A GLI1-p53 inhibitory loop controls neural stem cell and tumor cell numbers

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Supplementary Materials and Methods

Generation of transgenic mice

A tetracycline-regulated *GLI1* transgenic construct was made by subcloning a myc-tagged human *GLI1* into a vector containing the tetracycline-responsive element (TRE), consisting of seven copies of the 42-bp tetracycline operator sequence (tetO) flanked by two identical minimal cytomegalovirus promoter regions, one of which drives the expression of an enhanced green fluorescent protein (GFP) (Clontech Inc.). The construct was sequence-verified and tested for its tetracycline inducibility in Cos7 cells using a CMV-rtTA driver. The 6 kb transgene was isolated by digestion with NotI and injected into pronuclei of fertilized FVB oocytes. The putative founder transgenic mice were genotyped by PCR amplification. One founder transgenic mouse was obtained by random transgenesis and four at Nucleis, SA by homologous recombination in BPES ES cells, targeting the transgene into the *Hprt* locus. The random transgenic line was in a FVB genetic background. The targeted lines were in a C57Bl6 background. Transgenic mice were identified by PCR using the following primers: GLI1-F (5'-GGGATGATCCCACATCCTCAGTC-3'), GLI1-R (5'-CTGGAGCAGCCCCCCCAGT-3') and GFP-F: (5'-ATGGTGAGCAAGGGCGAGGAG-3'), GFP-R (5'-CGATGGGGGTGTTCTGCTGGTAGT-3'), mHprt-F (5'-CAGGGAGAAAAATGCGGAGTG-3'), mHprt-R (5'-CTCCGGAAAGCAGTGAGGTAAG-3'). The level of expression of *GLI1* was studied in three transgenic lines by crossing the BiTetO-GLI1/GFP F_1 mice (responder) with a Nestin-rtTA/IRESβgeo transgenic mouse (driver) (Mitsuhashi et al., 2001) (Suppl. Fig. 1) that is active in stem cells and precursors (Lothian and Lendahl, 1997). Genotyping of the Nestin-rtTA/LacZ mice was performed with the following primers: LacZ-F (5'-GTCGTTTTACAACGTCGTGACT-3'), LacZ-R (5'-GATGGGCGCATCGTAACCGTGC-3') or rtTA-F (5'-AGAGCTGCTTAATGAGGTCG-3'), rtTA-R (5'-GCGACTTGATGCTCTTGATC-3'). Doxycycline (West-ward Pharmaceutical Corp., Eatontown, NJ) was added freshly to the chow (6mg/gr) daily and fed continuously from E8.5 until the animal was sacrificed.

Neurospheres and cell lines

Neurospheres (NS) were prepared from E17.5 cortex, thalamus and cerebellum or from P3-5 SVZ of DT^{+dox} and DT^{-dox} as previously described in Palma and Ruiz i Altaba (2004) and incubated in defined serum-free NS media with EGF and FGF at 10ng/ml, with or without dox (2μg/ml; Sigma) renewed every two days. NS were dissociated and transduced with lentiviral vectors at MOI of ~10. Cell proliferation was evaluated by a BrdU-labelling assay (7 hr pulse at 4μg/ml; Sigma) approximately 4-5 days after lentiviral transduction. For clonal assays, transduced primary NS were dissociated and plated at 1 cell/well in 96-well plates in NS media with 1/3 conditioned media. The number and size of clones were counted after 2 weeks. Triplicates were performed for each experiments and each experiment was repeated at least three times. For differentiation assays, cortical, thalamic and cerebellar NS from DT^{+dox} and DT^{-dox} mice were dissociated and plated in polyornithine/laminin coated Lab-Tek chamber slides without FGF/EGF, with or without dox and incubated for 6 days. U87, U251, Daoy, HEK 293T, SKMel2 and Mewo cell lines were purchased from American Type Culture Collection (ATCC) and grown as specified. Glioma D54 cells were a kind gift of Dr. D. Bigner. Neurospheres from a glioblastoma multiforme (GBM12 and GBM13) were grown as in Clement et al. (2007).

