Supporting Information

Belgacem and Borodinsky 10.1073/pnas.1419690112

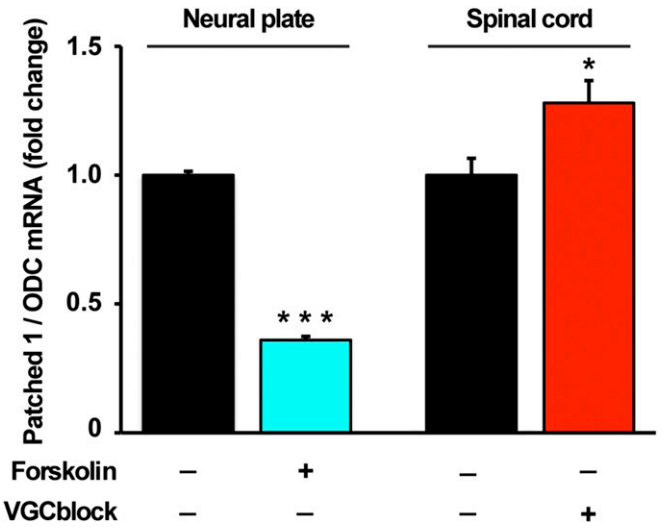

Fig. S1. Patched 1 expression is inhibited by PKA stimulation and electrical activity. Relative Patched 1 transcript levels from neural plates or spinal cords were quantified using qRT-PCR after 8 h incubation with 20 μM forskolin, voltage-gated Na^+ and Ca^{2+} channel blockers (VGCblock), or vehicle only (control). Primers specific for Patched 1 were used, and ODC gene transcript was used as normalizer. Graph shows mean ± SEM fold change of normalized transcript levels compared with control at each developmental stage; $n = 6$; $^*P < 0.05$ and $^{***}P < 0.00001$.

