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Recommended Citation

Yellajoshiyula, Dhananjay; Lim, Jong-won; Thompson, Dominic M.; Witt, Jacob S.; Patterson, Ethan S.; and Kroll, Kristen L., "Geminin regulates the transcriptional and epigenetic status of neuronal fate-promoting genes during mammalian neurogenesis." *Molecular and Cellular Biology*. 32, 22. 4549-4560. (2012).
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Mol. Cell. Biol. 2012, 32(22):4549. DOI: 10.1128/MCB.00737-12.

Published Ahead of Print 4 September 2012.

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Geminin Regulates the Transcriptional and Epigenetic Status of Neuronal Fate-Promoting Genes during Mammalian Neurogenesis

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Regulating the transition from lineage-restricted progenitors to terminally differentiated cells is a central aspect of nervous system development. Here, we investigated the role of the nucleoprotein geminin in regulating neurogenesis at a mechanistic level during both *Xenopus* primary neurogenesis and mammalian neuronal differentiation *in vitro*. The latter work utilized neural cells derived from embryonic stem and embryonal carcinoma cells *in vitro* and neural stem cells from mouse forebrain. In all of these contexts, geminin antagonized the ability of neural basic helix-loop-helix (bHLH) transcription factors to activate transcriptional programs promoting neurogenesis. Furthermore, geminin promoted a bivalent chromatin state, characterized by the presence of both activating and repressive histone modifications, at genes encoding transcription factors that promote neurogenesis. This epigenetic state restrains the expression of genes that regulate commitment of undifferentiated stem and neuronal precursor cells to neuronal lineages. However, maintaining geminin at high levels was not sufficient to prevent terminal neuronal differentiation. Therefore, these data support a model whereby geminin promotes the neuronal precursor cell state by modulating both the epigenetic status and expression of genes encoding neurogenesis-promoting factors. Additional developmental signals acting in these cells can then control their transition toward terminal neuronal or glial differentiation during mammalian neurogenesis.

Transcriptional and epigenetic control of neuronal gene expression plays a major role in the temporal and spatial regulation of nervous system development. Developmental genes, including those regulating neurogenesis and neuronal commitment and differentiation, are maintained in a repressive chromatin context through the activity of the Polycomb (PcG) repressor complex. PcG catalyzes a repressive chromatin modification (trimethylation of histone H3 lysine 27 [H3K27me3]) at genes involved in cell specification and differentiation to prevent their premature expression in embryonic stem (ES) cells (2). At developmental genes in both pluripotent stem and multipotent precursor cells, this repressive modification is frequently accompanied by the H3K4me3 histone modification, which is catalyzed by mixed-lineage leukemia (MLL) complexes and promotes active transcription (2, 16). Together, this bivalent combination of activating and repressive histone modifications retains developmental genes in a poised but repressed state (1, 14). During neurogenesis, this chromatin state changes at genes encoding factors that drive neuronal specification, commitment, and differentiation to promote their expression. Concomitant with high levels of transcription, the repressive H3K27me3 modification is lost, while the locus becomes highly enriched for H3K4me3 and histone acetylation, which promote target gene transactivation (16). These chromatin state changes are likely to facilitate the activities of the neural basic helix-loop-helix (bHLH) transcription factors, which play critical roles in activating gene programs that drive neurogenesis and neuronal differentiation.

Among the potential regulators of gene expression during neurogenesis, there is increasing evidence for the active involvement of the small nucleoprotein geminin (Gmnn or Gem). Gem was initially identified as a dual-function protein that promotes neural fate acquisition in the *Xenopus* embryo (9) and controls the fidelity of DNA replication (13), through its physical interaction with and functional antagonism of Cdt1 (25, 27). Gem is highly expressed in early embryonic tissues in vertebrates and plays a role in

regulating multiple developmental processes, including maintenance of the pluripotent cell state (5, 11, 28) and control of neural cell fate acquisition from pluripotent cells (9, 17, 20, 30). In the nervous system, Gem is highly expressed in early neural precursor cells (20, 23, 30). As committed neuronal precursor cells receive spatial and temporal cues to differentiate into postmitotic neurons, geminin expression is downregulated in concert with other neural progenitor-specific markers (20, 23). In *Xenopus*, forced expression of geminin at these stages antagonizes the expression of markers of terminal neuronal differentiation, such as neuron-specific β -III tubulin, while neural progenitor marker expression is retained (20). Therefore, in *Xenopus*, geminin plays a role in promoting initial neural fate acquisition while maintaining the neural precursor state and preventing terminal neuronal differentiation.

Studies to assess the role of geminin in mammalian neurogenesis and neural stem cell (NSC) maintenance have yielded mixed information. Loss of geminin in cortical neural progenitor cells (NPCs) surprisingly resulted in increases in NPC cell number and in decreased production of neurons in ventricular zone regions, suggesting a positive role for geminin in promoting cortical neu-

Received 1 June 2012 Returned for modification 25 June 2012

Accepted 30 August 2012

Published ahead of print 4 September 2012

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Supplemental material for this article may be found at <http://mcb.asm.org/>.

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doi:10.1128/MCB.00737-12

rogenesis (24). However, in another study, loss of geminin had no effect on the cycling NSC population in the subventricular region and hippocampal dentate gyrus, nor did it affect the ability of these NSCs to proliferate or further differentiate into neuronal and glial cell types *in vitro* (19). Another recent study reported complex actions of geminin during mammalian neurogenesis, where both knockdown and overexpression of geminin resulted in decreased numbers of Sox3-positive neural precursor cells in the mouse embryo. Likewise, geminin knockdown resulted in the premature appearance of β -III tubulin-positive cells, while overexpression of geminin also increased numbers of β -III tubulin-positive cells (3). These results suggest that geminin may have a context-dependent role in regulating the commitment and differentiation of mammalian neural progenitor cells, unlike *Xenopus* primary neurogenesis, where geminin unequivocally promotes neural precursor maintenance and blocks neuronal differentiation. It is difficult to understand the basis of the complex and dissimilar results obtained upon manipulating Gem's activities during mammalian neurogenesis, in part because of the gap in our understanding of the mechanism by which Gem regulates neurogenesis.