Plasmids, lentiviral vectors and RNA interference

Cells were transiently transfected with CMV expression vectors with Fugene 6 (Roche). Transfections were at equimolar amounts unless otherwise specified with different combinations of pCS2-Myc-tagged human GLI1, GLI1ΔN', GLI2ΔN', GLI3, GLI3R (GLI3C'ΔClaI), frog Gli1, NLSGli1 (Ruiz i Altaba, 1999), wt human p53 (a kind gift from J. Massagué), human dnp53 ($p53^{Thr280}$) (a kind gift of P. Vize), pCMV-EGFP or pCMV-LacZ.

Lentiviral vectors were produced in HEK 293T cells and used at the MOI of 10. pLV-CTH-shSMO was as described in Clement et al. (2007). pLV-CTH-shPTCH1 with targeting sequence 5'-GCACTATGCTCCTTTCCTC-3'; pLV-WPXL-shp53 (targeting human p53) was a king gift from D. Trono (Wiznerowicz and Trono, 2003) ; pLV-Sico-shp53 (targeting mouse p53) was obtained from Addgene (Ventura et al., 2004); pLV-KO.1-puro-shSHFUH (5 clones tested with similar results) was from Sigma Mission. Most experiments were done with a clone with targeting sequence:

5'-CCTTTCGTCTGAAGAGAGAAACT-3'. GLI1 lentivirus were in vectors pLV-TWEEN or pLV-Lox-CW (a kind gift from P. Salmon).

siRNA sequences for human GLI1 and control siRNA were as described in Sanchez et al., (2004). siRNAs were transfected with Oligofectamine (Invitrogen) for 48 hr.

Luciferase reporter assays

A GLI binding site-dependent (p8xGBS) Luciferase reporter (Sasaki et al., 1997) was used in combination with plasmids driving the expression of GLI proteins. U87 cells were serum-starved for 3 days, plated in 12-well plates and grown to 60% confluence with no FBS, whereas U251, Daoy and HEK 293T cells were plated with 2.5% FBS. Cells were transfected with Fugene 6 with 1 μg of DNA total, at a reagent: DNA ratio of 3:1 in Optimem medium (Invitrogen), containing a constant 100 ng of GLI1 plasmid and 200 ng of pLV-WPXL-shp53. Cells were then incubated for 36 hr with the transfected DNA and harvested with 200 μl of the passive lysis buffer (Promega). Cortical and SVZ NS were transfected by nucleofection (Mouse Neural Stem Cell Nucleofector kit, Amaxa) with equal amount of GLI1 plasmid and pLV-Sico-shp53 and incubated 16 hr. Luciferase activities normalized with Renilla pRL-TK vector were measured in triplicate with 50 μl of protein lysate by using the dual-luciferase reporter assay system (Promega) and a Perkin Elmer luminometer.

Isolation of Prominin1^{+} cells

DT^{+dox} and DT^{-dox} E17.5 bigenic brains were dissected and dissociated with 0.3% activated papain (Worthington Biochemicals). Cells were labeled with Prominin1-microbeads (Miltenyi Biotec) and separation of magnetic-beads was performed on a magnetic activated cell sorting column (Miltenyi Biotec) according to the manufacturer's instructions.

Synthetic RNA injection into frog embryos

Synthetic RNA microinjection into developing 4-cell frog (xenopus laevis) embryos with human *GLI1* and human *dnp53* ($p53^{Thr280}$) RNAs (at 2 ng/10 nl/embryo) was performed as previously described in Dahmane et al. (1997), targeting the animal region of ventral cells. Control and *Gli1* antisense morpholino oligonucleotides were microinjected at 20 ng/10 nl/embryo. *LacZ* RNA tracer was co-injected at 0.5ng/embryo.