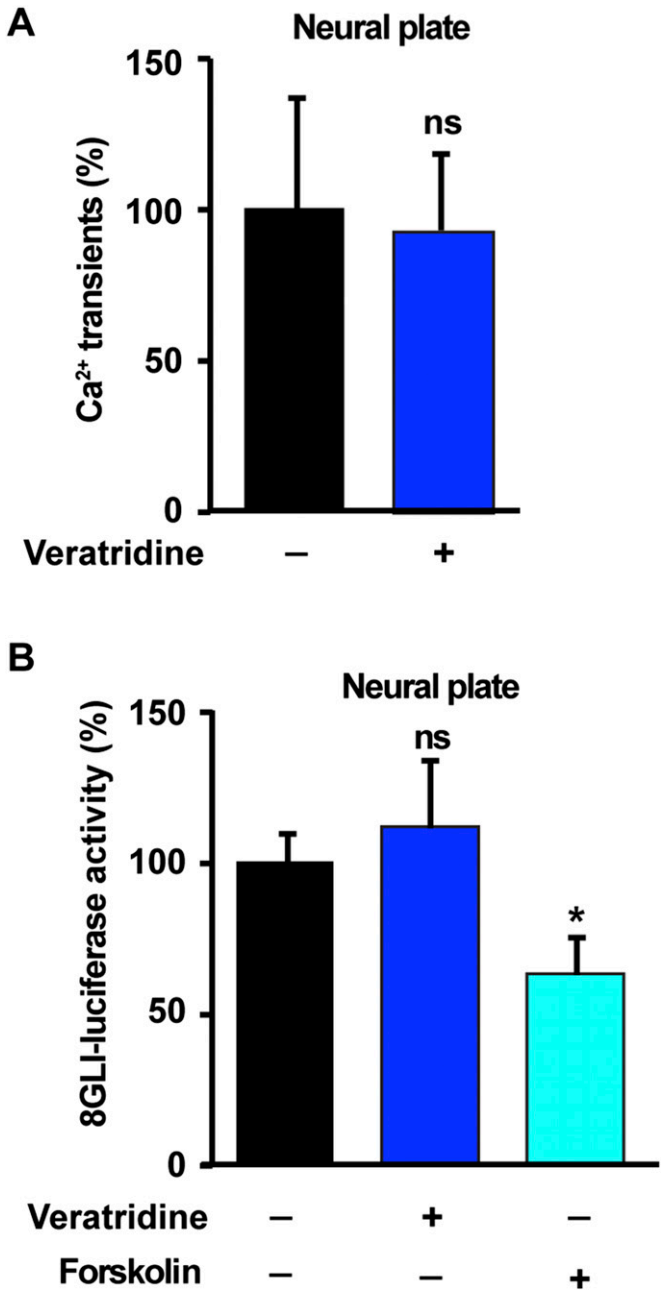

Fig. S2. Stimulation of voltage-gated Na^+ channels does not affect Ca^{2+} transients or Gli activity in the neural plate. (*A*) Two-cell-stage embryos were injected with the genetically encoded Ca^{2+} sensor, GCaMP6s, and time-lapse imaged at neural plate stages (stage 14, 16.25 hpf) for 30 min at 0.2 Hz in the absence or presence of 1 μM veratridine, voltage-gated Na^+ channel agonist. Graph shows mean ± SEM percentage of neural plate cells exhibiting Ca^{2+} transients in the presence or absence of veratridine; $n = 5$. (*B*) Neural plates from embryos expressing 8GLI-luciferase were incubated for 8 h with the indicated agents and processed for luciferase activity measurements. Graph shows mean ± SEM percentage of normalized luciferase intensity compared with control (incubated with vehicle only); $n = 5$; $^*P < 0.05$; ns: not significant.

