Supporting Information

Yellajoshyula et al. 10.1073/pnas.1012053108

SI Materials and Methods

Cell Culture. The A2lox mouse ES cell line and clonal derivatives were routinely propagated on feeder cells (mouse embryonic fibroblast cells; Chemicon) in DMEM (Invitrogen) supplemented with 15% FCS (HyClone), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 1 mM L-glutamine (Invitrogen), 10^{-4} M 2-mercaptoethanol (Sigma), and 10^3 units per milliliter of leukemia inhibitory factor, (ESGRO; Chemicon). Monolayer neural differentiation of ES cells was done as previously described (1). Briefly, ES cells cultured on feeder cells were dissociated and plated at $1 \times 10^5/cm^2$ for 1 d on 0.1% gelatin-coated tissue culture dishes. After 24 h, cells were plated onto 0.1% gelatin-coated tissue culture dishes at low density (1×10^4 cells/cm^2) in N2B27 medium and cultured for the time periods described in the results. Embryoid body formation was performed as previously described (2). hBMP-4, mWnt-3a, and Activin A were obtained from Peprotech.

Immunofluorescence. ES cells were fixed in 4% paraformaldehyde for 15 min followed by antibody staining (primary and secondary antibodies successively) in 1× PBS containing 1% BSA and 0.3% saponin (Sigma) for 1 h at room temperature. Images were collected using the Zeiss ApoTome epifluorescence microscope with a 20× objective lens. To determine the fraction of Geminin immunopositive cells that were also Oct4, Sox2, Pax6, or Sox1 immunopositive (Fig. 1 *C* and *D* and Fig. S1*C*), we used ImageJ to quantitate singly or doubly immunopositive cells, scoring at least 500 nuclei from three independent experiments.

TUNEL assay. ES cells were plated on gelatinized coverslips at low density (1×10^4 cells/cm^2) in N2B27 medium and cultured for 2 d, after which TUNEL assays were conducted as per the manufacturer's instructions (Roche; In Situ Cell Death detection kit, TMR Red).

Cell Viability Assay. For determining cell viability with Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma) ES cells were plated in 6-well dishes (Corning), in ES growth medium or N2B27 medium, and a final concentration of 0.5 mg/mL of MTT was added, followed by incubation for 3 h at 37 °C. The reaction was stopped by addition of equal volumes of isopropanol with 0.04 N HCl and the absorbance measured at a wavelength of 570 nm.

Quantitative Chromatin Immunoprecipitation. Quantitative ChIP was done with modifications to a standard protocol (Upstate) as follows. For each ChIP reaction, sheared chromatin (sonicated to 200–500 bp) from 2×10^6 mES cells fixed in 1% formaldehyde (Sigma) in DMEM + 5% FBS was incubated with 5 μg antibody using Dynabeads (Invitrogen) as per the manufacturer's instructions. Antibodies used for ChIP are listed in Table S2. After washing, elution and cross-link reversal, DNA from each ChIP sample and the corresponding input sample was purified and analyzed further using qPCR as follows: each ChIP sample and a range of dilutions of the corresponding input sample (0.01–5% input) were quantitatively analyzed with gene-specific primers using the 7500 FAST Real-time PCR detection system (ABI) and SYBR Advantage qPCR Premix (Clontech).

Microarray Analysis. Total RNA was isolated from GemKD cells differentiated in N2B27 medium for 2 d with or without 500 ng Dox and used for microarray analysis with the Affymetrix GeneChip Mouse Genome 430 2.0 array. Three independent experiments were conducted, using two GemKD lines with unique sequences targeting Geminin (shRNAmir nos. 9 and 11, Fig. S2) to control for off-target effects. Raw CEL and DAT files were analyzed with dChip software (http://www.bioinformatics.org/dchip) (3) after normalization to exclude probe sets that did not meet the following preliminary cutoff values: (*i*) expression, represented by model-based expression indices (MBEI), was changed more than 1.3-fold in treated versus untreated samples (E/B >1.3) groups, (*ii*) MBEI differences were larger than 30 (E/B > 30), (*iii*) expression was claimed as "present (P)" in at least one sample in each experiment. Probe sets that showed a change in expression upon Geminin knockdown that met cutoff values in at least two of the three experiments were considered for further single gene validation. Microarray data were deposited in the Gene Expression Omnibus as GSE25737.

qRT-PCR. Total RNA was extracted with Nucleospin II (Invitrogen) and used for cDNA synthesis with random primers (Invitrogen) and Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Clontech). qRT–PCR was performed with the 7500 FAST real-time PCR detection system (ABI) and SYBR Advantage qPCR Premix (Clontech). Primer sequences are in Table S1.

Purification of GST-Geminin and GST. GST and Geminin-GST were purified from BL21(DE3) bacterial cells transformed with pGEX-4T (Clontech) plasmid, which expresses GST, or with pGEX-4TmGem, which expresses GST fused with mouse Geminin at the N terminus, using Glutathione Sepharose 4B beads (GE Healthcare) as per the manufacturer's instructions. Molar amounts of purified proteins for in vitro experiments were defined by using the estimated molecular weight of the GST and Geminin-GST proteins determined from their amino acid sequence using the ExPASy Proteomic server and the protein concentrations determined using the Biorad protein assay.

Preparation of Total Histone and Chromatin from mES. Preparation of chromatin using micrococcal nuclease and total histones from mES nuclei was done as previously described (4). Briefly, nuclei were isolated from ES cells by lysis in homogenization buffer (1× nuclei wash buffer, 1 mM EDTA, 0.1 mM EGTA and 5% wt/vol sucrose) with a dounce homogenizer. Histones were extracted from these nuclei by solubilizing in 0.2 M sulfuric acid at 4 °C for 30 min, followed by precipitation with ammonium hydroxide and ethanol. Precipitated histones were resuspended in 10 mM Tris·HCl, pH 8.0, and their final concentration measured (Biorad protein assay kit).