Immunohistochemistry and in situ hybridization

DT^{+dox} and DT^{-dox} brains of E14.5- P0 were dissected in cold PBS and fixed in fresh 4% paraformaldehyde for 30 minutes at 4°C. Xgal staining was performed on frozen sections and counterstained with 0.1% eosin. Immunohistochemistry was performed on frozen sections or cells with the following primary antibodies: mouse anti-BrdU (1/200, Beckton Dickinson), rabbit anti-cleaved Caspase3 (1/200, Cell Signaling), mouse anti-Myc (1/500, Santa Cruz), rabbit anti-GFP (1/500, Molecular Probes), mouse anti-Nestin (1/1000, BD Pharmingen), mouse anti-GFAP (1/1000, BD Pharmingen), rabbit anti-Prox1 (1/2000, Chemicon), rabbit anti-TH (1/1000, Chemicon) mouse anti-53BP1 (1/20, a kind gift of T. Halazonetis), rabbit anti-phospho-Chk2 (1/1000, Cell Signaling), mouse anti-Tuj1 (1/1000, Covance), rabbit anti-O4 (1/5), mouse anti-CD34 (1/50, Zymed) and mouse anti-HP1γ (1/20,000 Chemicon) using FITC- or rhodamine-conjugated secondary antibodies (Molecular Probes). In situ hybridization was performed in frozen sections with digoxygenin-labeled antisense and sense RNA probes for *GLI1, Ptch1, Pdgfrα and NeuroD1* as previously described in Dahmane et al (2001).

Quantitative RT-PCR

The quantitative real-time PCR amplifications were carried out at 60°C using iQTm SYBR green mix (Bio-Rad). Primers for human GLI1 were as described in Clement et al (2007). Primer sequences for mouse genes were the following (5' to 3'): Abcg2-F, CATCAGCCTCGGTATTCCAT and Abcg2-R, AATCCGCAGGGTTGTTGTAG; Bax-F, GGAGACACCTGAGCTGACCT and Bax-R, GAAGTTGCCATCAGCAAACA; Bcl2-F, ATGTGTGTGGAGAGCGTCAA, Bcl2-R, CATGCTGGGGCCATATAGTT; Bmi1-F, GGTACTTACGATGCCCAGCA, Bmi1-R, CCATCCCTCTGGTGACTCAT; Bmp2-F, GTTTGGCCTGAAGCAGAGAC and Bmp2-R, CGTCACTGGGGACAGAACTT; Bmp4-F AGGAGGAGGAGGAAGAGCAG and Bmp4-R, CACCTCATTCTCTGGGATGC; BmpR-1a-F, GGACCAGAAGAAGCCAGAAA and BmpR-1a-R, CTTTCGGTGAATCCTTGCAT; BmpR-1b-F, TGACTCTGGAATGCCTGTTG and BmpR-1b-R, AGAGTGGGGTGGAGGTCTTT; Dl1-F, CCAGCGCTACATGTGTGAGT and Dl1-R, GTGGAGGCTGGTGTTTCTGT; EgfR-F, GGCCTATTCATGCGAAGACG and EgfR-F, CAGGAGGCGGCATACATGAG; Ezh2-F, ATCTGAGAAGGGACCGGTTT and Ezh2-R, TGTGCACAGGCTGTATCCTC; Gli1-F, GCAACCTTCTTGCTCACACA and Gli1-R, GAAGGAATTCGTGTGCCATT; Gli2-F, CGCACTCACTCCAATGAGAA and Gli2-R, GGACATGCACATCATTACGC; Gli3-F, GAGTTAGCTGGCAACACAGT and