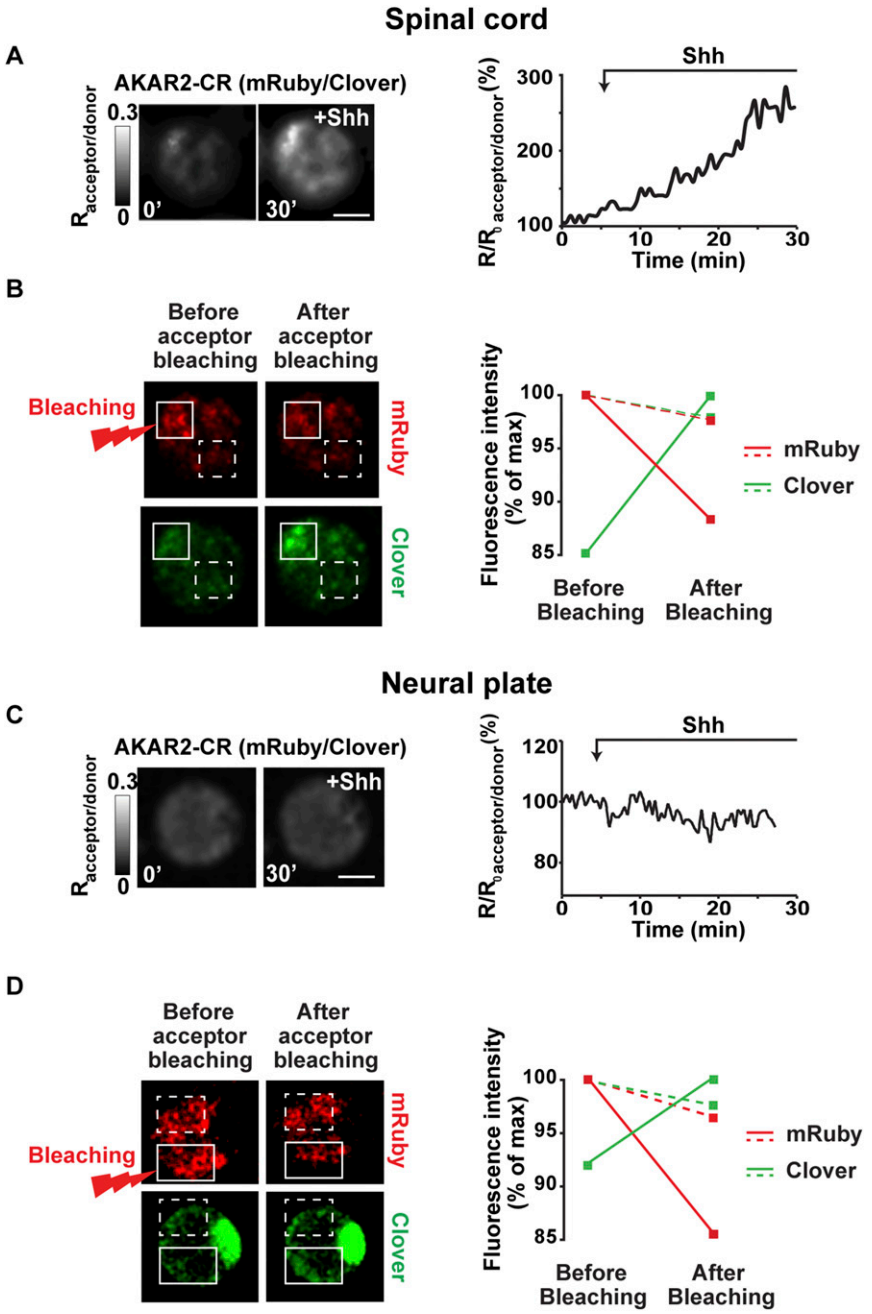

Fig. S3. FRET specificity of PKA reporter. (*A* and *C*) Dissociated spinal cord (*A*) or neural plate (*C*) cells from AKAR2-CR–expressing 21- or 14.75-hpf embryos, respectively, were time-lapse imaged every 30 s. Samples are illuminated with 488-nm laser and mRuby and Clover emitted fluorescence captured with spectral detector. (*Left*) Representative ratiometric (acceptor-mRuby/donor-Clover) images of cell before and 30 min after addition of 10 nM Shh. Grayscale bar represents acceptor/donor ratio increasing from black to white. (*Right*) Data are percentage change in emission ratio for cell on the *Left*. (Scale bar: 20 μm.) (*B* and *D*) FRET specificity is assessed by quantifying acceptor and donor emission fluorescence using a 488-nm laser before and after acceptor (mRuby) photobleaching. Shown is same cell as in *A* and *C* subjected to the positive control for FRET specificity before (*Left*) and after (*Right*) acceptor photobleaching using a 580-nm laser in indicated region of interest (ROI; solid line). Negative control is a random nonbleached ROI (dashed line). Graph represents average fluorescence intensity in indicated ROIs from mRuby (red lines) and Clover (green lines) emissions. Donor signal is increased only when acceptor is photobleached (compare solid and dashed lines), demonstrating FRET between Clover and mRuby. This protocol has been repeated in at least three cells in each imaged field. (Scale bar: 10 μm.)