For chromatin preparation, nuclei were isolated from 1×10^7 mES cells as previously described, washed in 1× nuclei wash buffer (10 mM Tris·HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) and digested at 25 °C for 10 min in 0.5 mL of MNase digestion buffer (1 mM $CaCl_2$, 4 mM $MgCl_2$, 0.3 M sucrose, 1% vol/vol thiodiglycol, 1 mM PMSF) supplemented with 1 unit/mL micrococcal nuclease (Sigma). Following digestion, nuclei were spun down, lysed in nuclei lysis buffer (Tris pH 8.0, 10 mM, 2 mM EDTA, 1 mM EGTA), and the resulting chromatin was dialyzed against 1 L of 20 mM Hepes containing 10% glycerol overnight and stored at 4 °C until further use.

Nuclear Extract. Nuclei were isolated as previously described and washed once in low salt buffer (20 mM Hepes 7.9, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA) followed by extraction of nuclear components in equal volume of low salt buffer and high salt buffer

(20 mM Hepes 7.9, 1.5 mM MgCl2, 1.2 M KCl, 0.2 mM EDTA). This nuclear extract was dialyzed overnight in dialysis buffer (20 mM Hepes 7.9, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) and stored at −80 °C until further use.

1. Ying QL, Smith AG (2003) Defined conditions for neural commitment and differentiation. *Methods Enzymol* 365:327–341.
2. Lugus JJ, et al. (2007) GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* 134:393–405.
3. Li C, Hung Wong W (2001) Model-based analysis of oligonucleotide arrays: Model validation, design issues and standard error application. *Genome Biol*, 2(8): RESEARCH0032.
4. Gunjan A, Sittman DB, Brown DT (2001) Core histone acetylation is regulated by linker histone stoichiometry in vivo. *J Biol Chem* 276:3635–3640.

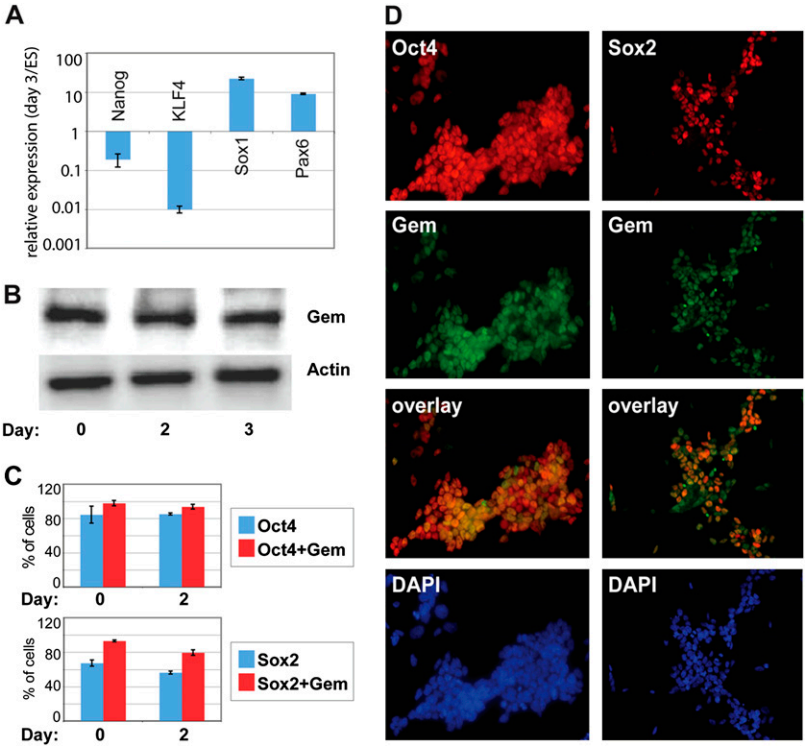
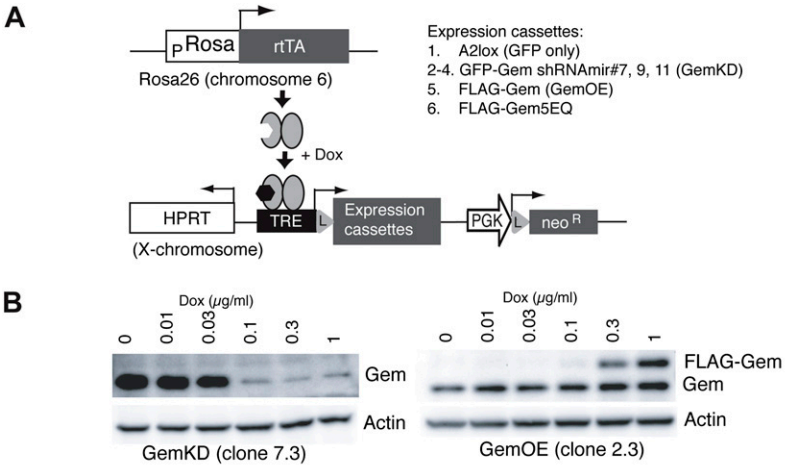


Fig. S1. ES cells undergo neural commitment in N2B27 medium. (*A*) Changes in the expression of pluripotency-associated (Nanog and KLF4) and neural (Sox1 and Pax6) genes after 3 d of culture in N2B27 medium. *Y* axis represents the RNA fold change at day 3 versus day 0 (ES cells) as measured by qRT-PCR. (*B*) Immunoblotting using an anti-Geminin antibody (N18; Santa Cruz) demonstrates that Geminin protein levels do not change substantially during 3 d of N2B27 differentiation. Actin serves as a loading control. (*C*) Quantitation of percentages of total ES cells and Geminin-positive ES cells in which Oct4 and Sox2 staining was detected by immunofluorescence during 2 d of N2B27 differentiation. Percentages were derived by counting at least 500 nuclei from multiple images and at least three experiments using the software ImageJ. (*D*) Immunofluorescence demonstrates Geminin colocalization with Oct4 and Sox2 after neural commitment in N2B27 medium for 2 d.