Here, we assessed geminin's activity in neural precursor cells at a mechanistic level, determining its effects on the transcriptional and epigenetic status of genes encoding transcription factors that promote neurogenesis and on the transcriptional activity of these factors. We found that geminin antagonizes the ability of neural bHLH transcription factors to activate transcriptional programs during neuronal commitment and differentiation. These effects are seen both during *Xenopus* primary neurogenesis and in mammalian neural precursor cells undergoing neurogenesis *in vitro*. At neural bHLH target genes, Gem regulates both the retention and levels of enrichment of PcG proteins and of the PcG-mediated repressive trimethylation of H3K27 (H3K27me3). Gem's ability to inhibit gene expression during neurogenesis correlates with an active role in maintaining a bivalent epigenetic state at these neural gene promoters, whereby they maintain high levels of both activating H3K4me3 and repressive H3K27me3 methylation. This epigenetic signature is characteristic of the poised but repressed state used to restrain the expression of developmental genes in undifferentiated stem and precursor cells (1, 16). However, maintaining geminin at high levels is not sufficient to prevent ES cell-derived neural precursor cells or NSCs obtained from mouse forebrain from undergoing terminal neuronal differentiation. Therefore, we propose that geminin contributes to restraining gene programs that drive neuronal commitment in mammalian neural precursor cells and that its activity is subject to additional molecular cues to control neuronal differentiation.

MATERIALS AND METHODS

Xenopus. Preparation of capped mRNAs encoding neurogenin (Ngnr1), Ngnr1-glucocorticoid receptor (Ngnr1-GR), and geminin Δ coil, *Xenopus* embryo microinjection, embryo *in situ* hybridization, and ectodermal explant isolation were performed as previously described (11, 21). For microarrays, 10 pg Ngnr1-GR with or without 100 pg Gem Δ coil RNA was injected into each cell of a 2-cell embryo and explant isolation, treatment, and subsequent microarray analysis were performed as previously described (21).

Cell culture. Culture of ES cells and generation of stable clonal lines, induction of Gem overexpression (OE) and knockdown (KD), and production of neuronal progenitors and differentiation into neuronal lineages (using N2B27 medium and monolayer culture) were performed as

previously described (30, 31). Culture of P19 cells and P19 cell differentiation into neuronal lineages using retinoic acid (RA) were done as previously described (29). P19 cells were transfected with US2MT-Ngn2, US2MT-Neurod1 (a kind gift of David L. Turner), or US2MT-geminin expression vectors; gene expression was driven from the human ubiquitin C promoter (US2), and proteins contained an amino-terminal 6 \times Myc tag (MT) as previously described (21). For generating P19 stable clonal lines overexpressing doxycycline (Dox)-inducible geminin, we used the Tet-Express inducible expression system (Clontech) to generate stable, clonal lines of P19 cells that carried both the prtTA and pTRE-geminin plasmids. Reverse transcription and quantitative reverse transcription-PCR (qRT-PCR) from *Xenopus* ectodermal explants and P19 and ES cells and immunocytochemistry (ICC) in ES cells were done as previously described (11, 21, 30). Primers for qRT-PCR and antibodies for ICC are described in Table S1 in the supplemental material.

Lentiviral production and transduction. Production of lentiviral particles from pGIPZ geminin microRNA-adapted short hairpin RNA (shRNAmir) (Open Biosystems) was done in 293T cells. The day before the transfection, 293T cells were seeded in a 6-well dish. Transfection was done with Fugene 6 (Roche) according to the manufacturer's instructions using 1 μ g of the lentiviral transfer vector (pGIPZ-geminin shRNAmir) and 1.1 μ g of the second-generation lentiviral packaging vector combination (1 μ g of delta-8.9 and 100 ng of pVSV-G) (Addgene). After 48 h, the supernatant containing the virus was collected, and 10^5 primary neural stem cells were infected with 100 μ l of lentiviral supernatant overnight, followed by medium change and puromycin (0.5 μ g/ml) selection to isolate a pure population of geminin knockdown primary NSCs.

qChIP. Quantitative chromatin immunoprecipitation (qChIP) was done as described in detail previously (30). Briefly, sheared chromatin (sonicated to 200 to 500 bp) was obtained from 2×10^6 ES or P19 cells that were fixed in 1% formaldehyde (Sigma) in Dulbecco modified Eagle medium (DMEM) plus 5% fetal bovine serum (FBS). Chromatin was incubated with 5 μ g antibody or with a corresponding isotype-matched non-specific antibody (IgG) using Dynabeads (Invitrogen) as per the manufacturer's instructions. Antibodies used for ChIP are described in Table S1 in the supplemental material. After washing, elution, and cross-link reversal, DNA from each ChIP sample and the corresponding input sample was purified and further analyzed using quantitative PCR (qPCR). Each ChIP sample and a range of dilutions of the corresponding input sample (0.01% to 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). qChIP data for each primer pair are represented as percent input by calculating amounts of each specific DNA fragment in immunoprecipitates (and in their corresponding isotype-matched nonspecific antibody [IgG] control immunoprecipitation) relative to the quantity of that fragment in input DNA. The final values represent the percent input for the antibody of interest after subtraction of the percent input value obtained for a control immunoprecipitation of the same sample with isotype-matched nonspecific antibody (IgG). Gene-specific primer sequences used for ChIP and the genomic location of the resulting amplicon with respect to the transcription start site (TSS) are indicated in Table S1 in the supplemental material.

RNA-seq. Total RNA was isolated from Gem KD cells differentiated in N2B27 medium for 5 days with or without addition of 500 ng/ml Dox for Gem knockdown from day 3. These samples were used for RNA-seq analysis (Illumina HiSeq2000 platform) by the Genome Technology Access Center (GTAC) at Washington University School of Medicine, St. Louis, MO. Three independent RNA-seq experiments were conducted to generate paired control (no-Dox) and geminin knockdown (plus-Dox) samples, and these were analyzed using TopHat and Cufflinks/Cuffdiff (26). Transcripts were considered differentially expressed upon Gem knockdown if data met statistical significance cutoffs in Cuffdiff (i.e., sufficient sequence alignments were obtained for analysis and the transcript had a significant change in FPKM value [normalized transcript abundance; fragments per kb of exon per million fragments mapped] between the

no-Dox and plus-Dox sample pairs) in at least two of the three independent experiments.