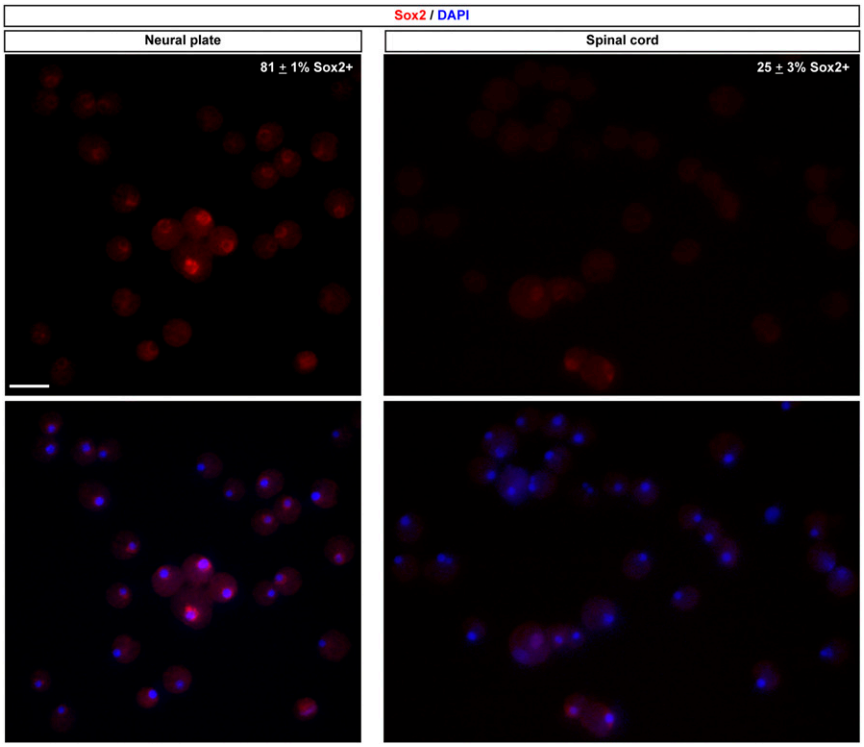

Fig. S4. Neural progenitors are predominant in neural-plate–derived cultures. Dissociated neural plate (stage 13 + 2 h in vitro) or spinal cord (stage 19 + 4 h in vitro) were processed for Sox2 immunostaining (red) and nuclear labeling (DAPI, blue). Numbers are mean ± SEM percentage of Sox2 immunopositive cells in the field of view; $n = 5$ independent experiments per stage corresponding to $n \geq 900$ cells per condition. (Scale bar: 20 μm.)

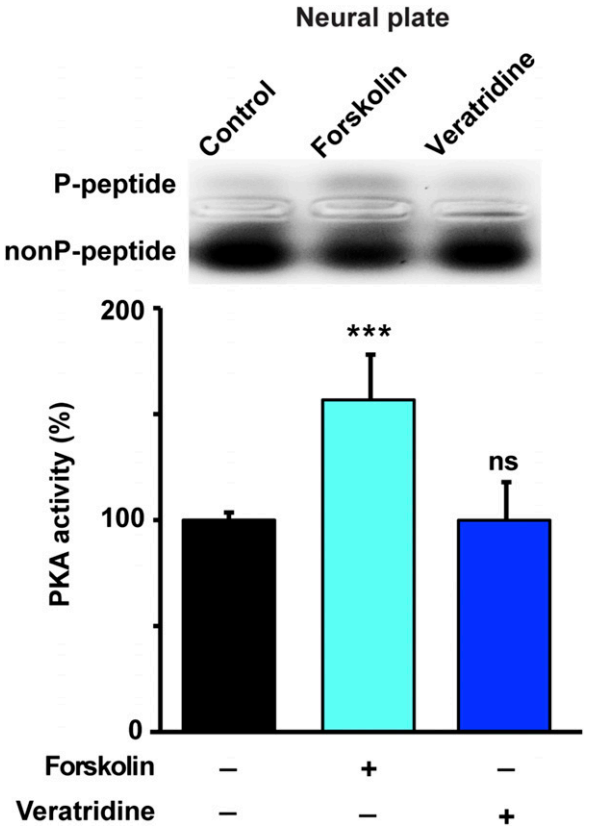

Fig. S5. Stimulation of voltage-gated Na^+ channels does not affect PKA activity in the neural plate. Neural plates were incubated for 30 min with indicated agents and processed for PKA activity measurements with a nonradioactive PKA assay. Image is a representative example of PKA activity assay. Graph shows mean ± SEM PKA activity (P-substrate/non–P-substrate optical density ratio) for the indicated treatments; $n \geq 5$, ns: not significant; ***$P < 0.0005$ compared with control (incubated with vehicle only).