C

	Antisense sequence	Total sequence (including mir30 sequence)
#7	TTACTTATAAGATCAAAGG	TGCTGTTGACAGTGAGCG CGCCTTTGATCTTATAAGTAA ATAGTGAAGCCACAGATGTAT TTACTTATAAGATC AAAGG CTTGCCTACTGCCTCGGA
#9	TATTCTCCTTTCTCAGGCG	TGCTGTTGACAGTGAGCG ACCGCCTGAGAAAGGAGAATA ATAGTGAAGCCACAGATGTAT TATTCTCCTTTCTC AGGCG GGTGCCTACTGCCTCGGA
#11	ATTCTTCACATTCTCTTGG	TGCTGTTGACAGTGAGCG ACCCAAGAGAATGTGAAGAAT ATAGTGAAGCCACAGATGTAT ATTCTTCACATTCT CTTGG GCTGCCTACTGCCTCGGA

Fig. S2. Construction of ES clonal lines for inducible Geminin overexpression and knockdown. (*A*) Schematic of clonal lines derived from A2lox mouse ES cells. The A2lox ES cell line is a derivative of E14Tg2a, which was generated by Michael Kyba (Department of Pediatrics, University of Minnesota, Minneapolis) and is very similar to Ainv15 (1); see also refs. 2–4). These lines carry a reverse tetracycline transactivator targeted to the Rosa26 locus and a cassette with a tetracycline response element, loxP-lox2272 sites, and a neomycin resistance gene targeted to the Hprt locus. We established multiple clonal lines carrying the expression cassettes indicated by Cre-mediated recombination. These permit tightly controlled, Dox dose-dependent inducible control of gene expression. The GemOE lines overexpress Geminin protein fused with a 3× FLAG peptide at the amino terminus. For Geminin knockdown (GemKD lines), we used the RNAi Codex and miR30-based shRNAmir (second generation design) to generate Dox-inducible shRNA expression cassettes after Hannon and Elledge/Open Biosystems. Each of these ES lines carries the GFP ORF with sequences encoding the shRNA expression cassette inserted into the GFP 3′ untranslated region. Expression of both GFP and the shRNA is Dox dependent (Tet On). (*B*) Immunoblotting with goat anti-Geminin (N18; Santa Cruz) demonstrates the dose-dependent knockdown or overexpression of Geminin for one clonal line. (*C*) To control for potential off-target effects, we generated lines carrying the three independent microRNA targeting sequences shown, which each effectively reduce Geminin levels (blue and red highlighted sequences). Consistent with prior work by others (3), treatment of the control (GFP only expressing) A2lox line with 500 ng/mL Dox did not result in cytotoxicity or alter cell proliferation or expression of the neural genes assessed here (for example, Fig. 1*G* and Fig. S6*B*).