NSC derivation and differentiation. Neural stem cells (NSCs) were derived from embryonic brain tissue as previously described (18). NSCs were derived from ES cells with modifications to the same protocol (18). Briefly, ES cells were plated for monolayer differentiation in N2B27 medium for 7 days as previously described (31). After 7 days, cells were collected and plated for neurosphere formation in suspension dishes in modified N2B27 medium supplemented with 2× N2 medium (Invitrogen) for 1 to 2 weeks. The neurospheres formed by this procedure were plated on laminin-coated dishes in NSC growth medium (18) supplemented with 20 ng/ml Fgf2 and 20 ng/ml epidermal growth factor (EGF) for 1 week. NSCs derived from these neurospheres were further propagated. Neuronal and glial differentiation of both primary and ES-derived NSC cells was done as previously described (18). Briefly, NSCs were plated in NSC growth medium with 2% FBS for 3 days to derive glial fibrillary acidic protein (GFAP)-positive glial cells. For neuronal cells, the NSCs were plated in DMEM-F12 medium supplemented with 1XB27, 1XN2, and 5 ng/ml Fgf2 for 3 days, after which the same medium without Fgf2 supplementation was used.

Accession numbers. The microarray and RNA-seq data have been deposited into the Gene Expression Omnibus under accession numbers GSE39657 and GSE39658 (SuperSeries GSE39673).

RESULTS

Geminin antagonizes neural bHLH transcription factor-regulated gene expression programs during neurogenesis. Prior work has demonstrated that geminin plays an important role in regulating neural development and neuronal differentiation during primary neurogenesis in the *Xenopus* embryo (9, 20). As Gem is a nuclear protein with known interactions with chromatin-regulatory proteins, we hypothesized that Gem's role involves effects on transcriptional regulation of genes that control neurogenesis. Therefore, we tested whether Gem could antagonize transcriptional responses to neural bHLH transcription factors in the *Xenopus* embryo. As in our prior work, we controlled transcription factor activity by using a variant of the *Xenopus* neurogenin (Ngnr1) transcription factor fused to the ligand binding domain of the human glucocorticoid receptor (GR) (21). Prior to addition of the ligand dexamethasone (DEX), this fusion protein is sequestered in the cytoplasm and remains transcriptionally inactive (8). Upon DEX addition, Ngnr1-GR translocates into the nucleus and regulates target genes. We induced Ngnr1-GR activity in the presence of the protein synthesis inhibitor cycloheximide, which blocks translation of secondary targets whose expression is regulated by primary target proteins. This approach allowed us to identify 57 direct transcriptional targets of Ngnr1 (21) (see Table S2 in the supplemental material).

Here, we combined this manipulation with overexpression of geminin, using a Gem variant (geminin Δ coil) that lacks the Cdt1 interaction domain. In our prior work, this variant maintained Gem's ability to inhibit neuronal differentiation in *Xenopus* and had no effects on cell cycle progression or cell death (11, 22). Therefore, it can be used to assess Gem's ability to antagonize Ngnr1-mediated transactivation of its targets, while precluding indirect effects stemming from perturbation of the cell cycle. Simultaneous coexpression of Ngnr1-GR with geminin Δ coil blocked Ngnr1-mediated activation of 44 of the 57 target genes (77%) (see Table S2 in the supplemental material). We confirmed Gem's ability to repress Ngnr1-mediated activation of a group of these genes in ectodermal explants (Fig. 1A). We also injected *Xenopus* embryos at the two-cell stage with RNA encoding Ngnr1

(non-GR fused), with or without Gem Δ coil RNA (and with β -galactosidase RNA coinjected as a lineage tracer, visualized as blue staining and oriented to the right in Fig. 1B). We assessed the *in vivo* effects at the early neurula stage (stage 14 to 15) by *in situ* hybridization for Ngnr1 target genes (Calponin, Dll1, Ebf3, Cbfa2t2, Myt1, Neurod4, and Amotl1 genes; purple stain) (Fig. 1B). Coinjection of Gem suppressed the Ngnr1-activated expression of all of these target genes in the *in vivo* context of the *Xenopus* embryo. From these data, we concluded that Gem antagonizes a significant portion of the Ngnr1-activated transcriptional program, which could account for Gem's ability to block primary neuronal differentiation in the *Xenopus* embryo.

In mammalian P19 embryonal carcinoma cells, overexpression of neural bHLH proteins such as Neurod1 and neurogenin 2 (Ngn2) (4) or treatment with retinoic acid (RA) (29) promotes neuronal commitment and differentiation. We wanted to test whether Gem's ability to antagonize neural bHLH-driven transcriptional programs and neuronal differentiation is a function also observed in mammalian cells. Transfection of P19 cells with expression plasmids for the neuronal bHLH protein Neurod1 or Ngn2 or differentiation in defined medium supplemented with 5 μ M RA (see Materials and Methods) induced the expression of previously defined Neurod1 and Ngn2 target genes (Fig. 1C to E) (21). However, simultaneous expression of Gem along with Ngn2, Neurod1, or RA treatment suppressed expression of all of these genes to various degrees (20 to 60%) (Fig. 1C to E). These results indicate that in mammalian cells, as in the *Xenopus* embryo, Gem antagonizes neural bHLH-driven transactivation of target genes that promote neurogenesis.

Changes in the epigenetic state of neurogenesis-regulatory genes during neuronal commitment. The ability of geminin to antagonize neural bHLH-driven neurogenesis, both in mammalian cells and in the *Xenopus* embryo, suggests a fundamental mechanism for Gem-mediated transcriptional regulation of genes that promote neurogenesis or are associated with neuronal commitment. Furthermore, we previously found that Gem regulates the chromatin structure of neural genes as pluripotent ES cells undergo the initial process of neural fate acquisition (30). Therefore, we hypothesized that Gem regulates chromatin structure at genes that promote neurogenesis, altering their ability to be transactivated by bHLH transcription factors. To test this hypothesis, we focused on chromatin-regulatory protein complexes and histone modifications that were likely to be involved in regulation of gene expression in neural precursors. We selected three candidate genes (Neurod1, Ebf2, and Dll1 genes) whose ability to be transactivated by neural bHLH proteins during neurogenesis was antagonized by Gem both in *Xenopus* and in P19 cells. Interestingly, all of these genes are in a bivalent state in pluripotent ES cells, indicating that their expression is subject to epigenetic regulation during development (1, 2).