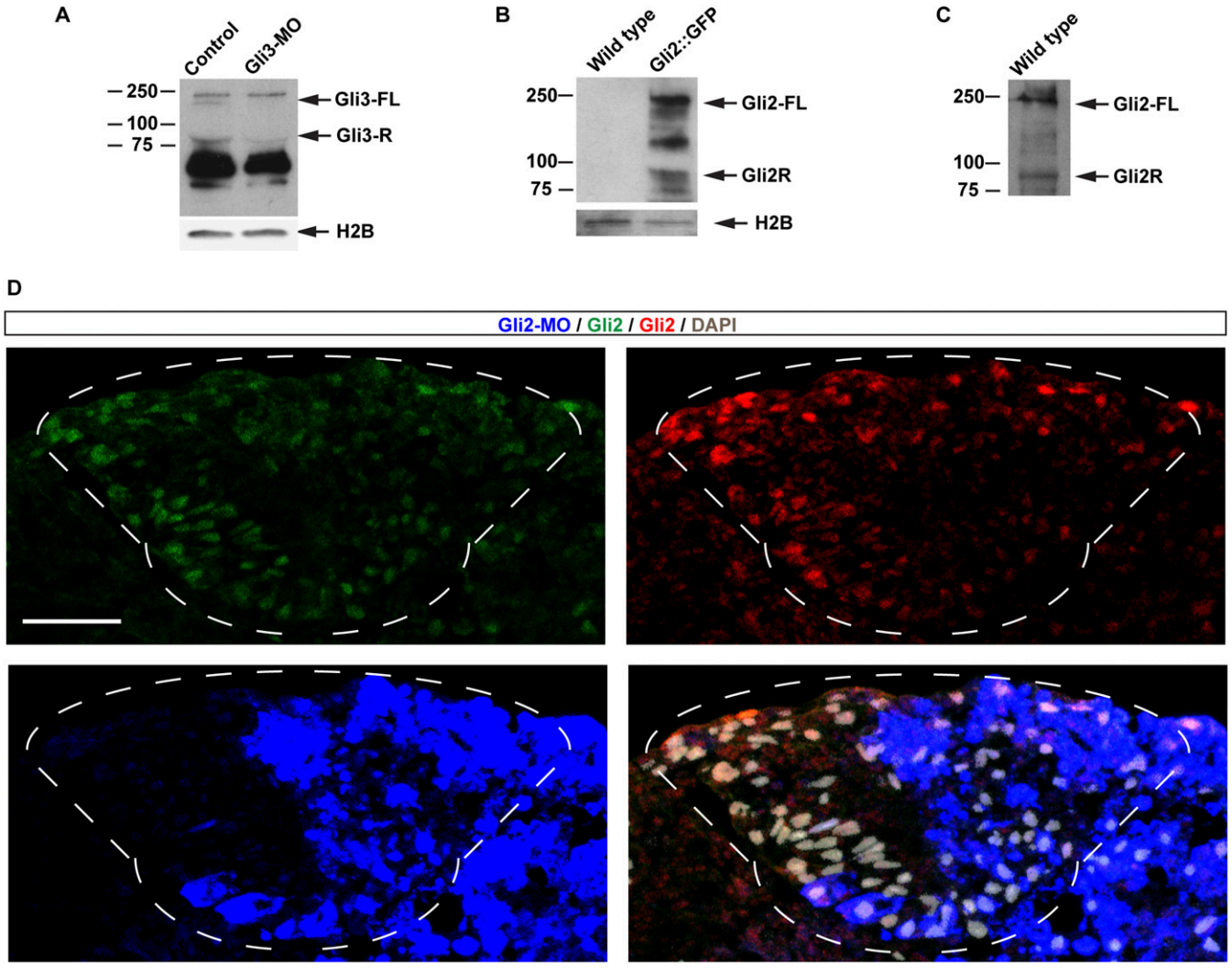

Fig. S6. Specificity of Gli2 and Gli3 antibodies. (*A*) Western blot assays from wild-type (Control) or Gli3-targeted–morpholino-injected (Gli3-MO) embryos at stage 20 (21.75 hpf) probed with antibody for Gli3 and H2B (loading control). Gli3-FL: Gli3 full length; Gli3-R: Gli3 repressor. Shown is a representative example. (*B* and *C*) Western blot assays from wild-type or mGli2-GFP–expressing embryos at stage 13 (14.75 hpf) probed with antibody for Gli2 (goat, R&D Systems) and H2B (loading control). Gli2-FL: Gli2 full length; Gli2-R: Gli2 repressor. Endogenous Gli2 is detectable (*C*) only when anti-Gli2 is incubated for ≥48 h and with longer exposure times than for detecting the exogenously expressed mGli2-GFP (*B*). Shown are representative examples. (*D*) Two-cell-stage embryos were unilaterally injected with Gli2-MO along with a dextran-Alexa fluor 647 conjugate (blue), grown until stage 20 (21.75 hpf), and then were processed for Gli2 [in green, goat anti-Gli2 (R&D Systems); in red, mouse anti-Gli2 (Genetex)] immunostaining and nuclear labeling (DAPI, gray). Shown is a representative example of a transverse immunostained section of the neural tube (outlined). (Scale bar: 30 μm.)