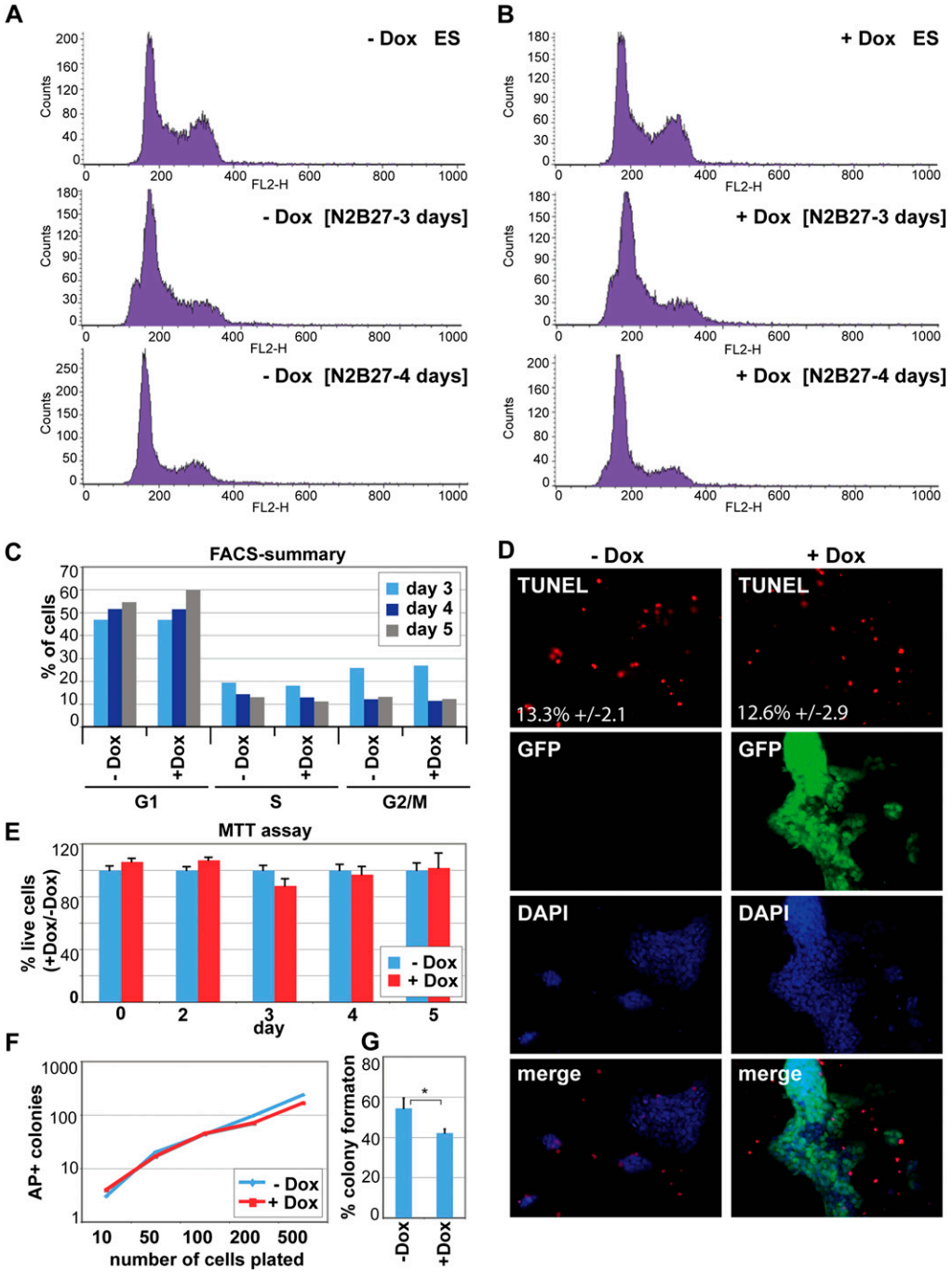


Fig. S3. Geminin knockdown does not affect cell cycle profile, cell ploidy, viability, or ES self-renewal. (*A* and *B*) Cell cycle analysis of ES cells undergoing neural commitment in N2B27 medium for 4 d with Dox-induced Geminin knockdown, compared with the same clonal ES line with no Dox treatment. The cells were stained with propidium iodide and analyzed by flow cytometry as described in *Materials and Methods*. The images show plots of forward versus side scatter. (*C*) Summary of flow cytometry data: percentages of GemKD cells in G1, S, and G2 phases of the cell cycle during days 3–5 of neural commitment in N2B27 medium. (*D*) TUNEL assays were used to score apoptotic cells on day 3 of commitment, with or without Geminin knockdown. At least 300 cells were counted to score percentages of TUNEL-positive cells (± SD) represented in the corresponding image panels. (*E*) MTT assays (*SI Materials and Methods*) were used to define percentages of viable cells in cultures with or without Dox treatment during days 0–5 of neural differentiation. The graph represents the viable cells upon Dox treatment during each day of N2B27 differentiation as a percentage of cells with no Dox treatment (*F* and *G*) Self-renewal of ES cells upon Geminin knockdown. (*F*) The *x* axis represents the number of GemKD cells plated on a 60-mm tissue culture dish for 5 d in ES growth medium +LIF (with or without Dox), and the *y* axis represents the number of colonies observed for the corresponding cells plated. (*G*) Percentages of GemKD cells forming colonies were scored 7–10 d after plating cells at a single-cell density in 96-well plates, with or without induction of Geminin knockdown. Percentages represent an average from three experimental replicates.

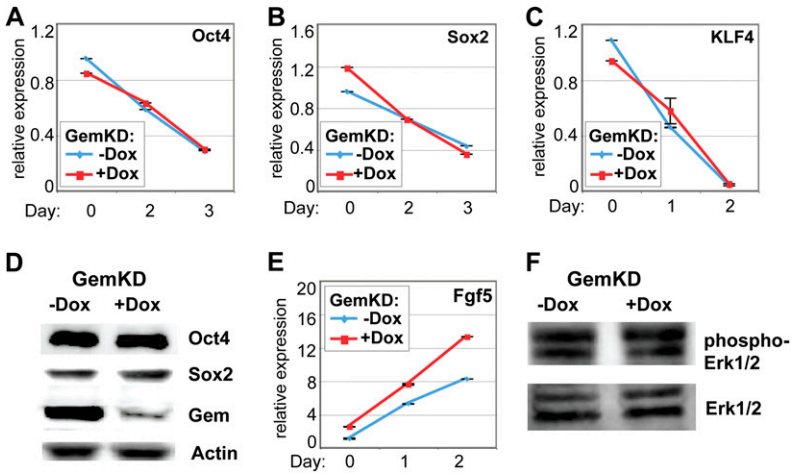


Fig. S4. Geminin knockdown does not affect the ability of ES cells to exit from pluripotency. (*A–F*) GemKD cells underwent neural fate acquisition in N2B27 medium for 3 d. (*A–C*) Quantitative RT-PCR and/or (*D*) immunoblotting showed no change in Oct4, Sox2, or KLF4 levels upon Geminin knockdown, relative to uninduced cells. Fold changes (*y* axis) are relative to day 0 minus Dox values. (*E* and *F*) Quantitative RT-PCR for the ectodermal marker Fgf5 (*E*) or immunoblotting for phosphorylated (activated) Erk1/2, normalized to total Erk1/2 (*F*). Neither Fgf5 expression nor phosphorylated (activated) Erk1/2 is reduced upon Geminin knockdown, relative to uninduced cells.