Using multiple primer pairs at a 1-kb resolution from 5 kb upstream to 5 kb downstream, relative to the transcription start site, we initially defined the location and temporal changes in regulatory protein occupancy and histone modification status at the Neurod1 locus in undifferentiated P19 EC cells (day 0) and at day 4 of RA-mediated neuronal differentiation (Fig. 2A). We found that the PcG repressor complex, as represented by the PcG subunits Suz12 and Mel18, was highly enriched at the Neurod1 transcription start site (TSS) in undifferentiated P19 cells, when the gene is not expressed. The PcG-mediated repressive histone mod-

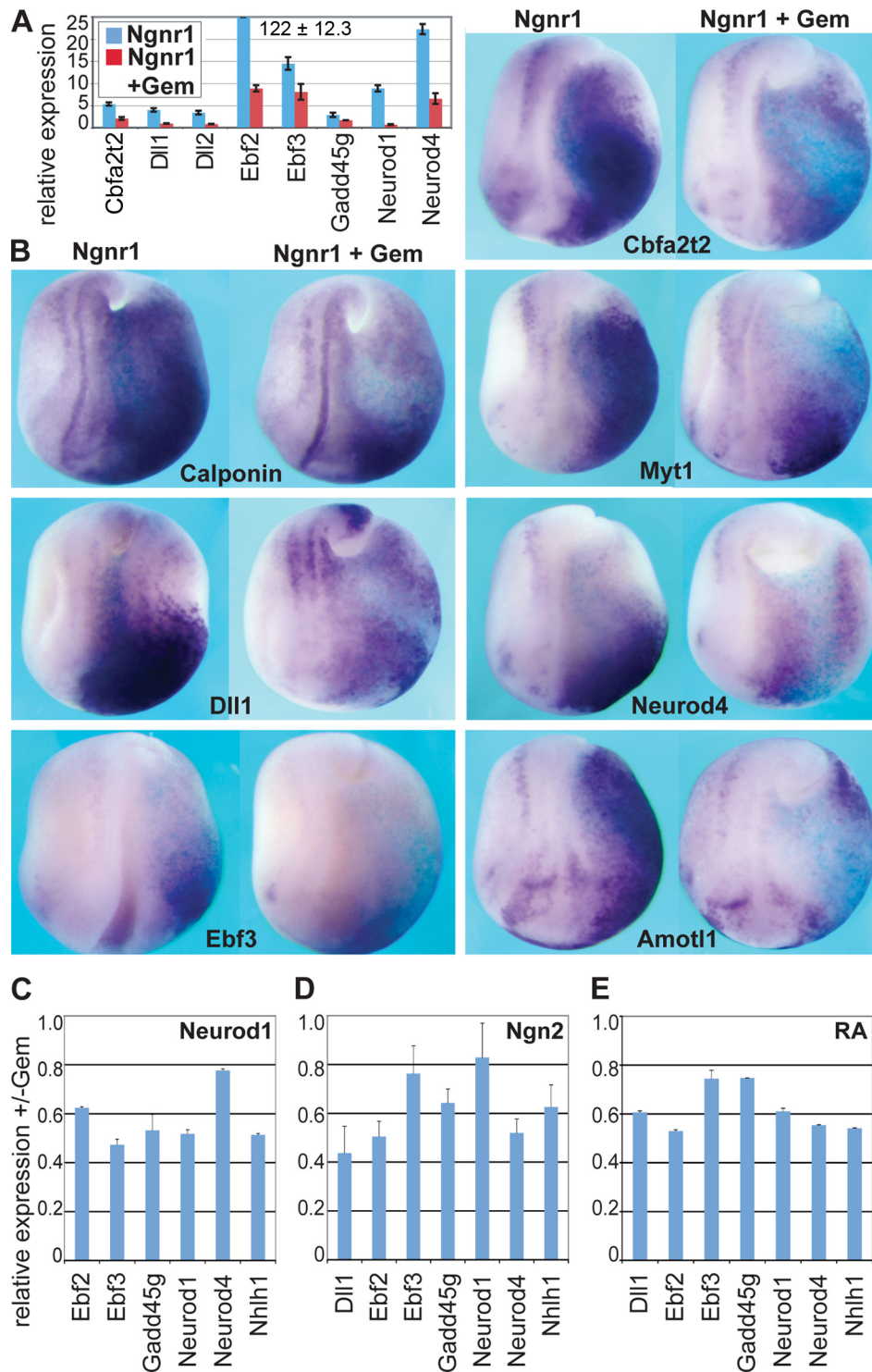


FIG 1 Geminin antagonizes neurogenin-dependent target gene transactivation during neurogenesis in *Xenopus* and in P19 EC cells. (A) qRT-PCR analysis of target gene activation by Ngnr1. *Xenopus* Ngnr1-GR was overexpressed in *Xenopus* embryonic ectoderm explants (animal caps), with or without coexpressed GemΔcoil. Upon DEX treatment, Ngnr1-GR induced expression of its target genes, as detected by quantitative RT-PCR. This was suppressed by coexpression of GemΔcoil. Expression is represented relative to values for uninduced (without DEX) Ngnr-GR-injected explants. (B) Gem suppresses Ngnr1-induced overexpression of its target genes in the *Xenopus* embryo. One bilateral half of each embryo was injected with capped mRNA encoding Ngnr1 with or without coinjection of GemΔcoil, with coinjection of a β-galactosidase lineage tracer mRNA (detected as blue X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] staining), by injecting one cell at the two-cell stage as previously described (20). Embryos were raised to neurula stages (stages 14 and 15), and *in situ* hybridization with the indicated probes was performed (purple stain). Ngnr1 induced ectopic expression of all 7 target genes tested on the injected side (oriented to the right), while GemΔcoil coinjection suppressed this Ngnr1-induced gene expression. (C to E) Geminin represses neural bHLH-driven target gene transactivation in P19 EC cells. Expression of neural bHLH target genes was induced in P19 cells by transfection of expression constructs for Neurod1 (C) or Ngn2 (D) or by treatment with 5 μM RA for 4 days (E), with or without overexpression of Gem (see Materials and Methods). qRT-PCR analysis defined relative expression levels for the genes shown, expressed as a ratio of their expression level with Gem overexpression versus the control (with Neurod1, Ngn2, or RA but without Gem overexpression). At least three independent experiments were performed for each gene analyzed, and a representative result, with the qRT-PCR performed in triplicate, is shown. Error bars indicate standard deviations.