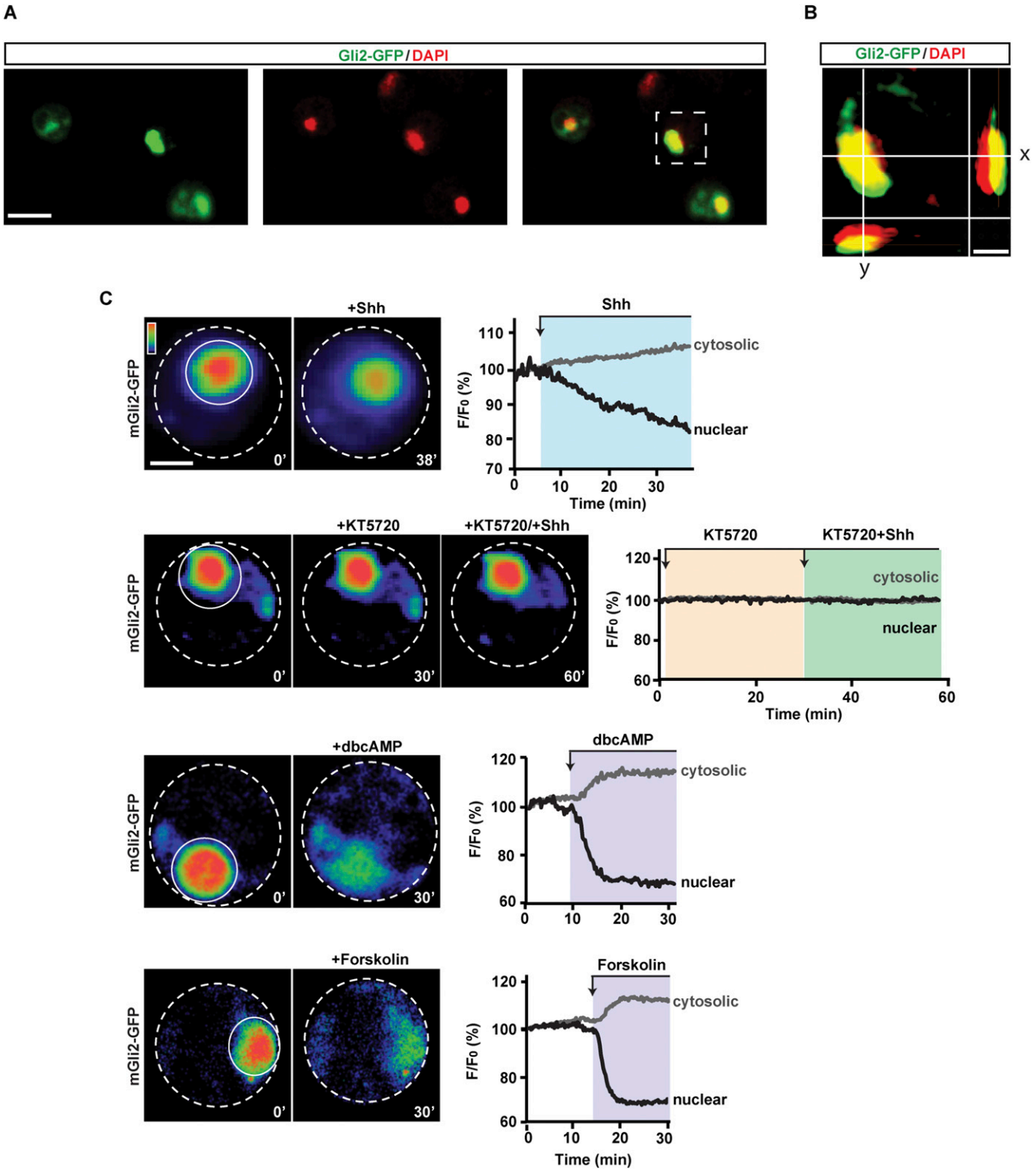

Fig. S7. Shh mimics PKA-induced regulation of Gli2 subcellular localization in spinal cord cells. Spinal cords from mGli2-GFP–expressing 21-hpf embryos were dissected, and cells were dissociated and plated. After 2 h, cultures were time-lapse imaged under a confocal microscope for a 1-h period every 15 s. (*A*) A representative region of a field of view of imaged cells that were fixed and labeled with the nuclear marker DAPI (in red) after imaging. (*B*) Orthogonal view of the z-stack projection of an imaged mGli2-GFP–expressing cell showing significant overlap between Gli2-GFP signal and DAPI. (*C*) Representative examples of imaged cells under indicated treatments. Contour of imaged cells and nuclei are indicated with dashed and solid lines, respectively. Color scale bar represents fluorescence intensity increasing from purple to red. Traces represent changes in nuclear (black) and cytosolic (gray) fluorescence over time for the given examples. $n \geq 27$ cells per condition. (Scale bars: 10 μm.)