A. GemKD up-regulated genes--top functions

Category	Function	Function Annotation	P-value	Molecules
Organismal Development	development	development of organism	1.19E-07	CHRD, COL2A1, CRABP1, EFNB1, FGFR1, GLI2, GNA12, HOXB2, KIF3B, LDB1, NANOG, NKX3-1, PIM1,
Organismal Development	developmental process	developmental process of organism	4.46E-06	CHRD, COL2A1, CRABP1, EFNB1, FGFR1, GLI2, GNA12, HOXB2, KIF3B, LDB1, NANOG, NKX3-1, PIM1, RARA, SFRP1, SKI, SUZ12, TRIM24, TYRO3, ZEB2
Cellular Growth and Proliferation	proliferation	proliferation of cells	9.77E-06	AXIN2, BAK1, CHRD, CITED2, COL2A1, EFNB1, EMP2, FGFR1, FSCN1, GLI2,
Embryonic Development	development	development of embryo	1.47E-05	CHRD, COL2A1, EFNB1, FGFR1, GLI2, GNA12, NANOG, SFRP1, SKI, ZEB2
Embryonic Development	specification	specification of body axis	1.82E-05	CITED2, KIF3B, LDB1, LEFTY1, PITX2

B. GemKD up-regulated genes--top canonical pathways

Pathway	-Log(P-value)	Ratio	Molecules
Wnt/Beta-catenin Signaling	3.05E+00	3.59E-02	SOX1, SFRP1, TCF7L2, AXIN2, LDB1, PITX2
Ephrin Receptor Signaling	2.94E+00	3.21E-02	GNA12, ITGA5, RAPGEF1, EFNB1, EPHA4, SHC1
BMP/TGF-Beta Signaling	2.31E+00	3.88E-02	CHRD, TYRO3, FGFR1, PITX2, CITED2, LEFTY1
RAR Activation	2.21E+00	2.67E-02	CRABP1, RDH10, CITED2, TRIM24, RARA
Axonal Guidance Signaling	1.91E+00	1.79E-02	GNA12, ITGA5, GLI2, EFNB1, EPHA4, SHC1, ABLIM1

C. GemKD down-regulated genes--top functions

Category	Function	Function Annotation	P-value	Molecules
Cellular Compromise	neurodegeneration	neurodegeneration of neurons	5.81E-05	BDNF, KIF1A, SNCA, TGFA
Neurological Disease	neurodegeneration	neurodegeneration of neurons	5.81E-05	BDNF, KIF1A, SNCA, TGFA
Neurological Disease	disease	disease of neurons	1.73E-04	BDNF, KIF1A, SNCA, TGFA
Neurological Disease	survival	survival of neuroblastoma cell lines	1.25E-03	BDNF, SNCA
Neurological Disease	hypertrophy	hypertrophy of cell body	3.81E-03	BDNF

D. GemKD down-regulated genes--top canonical pathways

Pathway	-Log(P-value)	Ratio	Molecules
Arachidonic Acid Metabolism	1.92E+00	1.42E-02	PLA2G10, CBR3, CYP4X1
Purine Metabolism	1.65E+00	9.57E-03	PDE1A, ENPP2, ADCY1, RECQL5
Linoleic Acid Metabolism	1.35E+00	1.61E-02	PLA2G10, CYP4X1
Huntington's Disease Signaling	1.33E+00	1.29E-02	BDNF, NEUROD1, SNCA
p38 MAPK Signaling	1.30E+00	2.08E-02	PLA2G10, MEF2A

E. Examples of GemKD up-regulated genes

Gene symbol: name	Role in early fate	Expression in early embryo*	References
Cited2: Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	Required for left-right axis establishment; regulates Nodal, Lefty2 and Pitx2 expression in the lateral mesoderm, and Lefty1 expression in the presumptive floor plate	At e7.0, highly expressed in anterior endoderm and low in the posterior endoderm, also expressed in nascent mesoderm and head process	Weninger et al. Development (2005) vol. 132 (6) pp. 1337-48; Bamforth et al. Nat Genet (2004) vol. 36 (11) pp. 1189-96
Pitx2: paired-like homeodomain transcription factor 2	Nodal signaling target, a Nodal-Pitx2 regulatory circuit is required to establish left-right asymmetry.	Expressed in node at e7.0, then left lateral plate mesoderm (e8.0)	Ryan et al. (1998) vol. 394 (6693) pp. 545-51; Logan et al. Cell (1998) vol. 94 (3) pp. 307-17
Mid1: midline 1	B-box protein, part of a microtubule-associated complex, mutations cause midline defects in embryogenesis and can lead to human Opitz syndrome (OS), a genetic disease characterized by deformities such as cleft palate. Required for left-right asymmetry establishment in chick, through action on the right side of the node.	In chick node, Mid1 expression is bilateral but then restricts to right side of the node. In mouse, may be ubiquitously expressed at e9-10	Buchner et al. Hum Mol Genet (1999) vol. 8 (8) pp. 1397-407; Granata et al. Dev Biol (2005) vol. 277 (2) pp. 417-24; Granata and Quaderi. Dev Biol (2003) vol. 258 (2) pp. 397-405
Lefty1: left right determination factor 1	Nodal signaling target and Nodal feedback inhibitor; inhibits Nodal signaling on the left side of the node to establish left-right laterality.	transient left-sided expression in mouse gastrula; at e7.0 in definitive endoderm and anterior visceral endoderm emerging from the anterior primitive streak,	Meno et al. Nature (1996) vol. 381 (6578) pp. 151-5; Meno et al. Cell (1998) vol. 94 (3) pp. 287-97
Kif3b: kinesin family member 3B	required for assembly of monocilia in the gastrula node; homozygous mutants have no node mono-cilia and left-right asymmetry is randomized.	present from 2 cell stage; expressed in the mesoderm of the gastrula node, where it localizes to monocilia.	Nonaka et al. Cell (1998) vol. 95 (6) pp. 829-37

*From Mouse Genome Informatics (MGI) annotated expression data

Fig. S5. (Continued)