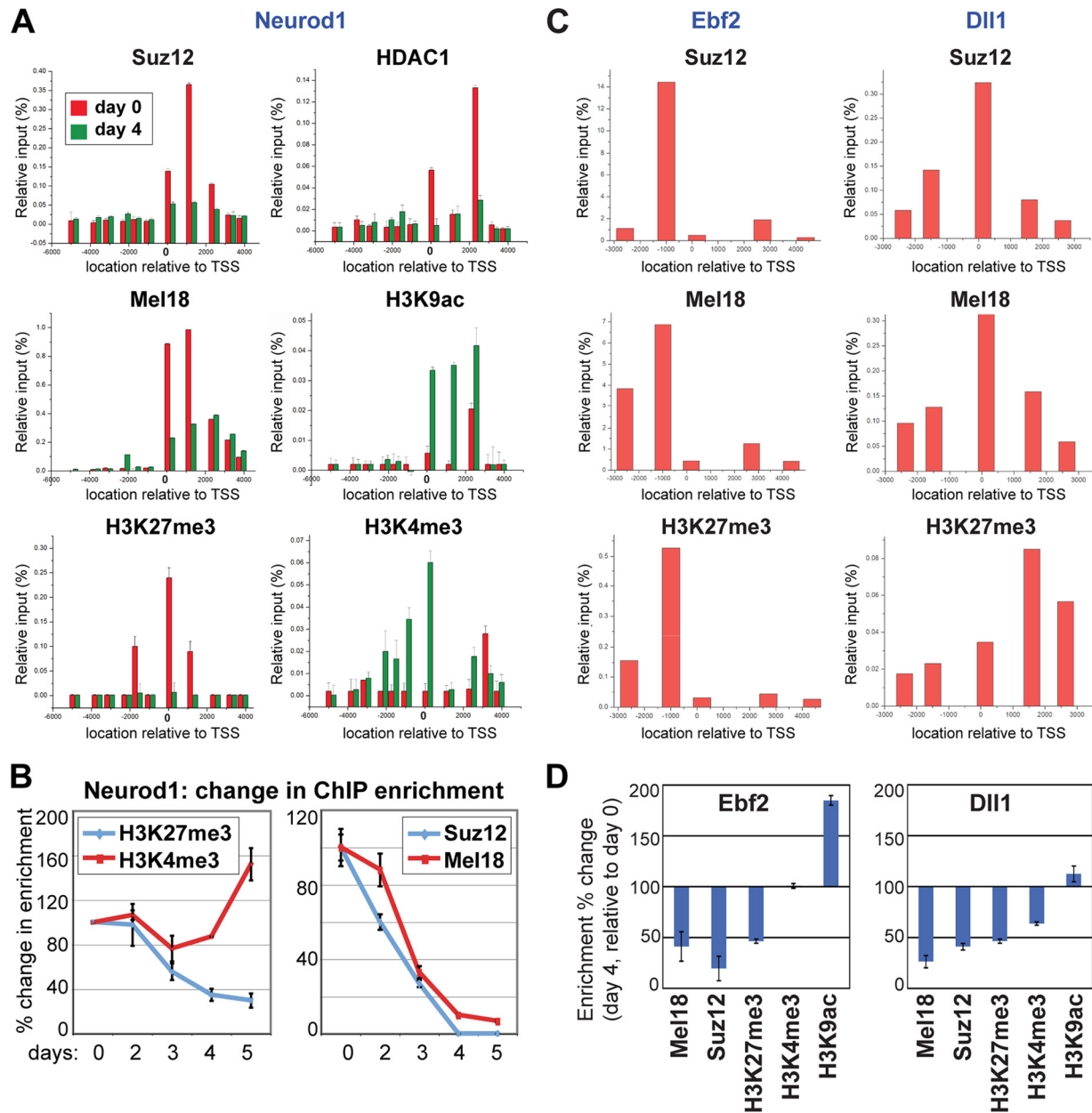


FIG 2 Epigenetic regulation of neurogenesis-related genes during neuronal commitment of P19 cells. (A) Chromatin immunoprecipitation for the proteins and histone modifications indicated. The x axis scans the *Neurod1* locus from 5 kb upstream to 4.5 kb downstream, relative to the transcription start site (TSS) (kb 0), and enrichment at each position is indicated as normalized (IgG-subtracted) percent input, as described in Materials and Methods. ChIP was performed in undifferentiated P19 cells (red) (day 0) or after 4 days of neuronal commitment induced by RA treatment (green). The locus is enriched for PcG proteins (Suz12 and Mel18), H3K27me3, and HDAC1 in undifferentiated cells, while becoming highly enriched for modifications associated with transcriptional activation (H3K4me3 and H3K9ac) during neurogenesis. (B) Temporal changes in relative levels of the bivalent histone modifications H3K27me3 and H3K4me3 and of PcG proteins (Suz12 and Mel18) at the *Neurod1* locus during days 0 to 5 of neuronal commitment of P19 cells. Percent change in enrichment at the promoter is shown, relative to day 0. (C) Chromatin immunoprecipitation for the PcG proteins (Suz12 and Mel18) and for H3K27me3 is shown for the neurogenesis-related *Ebf2* and *Dll1* genes in P19 EC cells. As above, the x axis scans the region flanking the TSS and enrichment at each position is indicated as normalized percent input. (D) P19 EC cells underwent neurogenesis in RA for 4 days, and changes in enrichment by day 4 are shown as a percent change relative to undifferentiated day 0 EC cells for the indicated proteins/histone modifications. Changes in Mel18, Suz12, H3K27me3, H3K4me3, and H3K9ac enrichment were assessed at kb -1.0 for *Ebf2* and 0 (TSS) for *Dll1*, corresponding to regions of strong PcG binding in EC cells (B).