Gli3-R, CTCAAAGCTGTCAACTGTGC; Hes1-F, AAAGACGGCCTCTGAGCACA and Hes1-R, TCATGGCGTTGATCTGGGTCA; Hes5-F, GCAGCATAGAGCAGCTGAAG and Hes5-R, TAGTCCTGGTGCAGGCTCTT; Hgf-F, AGGAACAGGGGCTTTACGTT and Hgf-R, GCTGCCTCCTTTACCAATGA; Hip-F, CCACTGACCTCCGATTGCTC and Hip-R, TGCAGCAGCACTTGCCAG; Igf2-F, GTCGATGTTGGTGCTTCTCA and Igf2-R, AAGCAGCACTCTTCCACGAT; Jagged1-F, GGAAGTGGAGGAGGATGACA and Jagged1-R, TGGGCACTTTCCAAGTCTCT; Jagged2-F, GCGCTATCTGCTCTGGAATC and Jagged2-R, CTGAGTGATAGCAGCCACGA; Klf4-F, CTGGCCATCGGACCTACTTA and Klf4-R, GTACCTGAGCCCCAAAGTCA; Lgr5-F, CTGCCCATCACACTGTCACT and Lgr5-R, GCAGAGGCGATGTAGGAGAC; Maml1-F, AGCTGCCCCATCTGAGTAAC and Maml1-R, GAGGTATGGCGAGCTGTTGT; Maml2-F, TTATCAAGCCAGCCTTTGCT and Maml2-R, GGACTGCTGGGAGTTCATGT; Math1-F, ACATCTCCCAGATCCCACAG and Math1-R, ACAACGATCACCACAGACCA; Mdm2-F, CTTCGTGAGAACTGGCTTCC and Mdm2-R, CTGTCAGCTTTTTGCCATCA; cMet-F, TGAGAAACTCTTCCGGCTGT and cMet-R, GTGAGGTGTGCTGTTCGAGA; Musashi-F, GAGGACTCAGTTGGCAGACC and Musashi-R, CTGTGCTCTTCGAGGAAAGG; Nanog-F, ATGCCTGCAGTTTTTCATCC and Nanog-R, ACAGTCCGCATCTTCTGCTT; Nestin-F AAGAACCACTGGGGTCTGTG and Nestin-R, TCCCACCTCTGTTGACTTCC; Nodal-F, GGCGTACATGTTGAGCCTCT and Nodal-R, CGCCCATACCAGATCCTCT; Notch1-F, TGAGACTGCCAAAGTGTTGC and Notch1-R, GTGGGAGACAGAGTGGGTGT; Notch2-F CCCTTGCCCTCTATGTACCA and Notch2-R, AGGAGGCGTAACTGTGTTGG; Nst-F, CTGACGAGCCCAAAAGAAAG and Nst-R, ATCTGAGGACACCTGCAACC; Numb-F, CAGTTGCAAGATGCCAAGAA and Numb-R, TGGCGTTTAAAGGGTGACAT; Olig2-F CTGGTGTCTAGTCGCCCATC and Olig2-R, AGGAGGTGCTGGAGGAAGAT; Prominin1-F, TGGCCCTCTCTACAAAATGG and Prominin1-R, CGGCACATACAAAAGAAGCA; Ptch1-F, ATGGTCCTGGCTCTGATGAC and Ptch1-R, TAGCCCTGTGGTTCTTGTCC; Ptch2-F, GTGCCCTACTTGGCATCACT and Ptch2-R, GAAAAAGGCGACCATGTTGT; Pten-F, TGTGGTCTGCCAGCTAAAGGT and Pten-R, ACATGAACTTGTCCTCCCGC; PUMA-F, GCCCAGCAGCACTTAGAGTC and PUMA-R, TGTCGATGCTGCTCTTCTTG; p16 –F, GTCGCAGGTTCTTGGTCACT and p16-R, CGAATCTGCACCGTAGTTGA; p21-F, AATCCTGGTGATGTCCGACC and p21-R, TCAAAGTTCCACCGTTCTCGGG; p53-F,