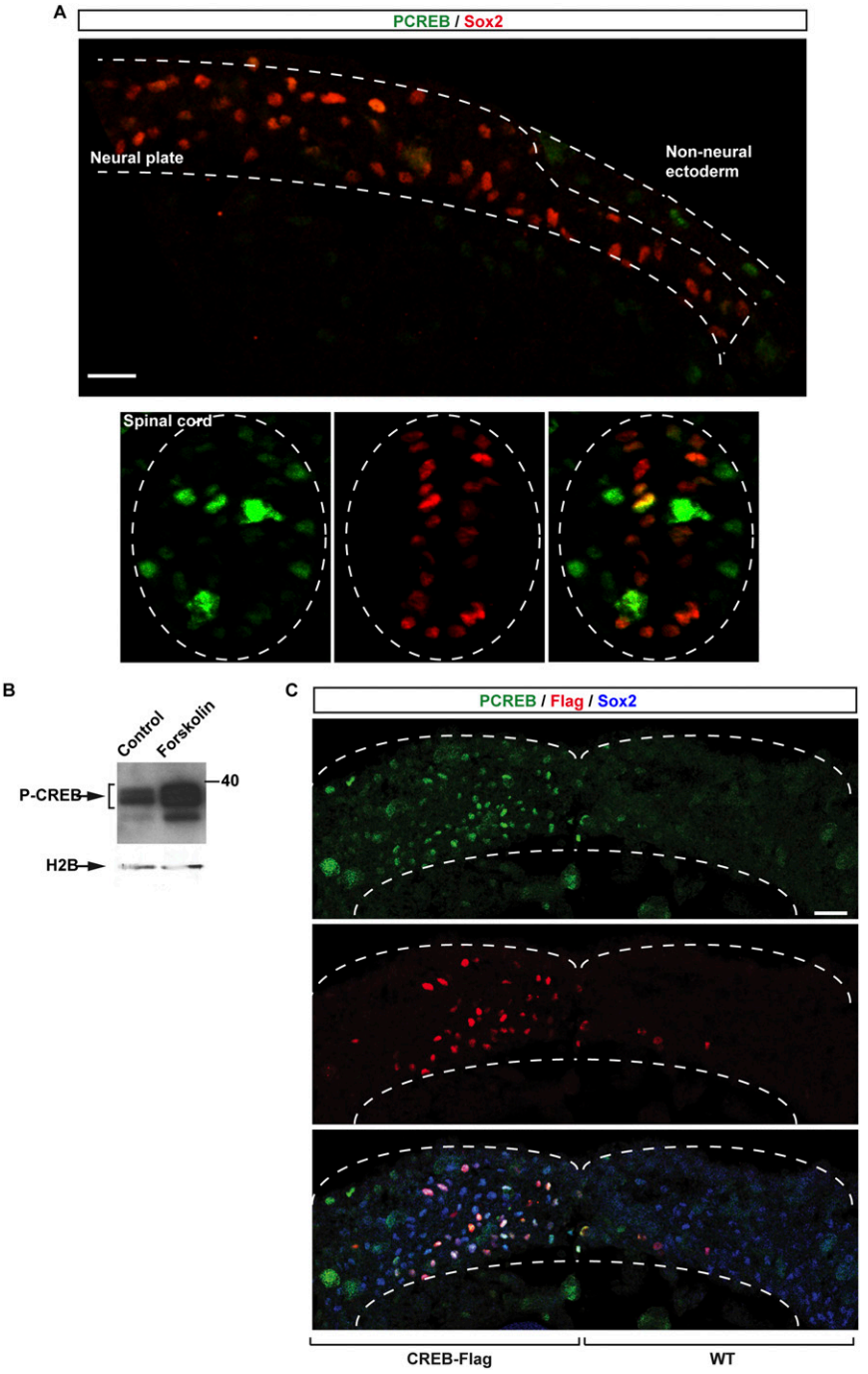

Fig. S8. PKA stimulation and CREB overexpression enhance P-CREB levels in the developing spinal cord. (*A*) CREB activation is apparent in the developing spinal cord. Neural plate and spinal cord transverse sections from wild-type embryos were processed for P-CREB (green) and Sox2 (red, neural progenitor marker) immunostaining. Shown are representative examples of transverse immunostained sections of the neural plate (*Top*) and spinal cord (*Bottom*) (outlined). (Scale bar: 20 μm.) (*B*) PKA activates CREB in the embryonic spinal cord. Western blot assays from spinal cord homogenates probed with antibodies for P-CREB and H2B (loading control). Samples were incubated in the absence or presence of 20 μM forskolin for 40 min. Shown is a representative example. (*C*) Expressing CREB induces enhanced levels of P-CREB in the neural plate. Two-cell-stage embryos were unilaterally injected with CREB-flag mRNA, grown until neural plate stages, and then were