ification H3K27me3 and the histone deacetylase 1 (HDAC1) were also enriched at the locus at this time (Fig. 2A). *Neurod1* expression increases between days 2 and 5 of neuronal differentiation, and this corresponds to a 90% loss in enrichment of these PcG proteins (Suz12 and Mel18) and HDAC1 and a 60% decrease in

H3K27me3 enrichment, relative to those in undifferentiated P19 cells (Fig. 2A and B). *Neurod1* is bivalently marked with both the activating H3K4me3 and the repressive H3K27me3 modification in undifferentiated P19 cells (Fig. 2A and B), and enrichment of the locus for histone modifications that correlate with active gene

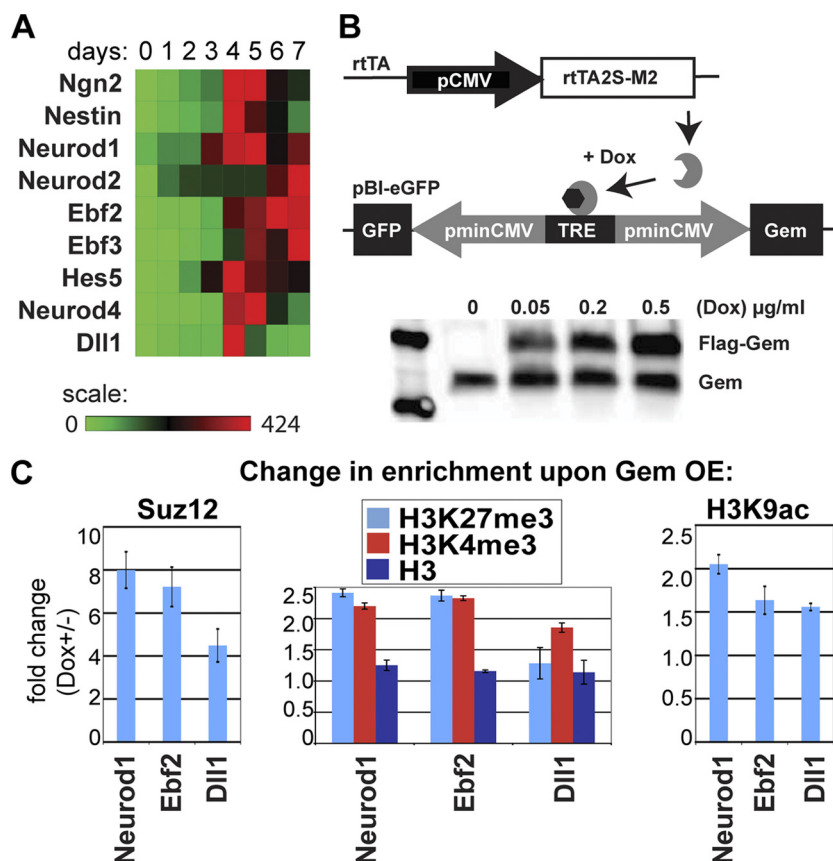


FIG 3 Geminin regulates the epigenetic state of neural genes during neurogenesis in P19 cells. (A) qRT-PCR was performed for the genes indicated during 7 days of neuronal commitment of P19 EC cells, and the results were represented as a heat map using Spotfire. Expression levels of neurogenesis-related genes, including those selected for ChIP analysis (Neurod1, Ebf2, and Dll1 genes), increase by day 4 of neuronal commitment. (B) Generation of doxycycline-inducible P19 EC cell lines. Stable clonal lines of P19 cells that overexpress Gem in a Dox-inducible manner were created using a doxycycline-inducible expression system (Clontech). Stable lines of P19 cells that express reverse Tet transactivator protein (rtTA) from the CMV promoter were transfected with a bidirectional vector (pBI-eGFP) that expresses Flag-tagged Gem and enhanced green fluorescent protein (eGFP) under control of a tetracycline-response element (TRE) and neomycin resistance under control of a simian virus 40 (SV40) promoter. Multiple stable clonal lines were selected (using G418 for neomycin resistance). These stable P19 lines were tested for Dox-dependent inducible overexpression of Flag-tagged Gem. (C) Gem overexpression during 4 days of neuronal commitment of P19 cells increases enrichment for both modifications associated with gene repression (PcG protein Suz12 and H3K27me3), and modifications associated with transcriptional activation (H3K9ac and H3K4me3), while enrichment for unmodified histone H3 is unchanged by Gem OE. Fold change in enrichment at the promoter with (+Dox) versus without (–Dox) Gem overexpression is shown for three neural genes.

transcription (H3K4me3, acetylation of histone H3K9 [H3K9ac]) increases prominently by day 4 to 5 (Fig. 2A and B). Therefore, activation of Neurod1 during RA-mediated P19 differentiation is associated with the loss of PcG protein binding and H3K27me3 histone modification followed by the transition to an H3K4me3-only monovalent state. Several genome-wide studies utilizing human and mouse ES cell systems have demonstrated that PcG complexes and the PcG-regulated H3K27me3 histone modification constitute an epigenetic mechanism for regulating the expression of developmental genes, including all of our candidate neurogenesis regulatory genes (NeuroD1, Ebf2, and Dll1 genes) (1, 2, 15). For the Ebf2 and Dll1 genes, we likewise confirmed that PcG and H3K27me3 were enriched around the TSS in undifferentiated P19 EC cells, while this enrichment decreased during 4 days of neuronal commitment (Fig. 2C and D). This decrease in PcG protein binding and H3K27me3 modification corresponds with increases in expression of these and other bHLH target genes by day 4, relative to levels found in undifferentiated P19 cells (Fig. 3A). Therefore, activation of expression of these neurogenesis-regula-

tory genes involves loss of PcG-mediated repression and a transition from a bivalent modification state to a chromatin state where the locus is enriched for modifications (H3K4me3 and H3K9ac) that are conducive for active gene transcription.