GCAACTATGGCTTCCACCTG and p53-R, CTCCGTCATGTGCTGTGACT; rtTA-F, AGAGCTGCTTAATGAGGTCG and rtTA-R, GCGACTTGATGCTCTTGATC; Shh-F, GCAGGTTTCGACTGGGTCTA and Shh-R, GAAGGTGAGGAAGTCGCTGT; Snail1-F, AAACCCACTCGGATGTGAAG and Snail1-R, GAAGGAGTCCTGGCAGTGAG; Sox2-F, GGCAGCTACAGCATGATGCAGGAGC and Sox2-R, CTGGTCATGGAGTTGTACTGCAGG; Sufu-F, GGCCTAGCCCGATATGTCTT and Sufu-R, CTCCTCAGTGCAGACACCAA; Tert-F, CTGGCTGATGGACACATACG and Tert-R, GTAGCCGCACTCTCTCAAGG; Wnt5A-F ATTGTCCCCCAAGGCTTAAC and Wnt5A-R, ACTTGGAAGACATGGCACCT; Wnt7B-F, TACTACAACCAGGCGGAAGG and Wnt7B-R, GTGGTCCAGCAAGTTTTGGT; Yap1-F, CCCGACTCCTTCTTCAAGC and Yap1-R, TGTGAGTGTCCCAGGAGAAA. Primers for mouse reference genes were: Gapdh-F, CGTCCCGTAGACAAAATGGT and Gapdh-R, GAATTTGCCGTGAGTGGAGT; β-actin-F, GCTGTATTCCCCTCCATCGTG and β-actin-R, CACGGTTGGCCTTAGGGTTCAG; EF1α-F, GCATGGTGGTTACCTTTGCT and EF1α-R, CAGCAACATTGCCTCGTCTA.

Intracranial xenografts

U87 glioblastoma cells were transduced with lentiviral vectors pLV-TWEEN, pLV-TWEEN-GLI1, pLV-WPXL, pLV-WPXL-shp53. 72-96 hr after transduction cells were dissociated and were resuspended (500.000 cells/5μl) in Hank's balanced saline solution (HBSS) and injected with a stereotaxic apparatus with coordinates L2,P1,D2 relative to Bregma.

Western blotting

Exogenous and endogenous GLI1 proteins were visualized by 7.5% SDS-PAGE Western blotting with mouse anti-myc tag monoclonal or rabbit polyclonal anti-GLI1 affinity-purified antibodies made against aa 412-427 of GLI1, and chemiluminescence ECL kit (Amersham) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) for detection of endogenous GLI1. The GLI1 peptide had a C-terminal cysteine for coupling to activated KLH (Pierce) and adjuvant immunization. Anti-GLI1 antibodies were purified in a glycine-blocked affinity column containing peptide coupled to Protein A. Treatments were with cycloheximide (80μg/ml Sigma), okadaic acid (10nM Alexis), oxaliplatin (1-5μM Sigma) and forskolin (50μM). Phosphoprotein selection was performed in U87 cells by the Pro-Q Diamond Phosphoprotein enrichment kit (Invitrogen). Cell extracts in

RIPA buffer were centrifuged at 16K rpm in a microfuge for 20 min and the resulting supernatants subjected to immunoblotting using mouse anti-HSP90 (1/4000 Santa Cruz), mouse anti-Myc (1/2000 mAb 9E10 Santa Cruz), mouse anti-p53 (1/2000 DO-1, Santa Cruz), rabbit anti-phospho p53 (Ser15) (1/2000 Cell Signaling), goat anti-SUFUH (1/200 C-15 Santa Cruz), mouse anti-Mdm2 (1/1000 SMP14 Santa Cruz), and our rabbit anti-GLI1 (1/2000). Extracts had a protease inhibitor cocktail (Sigma), PMSF (Fluka), plus phosphatase inhibitors (Thermo Scientific). The latter was omitted in extracts to be treated in vitro with 100U of recombinant λ protein phosphatase (λPPase) (New England BioLabs) at 30°C for 15 min. Commercial Westerns were purchased from DNA technologies. The following commercial antibodies did not recognize endogenous human GLI1 in Western blots in our hands: mAb L42B10 (2643), rabbit Ab 2553 (aa ~420) both from Cell Signaling; rabbit Ab GTX27523 (aa 803-818) from GeneTex, Inc; rabbit Abs ab7523 (aa 803-818) and ab49314 both from Abcam; rabbit AB3444 (aa 805-820) from Chemicon; and rabbit Abs H-300 sc-20687 (aa 781-1080) and N-16 sc-6153 both from Santa Cruz.

