. Four or five target sequences were used for each gene.

Table S2 (related to Figure 1) - Primary Screen. Individual genes identified as positive hits in an shRNA screen for phosphatases that affect Shh signaling in ShhLightII cells as assessed by Gli promoter-luciferase expression.

Table S3 (related to Figure 1) – Genes Previously Identified in Shh Signaling. Genes identified in the primary screen previously identified as impinging on Shh/Hh signaling. References are provided.

Table S4 (related to Figure 1) - Rescreen Hits. Genes identified in the primary screen that had not been previously identified as impinging on Shh signaling were rescreened. Only those genes where two or more of the target sequences tested altered Shh responses by more than 4-fold were scored as hits in this more rigorous analysis.

Supplemental Experimental Procedures

Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted using Trizol (Invitrogen) according to manufacturer's protocol. Reverse Transcription was performed using the cDNA archive kit (Applied Biosystems) according to the manufacturer's specifications. Quantitative real-time RT-PCR was performed using Taqman gene expression assays to assess the expression of: *Eya1* Mm00438796_m1, *Eya2* Mm00802561_m1, *Smo* Mm01162710_m1, *Gli1* Mm00494645_m1, *Ptch1* Mm00436026_m1, *Gli2* Mm01293117_m1, *c-fos* Mm00487425_m1, *Nrp1* Mm00435371_m1, *Nrp2* Mm00803099_m1. Values were normalized to *gapdh* levels.

Western Blotting

Cells were lysed in modified RIPA buffer and protein lysates were separated by 4-12% SDS-PAGE and blotted with primary antibodies. Bands were visualized with secondary antibodies conjugated to HRP (1:10,000: Bio-Rad) and SuperSignal chemiluminescent substrate kit. For western blot quantification, film was scanned using Epson perfection V750 pro scanner and Epson scan software. Background-subtracted band density was measured in ImageJ, and normalized to actin as a loading control.

Antibodies: Actin (Cell Signaling #4968), H2AX (Cell Signaling #2595, #5438, #2577), Sufu (Cell Signaling #2520S), Gli1 (Cell Signaling #2534), V5 (Invitrogen, #R960), Olig2, Islet1 (Developmental Studies Hybridoma Bank #40.2D6-c), MNR2 (Developmental Studies Hybridoma Bank #81.5C10) and NKx2.2 (Developmental Studies Hybridoma Bank #74.5A5) and Nkx6.1 (Developmental Studies Hybridoma Bank #F55A10).

Statistics

Within each experiment, mRNA or protein values for each condition were normalized to an internal standard (*gapdh* or actin). To average results across multiple independent experiments, values were normalized to the results obtained with a control virus in that experiment (LacZ). Statistical significance was determined using a z-test with Bonferroni correction for multiple comparisons, or by Student's t-test with Bonferroni correction in Microsoft Office

Excel, or by ANOVA with Tukey's post-test analysis in Prism as indicated. All experiments were done with at least three independent biological replicates.