Geminin maintains the chromatin of neurogenesis-related genes in a bivalent state. Having established how regulatory proteins and histone modifications change at these loci during neuronal differentiation, we tested the effects of maintaining high Gem levels in these cells while inducing them to undergo neuronal commitment. We constructed stable clonal lines of P19 EC cells enabling doxycycline (Dox)-inducible Gem overexpression (Gem OE) (Fig. 3B). We performed Gem OE during 4 days of neuronal commitment and assessed enrichment of Suz12, H3K27me3, H3K4me3, and H3K9ac at these loci with Gem OE versus without (with or without Dox). Gem OE resulted in a 4- to 8-fold increase in Suz12 enrichment at the three loci tested and a 2-fold increase in H3K27me3 (for Neurod1 and Ebf2) (Fig. 3C). Maintaining high levels of Gem during days 0 to 4 of neuronal commitment also consistently increased H3 acetylation (H3K9ac) and

H3K4me3 modification (Fig. 3C). In the same experiments, Gem overexpression did not affect levels of unmodified histone H3 protein enrichment on chromatin at these loci (Fig. 3C). These data suggest that Gem acts in this context by enhancing the enrichment of specific epigenetic modifications that play a regulatory role in controlling gene expression, rather than by generally increasing overall chromatin accessibility. Together, these results demonstrate that Gem can promote a bivalent histone modification state at genes that promote neurogenesis, with high levels of both activating H3K4me3 and repressing H3K27me3 histone modifications. Developmental genes that promote cell specification, commitment, and differentiation often have this characteristic histone modification status in undifferentiated stem and progenitor cells. Therefore, Gem's ability to promote this epigenetic modification state could contribute to Gem's ability to restrain neural bHLH-driven transactivation of these loci during neuronal commitment.

As described above, we found that Gem blocks bHLH-driven transcriptional programs in both *Xenopus* embryos and P19 cells. We wished to see whether Gem could likewise regulate neuronal commitment and differentiation in neural stem cells (NSCs) that were derived from ES cells or obtained from the mouse embryonic forebrain (embryonic day 12.5 [E12.5]). These NSCs can be grown as a self-renewing cell population in culture or can be efficiently driven to undergo terminal neuronal or glial differentiation. Therefore, they represent ideal models for assessing regulation of differentiation from lineage-restricted but undifferentiated neural precursors. Mouse ES cells plated in serum-free medium (N2B27) as a monolayer for 21 days undergo terminal differentiation into neurons, as indicated by staining for β -III tubulin, a marker for mature neurons (31). Reproducible transcriptional and phenotypic changes occur during this time: By day 4, approximately 60% of cells in the culture are immunopositive for Sox1 and Pax6, markers of neural precursors, and most cells lose expression of Oct4, a marker of the pluripotent ES state (Fig. 4A to D). Relative to levels found in ES cells, Gem expression decreases considerably at the protein level by day 4, while Gem expression levels are <10% of expression levels found in ES cells by day 7 (Fig. 4E and F). During the same time window, expression of all of our candidate neurogenesis-regulatory genes increases exponentially (Fig. 4G). Plating these neural precursors at day 7 onto poly-D-lysine/laminin-coated dishes and further culture (for 2 weeks) result in phenotypic changes characteristic of neuronal differentiation, with neurite outgrowth and staining for mature neuronal fate markers such as β -III tubulin (Fig. 4H).

We overexpressed Gem during days 3 to 7 of neurogenesis in this system to test whether maintaining high Gem levels would repress expression of neurogenesis-related genes, as was seen in P19 cells and *Xenopus* embryos. For this purpose we used clonal ES cell lines that overexpress Gem by 2- to 4-fold in a doxycycline (Dox)-inducible manner as previously reported (30). Gem overexpression (OE) between days 3 and 7 suppressed the expression of many of our candidate neurogenesis-promoting genes (Fig. 5A). Using corresponding cell lines for Dox-inducible expression of shRNAmirs directed against Gem (Gem knockdown [KD]) (30), we also performed Gem knockdown (by 80%) during days 3 to 7 of neurogenesis. This resulted in increased expression of the same neurogenesis-regulatory genes (Fig. 5A). To analyze these phenomena in a comprehensive manner, we next performed tran-

scriptome analysis by deep sequencing (RNA-seq) in Gem knock-down cells. RNA was extracted from Gem KD cells after cells underwent neurogenesis for 5 days in N2B27 medium with or without Dox-induced Gem knockdown from day 3 to 5. Gem-regulated genes were then identified using RNA-seq (Materials and Methods). A total of 388 genes were differentially regulated by Gem KD, which represented 273 significantly upregulated and 115 downregulated genes (see Table S3 in the supplemental material). We used Gene Ontology (GO) enrichment analysis to assess functional roles of the genes that were upregulated upon Gem knockdown in order to understand the biological significance of gene regulation by Gem in neural progenitors. Gem KD-upregulated genes were highly enriched for genes involved in neuronal commitment, differentiation, and function (e.g., neuron projection) and transcription factor activities, including bHLH transcription factors ($P = 6.5E-06$) (Fig. 5B). Examples of these genes are shown in Fig. 5C and include a number of transcription factors with known roles in neurogenesis (Ascl1, Ebf1/3, Hes6, Myt1, Neurod4, Ngn1, and Nhlh1/2) as well as other molecules with known roles in neuronal development and function (Fig. 5C). Some of these were Ngn1 targets also repressed by Gem in *Xenopus* (Ascl1, Ebf3, Hes6, Myt1, and Neurod4) (Fig. 1; see Table S2 in the supplemental material). In contrast, a distinct group of genes showed decreased expression upon Gem knockdown; these included genes involved in cell adhesion/epithelial function. Therefore, global gene expression analysis of Gem-regulated genes supported our hypothesis that Gem's major role in cells at these stages is to restrain the expression of transcription factors involved in neurogenesis to maintain the progenitor cell state and control neuronal differentiation.