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Supplementary Figures

Supplementary Table 1
Summary of the number of mice analyzed and phenotypes of bigenic lines.

Supplementary Figure 1. Bigenic model and overall brain phenotype.
A) Schematic representation of the bigenic system used in this study showing the diagrams of driver and responder constructs. We have used both male and female random and male targeted transgenics (as the *hprt* locus in the X chromosome). The *Nestin* regulatory regions used are those in the second intron plus the HSV tk promoter (Mitsuhashi et al., 2001). B) RT-PCR analyses of transgene (*rtTA, GLI1, GFP*) and of *Ptch1*, an endogenous Hh-Gli target in dissected P0 cortex (C), thalamus (T) and cerebellum (Cb) as indicated. GFP and GLI1 are only expressed when dox is administered to double transgenic (DT) mice. GLI1 is functional as it augments the level of *Ptch1*. The expression of the housekeeping gene *Hprt* is shown as control. Empirically we found that the best induction in mid to late gestation embryos was achieved by commencing administration at E8.5-E9. Starting induction postnatally was insufficient to drive effective levels of transgene expression in the brain. C) Half dorsal views of normal DT^{-dox} (left) and DT^{+dox} (right) P0 brains showing the relative proportions of the different brain regions. The dotted line silhouettes at right highlights the larger thalamus (Thal), tectum or dorsal midbrain (Mid) and cerebellum (Cb). Scale bar = 0.75mm (C).

Supplementary Figure 2. Brain phenotypes of DT^{+dox} embryos.
A-D) Expression of *GLI1* in DT^{+dox} mice boosts endogenous *Ptch1* expression in the cerebral cortex, striatum (A,B) and SVZ (C,D) of E16.5 (A,B) and P0 (C,D) brains as compared wth DT^{-dox} brains. Note the accumulation of high $LacZ^{+}$, $GLI1^{+}$, $Ptch1^{+}$ cells in clusters in these regions. E) Confocal image of streaming GFP^{+} cells co-expressing Nestin in the P0 DT^{+dox} cingulate cortex. F) Phenotypes of cerebellum (Cb) and medulla oblongata (Md) at E16.5 shown histologically and expression of *GLI1* and *Ptch1* as noted in DT^{+dox} and DT^{-dox} mice. G,H) Cross sections of P0 thalamus of DT^{-dox} (G) and DT^{+dox} (H) brains showing *LacZ*, BrdU and GFP expression as indicated. Note the large hyperplastic thalamus in DT^{+dox} brains. I) Detailed high-power view of the cortical wall at E16.5 DT^{+dox} embryos in a cross section showing the heterogenous high expression of endogenous *Ptch1* in cells that appear to be migrating out of the germinal zone. J) Longitudinal sections of the ventral midbrain region in DT^{-dox} and DT^{+dox} E14.5 embryos labeled with anti-tyrosine hydroxylase antibodies marking dopaminergic neuronal differentiation. Regions such as this one, where transgene expression is low, maintain differentiation. Nuclei are counterstained with DAPI. Scale bar = 0.5mm (A,B,G,H), 360µm (C,D) 15µm (E) 500µm (F), 80µm (I, H inset), 300µm (J).