processed for P-CREB (green), flag (red), and Sox2 (blue) immunostaining. Shown is a representative example of a transverse immunostained section of the neural plate (outlined). (Scale bar: 30 μm.)

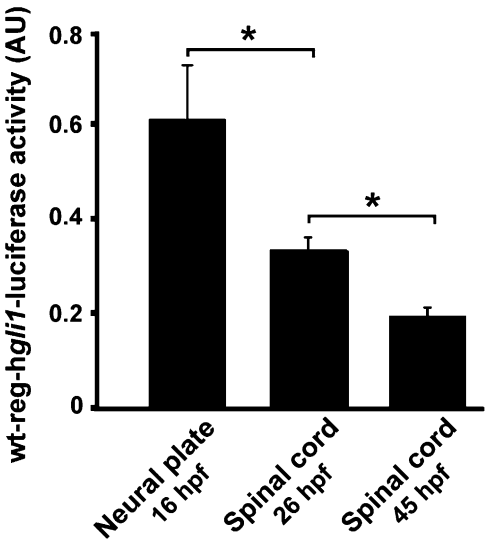

Fig. S9. *gli1* transcription decreases as spinal cord development progresses. Dissected neural plates and spinal cords from embryos at different developmental stages expressing wt-reg-h*gli1*-luciferase were processed for luciferase activity measurements. Graph shows mean ± SEM firefly luciferase activity levels normalized to Renilla luciferase activity levels; $n \geq 5$; $^*P < 0.01$.

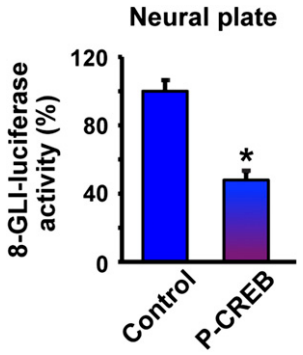

Fig. S10. P-CREB inhibits Gli activity. Neural plates from embryos expressing 8GLI-luciferase in the absence or presence of CREB overexpression were processed for luciferase activity measurements. Graph shows mean ± SEM percentage of normalized luciferase intensity compared with control (incubated with vehicle only); $n = 5$; $^*P < 0.05$.