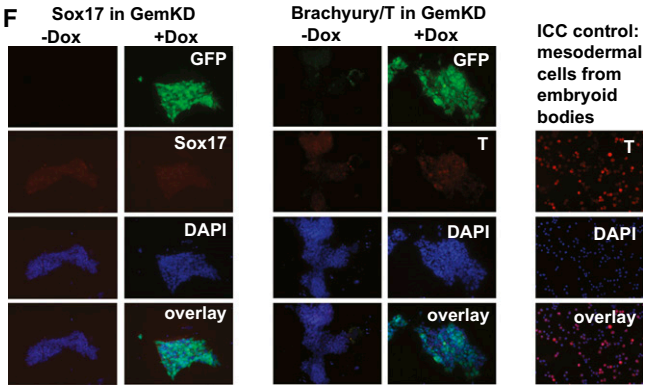


Fig. S5. Summary of Ingenuity Pathway Analysis (IPA) of differentially regulated genes upon Geminin knockdown. GemKD cells differentiated in N2B27 medium for 2 d with or without Dox treatment were used for microarray analysis using Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Genes that met threshold cutoffs and showed differential regulation in two out of three arrays were further analyzed with the IPA software suite. (*A*–*D*) Top functional categories and canonical pathways represented among genes that were up-regulated or down-regulated upon Geminin knockdown are shown. (*E*) Group of genes up-regulated under conditions of Geminin knockdown, as defined by microarray analysis. These include genes that function or are expressed in the mouse gastrula node. Expression data are derived from the Mouse Genome Informatics database. (*F*) Cells underwent N2B27-mediated neural fate acquisition for 3 d, with or without Geminin knockdown, and then were immunostained for endodermal (Sox17, *Left*) or mesodermal [Brachyury (T), *Center*] markers, to determine whether loss of neural fate resulted in up-regulation of mesendodermal gene expression. No staining above background was observed either under uninduced or Geminin knockdown conditions. (*Right*) as a positive control for the immunocytochemistry procedure, ES cells were induced to form embryoid bodies in serum-containing medium, after ref. 2, were dissociated after 4 d, and were immunostained for Brachyury, which is highly expressed in many of these cells.

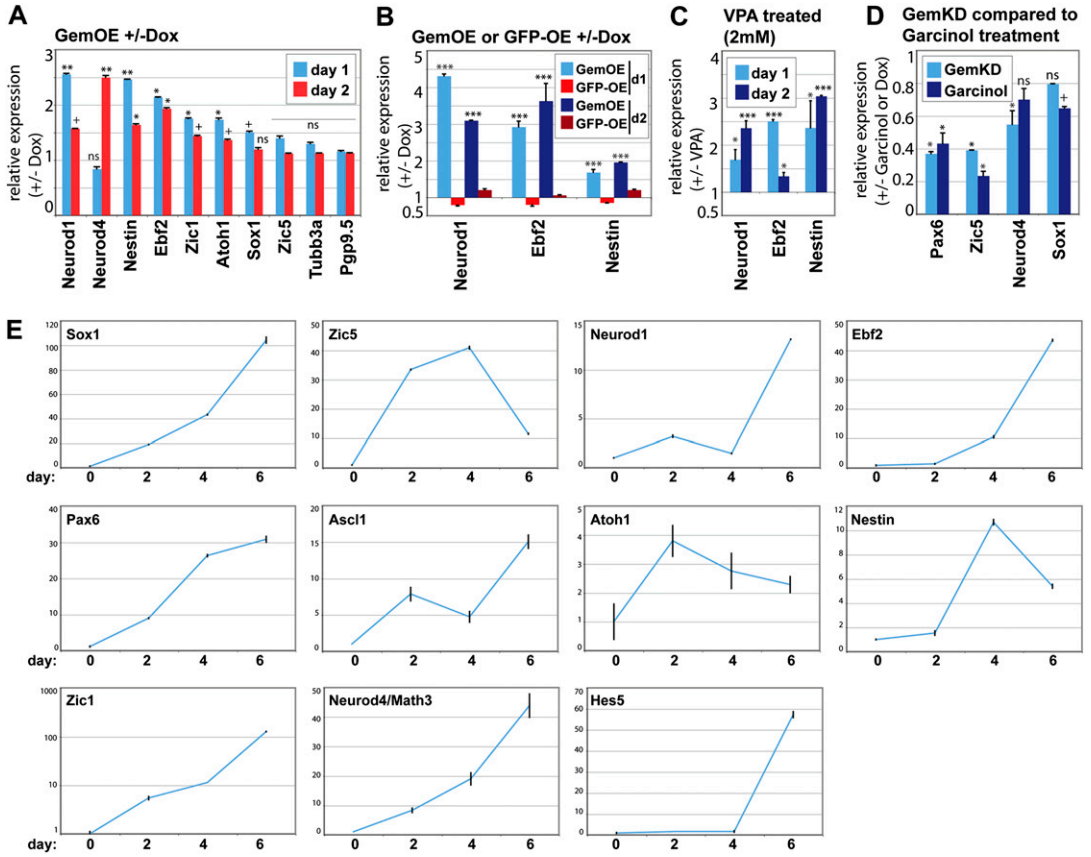


Fig. S6. Early activation of neural gene expression by Geminin overexpression or valproic acid treatment. (*A*) GemOE cells underwent neural commitment in N2B27 medium with Dox-induced Geminin overexpression for 1 or 2 d and changes in neural gene expression (relative to the uninduced −Dox condition) were analyzed by qRT-PCR. (*B* and *C*) GemOE cells were differentiated in N2B27 medium with or without (*B*) Geminin overexpression by the addition of 500 ng Dox for 24 h or (*C*) 2 mM VPA treatment for 8 h. Cells were harvested on days 1 and 2 (d1/d2) after either Geminin overexpression or VPA treatment, and expression levels of the neuronal genes Neurod1, Ebf2, and Nestin were analyzed by qRT-PCR. The *y* axis represents the fold change in gene expression after Dox or VPA treatment compared with untreated cells. In *B*, effects of Dox-induced Geminin overexpression are compared with Dox treatment of the parental A2lox line, which induces GFP overexpression (GFP-OE). Whereas Geminin overexpression increases neural gene expression, Dox-induction of GFP in the A2lox parental line does not affect neural gene expression. (*D*) GemKD cells were differentiated in N2B27 medium with Dox-induced Geminin knockdown for 2 d or treatment with 10 μM garcinol for 4 h. Cells were harvested and the expression levels of the neural genes Pax6, Zic5, Neurod4, and Sox1 were analyzed by qRT-PCR. The *y* axis represents the fold change in gene expression after Dox or garcinol treatment compared with untreated cells. (*E*) ES cells were plated on 0.1% gelatinized dishes at a density of 1×10^4 cells/cm^2 in N2B27 medium and harvested on days 0, 2, 4, and 6. Total RNA was extracted and the expression pattern of neural genes was analyzed by qRT-PCR and represented relative to expression at day 0.