Supplementary Figure 3. Postnatal DT^{+dox} brain phenotypes.
A) Sagittal P0 whole-brain sections of a weak DT^{+dox} phenotype showing limited hyperplasias in the thalamus (Thal), cerebellum (Cb) and hindbrain as well as the presence of clusters in the cortex (Ctx). These effects are seen with transgenic *LacZ* (Xgal) labeling, *GLI1* and endogenous *Ptch1* expression as noted. B) Comparison of normal (DT^{-dox}) and induced (DT^{+dox}) postnatal cerebella highlighting the hyperplastic ventricular zone of the cerebellum seen in sagittal sections. The images show expression of endogenous *Ptch1*. Note that its endogenous high expression in the external germinal layer (EGL) is largely unaltered but the expression in the Purkinje layer (PL) is greatly diminished in induced brains, in accordance with the defective differentiation of the hyperplastic ventricular zone

(VZ), which is the source of PL cells. C) High-magnification images of DT^{+dox} cerebella showing expression of Nestin by GFP^+ cells and the absence of activated Caspase3 labeling in hyperplastic regions expressing the GLI1 transgene. D) Details of P0 cross hemisections of the thalamic region of DT^{+dox} pups with a weak phenotype showing the expression of the *LacZ* transgene by Xgal staining and coincident expression of *GLI1* and *Ptch1*. *NeuroD*, in contrast is expressed in a complementary pattern to *GLI1*, being excluded from induced cells, whereas *Pdgfr alpha* is enhanced. For comparison, a lower magnification image of the thalamic region of a DT^{-dox} mice at the same stage is shown at right as indicated. Note the even distribution of *Pdgfr alpha*$^+$ oligodendrocyte precursors throughout the CNS and thalamus (Thal). The box denotes the position of the images at left. Scale bar = 0.8mm (A), 300µm (B,D), 200µm (C).

Supplementary Figure 4. DT^{-dox} and DT^{+dox} spinal cord phenotype.
Cross sections of the cervical spinal cord of DT^{-dox} and DT^{+dox} embryos at E17.5 showing the expression of *LacZ* (through the Xgal reaction for βgal), GFP, Nestin, *GLI1* and *Ptch1* as indicated. Note the hyperplastic ventricular and subventricular zone and the invasion of precursors reaching in some areas the pial surface. Nestin->*rtTA-IRES-LacZ* is active in a very small population of precursors in the normal spinal cord. Scale bar = 150µm.

Supplementary Figure 5. Numerical values of RT-qPCR analyses shown in Figure 2A and gene expression values in E17.5 NS.
The numbers denote fold increase or decrease (negative values) and are the values of DT^{+dox}/DT^{-dox} ratios obtained with individual gene expression ct values normalized by the geometrical mean of the housekeeping genes *Gapdh*, *βactin* and *EFIα*. In addition, all DT^{+dox} values were normalized by the expression of the *GLI1* transgene to be able to compare across brain regions. The comparative levels of *GLI1* are shown at the bottom.

Supplementary Figure 6. Neurosphere differentiation and gene expression in Prominin1$^+$ cells of DT^{+dox} cerebella.
A) Top- Representative images of differentiation markers are noted of neurospheres (NS) derived from different brain regions of DT^{+dox} and DT^{-dox} E17.5 brains. Note the typical punctate staining of the oligodendrocyte marker O4. An increase in neuronal differentiation observed in DT^{+dox} NS parallels that detected in *p53*$^{-/-}$ NS (Gil-Perotin et al., 2006). Bottom- Quantification of the differentiation of neurons ($Tuj1^+$) and astrocytes ($GFAP^+$) from induced and uninduced NS as shown above. B) Fold upregulation or downregulation (negative numbers) of gene expression as shown in Fig. 2I. Numbers refer to ratios of DT^{+dox}/DT^{-dox} values for each gene normalized with housekeeping genes as in Suppl. Fig. 4. The enrichment of *Prominin1* mRNA expression is shown at the bottom. Scale bar = 20µm (A).

Supplementary Figure 7. Induction of hallmarks of DNA-replication stress by GLI1. A) Induction of $53BP1^+$ DNA replication stress foci (arrow) in 293T cells by transduced GLI1. B). Increased number of $P\text{-}Chk2^+$ cells (arrows) in DT^{+dox} vs. DT^{-dox} cerebellum and thalamus. Scale bar = 15µm (A), 120µm (B).

Supplementary Figure 8. RT-qPCR analyses in neurospheres from cortex, thalamus, cerebellum and SVZ expressing GLI1 and GLI1 + shp53.
RT-qPCR analyses of NS from four different regions shown as GLI1+shp53/GLI1 ratios. The left part shows a heat map and the right side the actual values. The numbers denote fold increase or decrease (negative values) and are the ratios of values of neurospheres from E17.5 cortex (Ctx), thalamus (Thal) or cerebellum (Cb) and those of postnatal; forebrain subventricular zone (SVZ) transduced with GLI1 plus shp53 lentivectors over those transduced with GLI1 lentivectors only. Values of each individual gene were obtained after normalization with housekeeping genes as in Suppl. Fig. 5.

Supplementary Figure 9. Effects of altering p53 levels on GLI1 transcriptional activity and GLI1 levels and expression of GLI1 in human tissues and tumors.
A,B) Gli binding site reporter activity in 293T (A) and Daoy (B) cells as indicated. ns: not significative. The gradient of p53 in (B) denotes increasing amounts of *p53* expression plasmid included in the same total amount of transfected DNA, with ration of 1/0.125, 1/0.25, 1/1 in relation to *GLI1*.
C) Western blot of exogenous Myc-tagged GLI1 protein in control-transfected U87 cells (wt), p53 kd and dominant negative p53 (dnp53)-expressing cells. HSP90 is used as internal control. Only the full-length protein is readily detected in these cells.
D) Endogenous GLI1 isoforms in human U87 cells are only recognized by our α-GLI1$^{412-427}$ antibody and not by α-GLI1$^{\sim 420}$. Asterisk denotes a ~115KD band of unknown nature recognized by α-GLI1$^{\sim 420}$
E) 7.5-20% gradient SDS-PAGE commercial membranes with human tumor and normal samples as indicated probed with affinity-purified anti-GLI1 antibodies. Endogenous GLI1 is detected in all cases with predominant presence of a ~130-140 KD form running lower than the full-length form in human cell lines or in transfected cells (compare with Fig. 6A,B, D), which runs close to 180KD. These Westerns are shown as validation of the expression of GLI1 in normal and tumor tissues but not for quantification purposes.
F) One breast cancer, but not the normal breast tissue of the same patient, showed an altered pattern, suggesting a tumor-specific modification of GLI1. The sizes of markers used are shown on the right.
G) Western blot analyses of exogenous myc-tagged GLI1 revealed with anti-Myc antibodies. HSP90 is used as control. Treatments were: untransfected U251 cells (untransf.), U251 cells transfected with GLI1 (GLI1), and U251 cells transfected with GLI1 followed by lambda phosphatase treatment in vitro for 15 min (+λPPase).
H) Western blot analysis for endogenous GLI1 with α-GLI1$^{412-427}$ of total lysates of LV-control-transduced or LV-shp53-transduced U87 cells and of selected phosphoproteins from LV-control U87 cells. GLI1^{FL} and GLI1^{130P} but not GLI1^{130} are detected in the phosphofraction.

Supplementary Table 1.

a) Number of mice analyzed taken at the end of the treatment period.

	Random integration				HPRT targeted integration			
Treatment period	ST^D	ST^R	DT^{-dox}	DT^{+dox}	ST^D	ST^R	DT^{-dox}	DT^{+dox}
E8.5-14.5 /16.5 /17.5	70	60	60	75	18	18	20	24
E8.5-P0	30	30	40	40	23	22	25	30
E8.5-P21 /30	32	32	35	45	18	18	22	25

b) Frequency of DT^{+dox} phenotypes.

	Phenotype (random integration)		Phenotype (HPRT targeted integration)	
Treatment period	Weak	Strong*	Weak	Strong*
E8.5-14.5 /16.5 /17.5	61%	39%	80%	20%
E8.5-P0	75%	25%	90%	10%
E8.5-P21 /30	0%	0%	0%	0%

* Overtly enlarged brain, dilated ventricles, hyperplastic thalamus and cerebellum.
ST^D, single transgenic driver; ST^R, single transgenic responder.