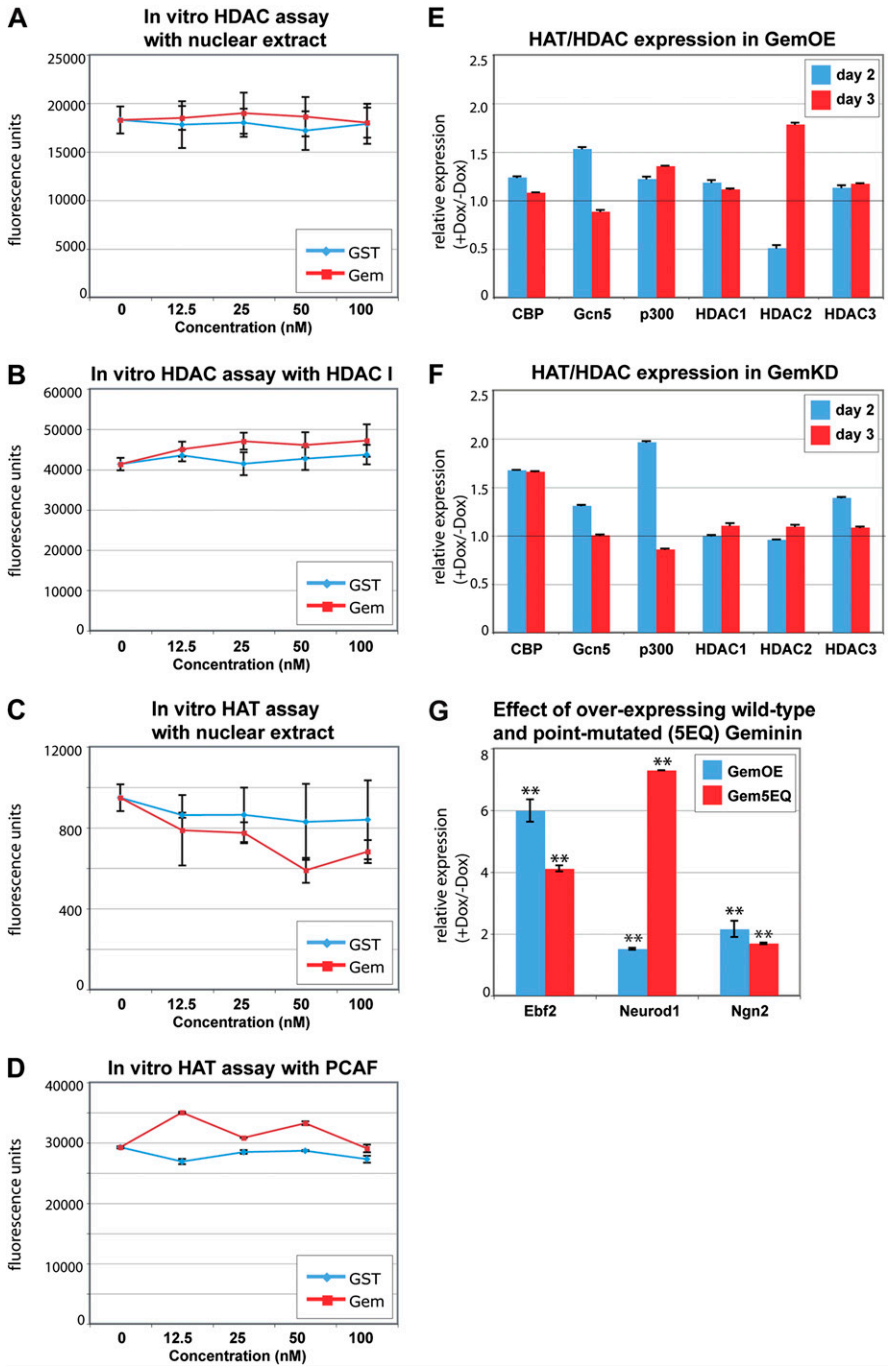


Fig. S7. Geminin does not affect expression levels or in vitro enzymatic activity of HDACs and HATs. The effect of increasing concentrations of Geminin-GST or GST (0, 12.5, 25, 50, or 100 nM) on HDAC activity was assayed, using (*A*) mammalian nuclear extracts (incubated with 20 mM garcinol) or (*B*) purified HDAC I (Cayman) with an HDAC activity assay kit (Cayman). The HDAC assay was done as per the manufacturer's instructions. Similarly, the effect of increasing concentrations of Geminin-GST or GST (0, 12.5, 25, 50, or 100 nM) on HAT activity was assayed, using (*C*) mammalian nuclear extracts (incubated with 100 nM TSA) or (*D*) purified PCAF (Cayman) with a HAT activity assay kit (Cayman), performed per the manufacturer's instructions. (*E* and *F*) Changes in mRNA expression levels of three HATs (CBP, Gcn5, and p300) and three HDACs (HDAC1–3) were assessed by qRT-PCR under conditions of Geminin overexpression and knockdown by Dox addition to GemOE (*E*) and Gem KD (*F*) lines. Altering Geminin levels did not consistently alter expression levels of any of the HAT/HDACs tested. (*G*) Loss of Brg1 binding does not affect Geminin's ability to increase neural gene expression. Clonal lines of ES cells were differentiated for 2 d in N2B27 medium with or without the Dox-induced overexpression of wild type Geminin or its mutant form (an animo acid substituted variant, 5EQ) that does not bind to Brg1 (5). Total RNA was extracted and the genes depicted in the graph were analyzed using qRT-PCR.

1. Kyba M, Perlingeiro RC, Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109: 29–37.
2. Lugus JJ, et al. (2007) GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* 134:393–405.
3. Lee D, et al. (2008) ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell* 2:497–507.
4. Lindsley RC, Gill JG, Kyba M, Murphy TL, Murphy KM (2006) Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133: 3787–3796.
5. Seo S, et al. (2005) Geminin regulates neuronal differentiation by antagonizing Brg1 activity. *Genes Dev* 19:1723–1734.

Table S1. Primers used for quantitative RT-PCR

	Primer sequence	
Gene name	Forward	Reverse
Rpl19	TCAGGCTACAGAAGAGGCTTGC	ACAGTCACAGGCTTGCGGATG
Sox1	CACGCGCATCCTCACAA	CATGTAGCCCTGAGAGTTGGA
Pax6	CGGTGAGCAGATGTGTGAG	ACTCCGCTGTGACTGTTCTG
Oct3/4	AAGAGGATCACCTTGGGGTA	CCGCAGCTTACACATGTTCT
Sox2	AGCTCGCAGACCTACATGAA	CCTCGGACTTGACCACAGA
Nanog	CCAGTCCCAAACAAAAGCTC	TGAAACCTGTCCTTGAGTGC
Klf4	GCCCCAAGATTAAGCAAGAG	GGGAAGTCGTGTGTGTTGG
Zic5	TGGTGTTCACTCTGCCAATCGC	TGTGTCCGGGCCTCCTAGCTG
Neurod1	CTCAACCCTCGGACTTTCTT	CCAGGGGACTGGTAGGAGTA
Ebf2	AGGATACATCCGCAACACAA	CTGTAGCCGTTCATGCTGTT
Nestin	CTTGCAGACACCTGGAAGAA	AAGGGGGAAGAGAAGGATGT
Ascl1	GCCAACAAGAAGATGAGCAA	TGGAGTAGTTGGGGGAGATG
Atoh1	GCAAGGGAACGGCGCAGGAT	TTGCAGGAAGCTGTGGGCGG
Zic1	GTCCTCTTCTCAGGGCTCAC	TGTTGTGGGAGACACGATG
Neurod4	TCTGGGCCTTGTCTGAAGTC	TCCAGGAGGGTAGATTGAGG
Hes5	TGAAACACAGCAAAGCCTTC	GTGCAGGGTCAGGAACTGTA
Tubb3a	CCCAAGTGAAGTTGCTCGCAG	ACAGAGCCAAGTGGACTCACAT
Pgp9.5	GCTCCCGTCTCCCCTGCTCA	TTCGCGGATGGCACCTGCAG
CBP	AGACCCCAGTGCAGCCACCA	AAAGCGGTGTGCCAGGAGGC
Gcn5	GGACTGAGCCCATGCCAGGGGAG	GGGATGTCCCCCATCACGCGG
P300	GCTGGGGACTGCGTCTGTAGAGC	TAAACCAGGCCGGGAGGAGGGT
HDAC1	TCTGACCATCAAAGGACACG	AACATTCCGGATGGTGTAGC
HDAC2	TCTGTGCCCTACGGCCCTCC	CTGCAGCCCTCGGAGCAAGG
HDAC3	GCTCCCGTTACACAGGCGCA	CCACCGGCCCAGTTGATGGC

Table S2. Antibodies used for immunofluorescence, immunoblotting, and/or quantitative ChIP

Protein	Company	Catalog no.
Geminin (N18)	Santa Cruz	sc-8449
Geminin (FL209)	Santa Cruz	sc-13015
Sox2	Millipore	AB5603
Pax6	Millipore	AB2237
Sox1	R&D Systems	AF3369
Oct3/4	Santa Cruz	sc5279
Erk1/2	Cell Signaling	9102
Phosphorylated Erk1/2	Cell Signaling	4695
Brachyury	R&D Systems	AF2085
Sox17	R&D Systems	AF1924
Actin	Santa Cruz	sc-10731
ACH3	Millipore	06–599
ACH4	Millipore	06–866
H3K9Ac	Abcam	Ab10812
H4	Millipore	07–108
IgG	Millipore	12–370
H3K27 (me3)	Abcam	ab002
H3K4 (me3)	Cell Signaling	9751S
Pol II	Santa Cruz	sc-56767

Table S3. Primers used for chromatin immunoprecipitation and nuclease accessibility assay

Gene name	Primer sequence		Position (TSS)
	Forward	Reverse	
Sox1	TTTGCACAGTTCAGCCCTGAGTGA	GGTGCACAAACCACTTGCCAAAGA	−313
Pax6	CTTGCGAAAGTTGGTGTGTTCCCT	CTTGGTCAATGGAGACACGGGAAA	342
Atoh1	TGCAGAAGAGTGGGCTGAGGTAAA	TGCTATCCAGGAGGGACAGTTCT	187
Neurod1	CTTTGCATGCGCACATTTGTGGAG	TACGTGCGAGTACTTGTGGGCAAT	155
Ebf2	AAGGCACCCACACACTATCTTCCA	AACAGCTGCCGCCCAAATTATACC	−987
Nonpromoter control: −4,870 from Neurod1 transcription start site	CGCGTTTCTTTGATCAATCC	GAGCAAGCACCCTTAAACCA	−4,870

TSS, transcription start site.