We next used chromatin immunoprecipitation to evaluate this regulation at the chromatin level. During neuronal differentiation of ES cells, we compared enrichment for Suz12, H3K27me3, and H3K4me3 at the promoters of candidate neuronal genes on day 0 (ES) cells versus day 7. We observed a 90% reduction in enrichment of Suz12 and a 60 to 80% reduction in enrichment of H3K27me3 by day 7 of neurogenesis at the Ascl1, Dll1, Ebf2, Ebf3, Hes5, nestin, and Neurod1 loci (Fig. 6A and B). There was a corresponding gene-dependent 1.5- to 5-fold increase in the activating histone mark H3K4me3 at these loci (Fig. 6C). We also performed ChIP under conditions of Gem overexpression and knockdown in ES-derived neural precursor cells. Similar to the results for P19 EC cells, Gem overexpression in neural precursor cells from day 3 through day 7 increased H3K27me3 enrichment 1.5- to 4-fold at these neurogenesis-related genes, relative to the control without Dox (Fig. 6D). In contrast, Gem knockdown in ES-derived neural precursor cells resulted in a 2-fold decrease in H3K27me3 enrichment (Fig. 6D). We also analyzed the activating mark H3K4me3 under conditions of Gem OE and KD during neuronal differentiation and observed no strong change in enrichment at our candidate genes (Fig. 6E). These observations were consistent with our findings in P19 cells and with the RNA-seq data, which indicated that many transcription factors with roles in neurogenesis were under negative regulation by Gem in neural precursor cells. Therefore, high Gem levels contribute to repressing the expression of genes that promote neurogenesis and neuronal differentiation, and this correlates with Gem's ability to promote retention of the H3K27me3 modification in neural precursor cells, which would be expected to restrain gene expression.

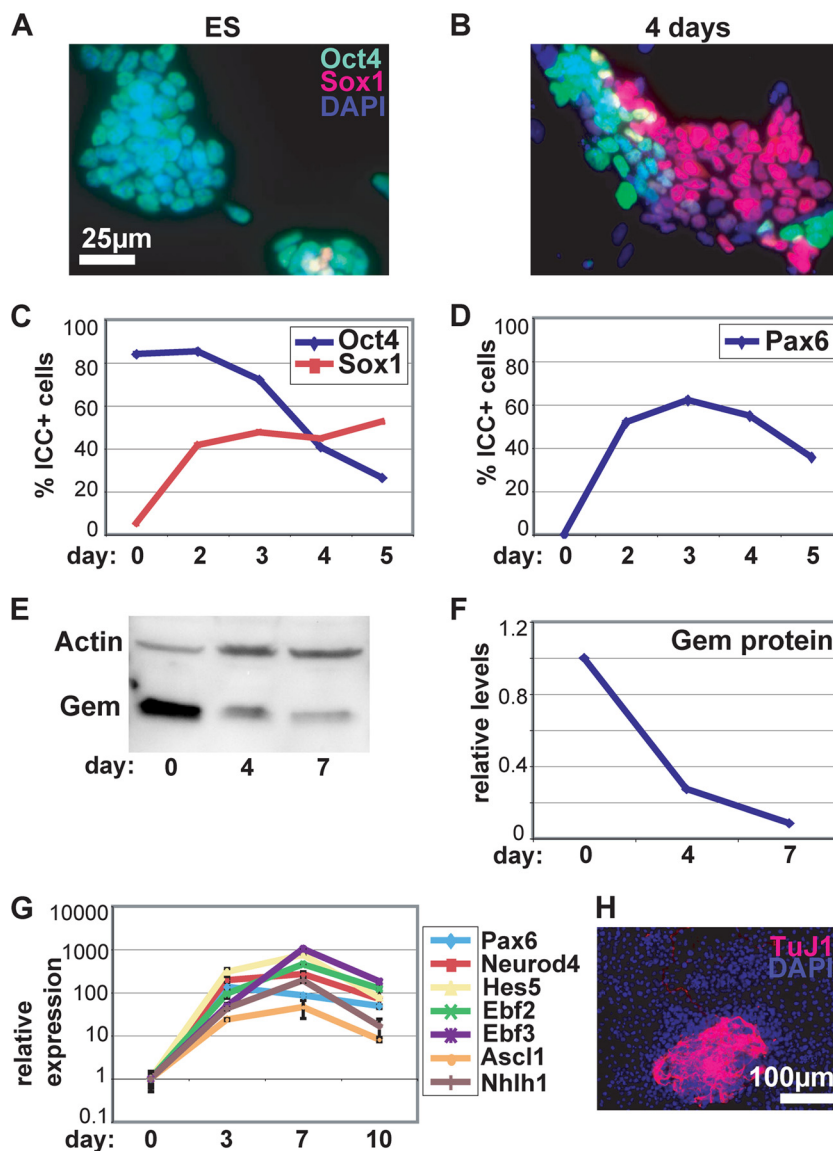


FIG 4 ES cells undergoing neurogenesis and neuronal differentiation in N2B27 monolayer culture. (A to D) By 5 days of culture in N2B27 medium, most ES cells have lost pluripotency marker expression (fewer than 20% Oct4-positive cells) and a majority of cells have been converted into neural precursors expressing Sox1 and Pax6 (40 to 60% Sox1-positive and 60% Pax6-positive cells) as shown by immunocytochemistry (A and B) and quantitated in the graphs (C and D). (E and F) During neurogenesis in this system, Gem levels decline steadily, with more than an 80% decrease by day 7, as shown by Western blotting (E) and quantitated in the graph (F). (G) During 10 days of N2B27 culture, expression of neurogenesis-regulatory genes increases exponentially, as demonstrated by qRT-PCR analysis. Increased expression of these genes is accompanied by downregulation of Gem. (H) After 2 weeks of differentiation in N2B27 medium, cells expressing the neuron-specific β -III tubulin marker (TuJ1) are apparent.

Geminin is dispensable for controlling neuronal differentiation of ES- and brain-derived neural precursor cells. We next induced these ES-derived neural precursor cells to undergo neuronal differentiation in monolayer culture under conditions in which Gem expression was unperturbed versus conditions of Gem overexpression. However, despite Gem's effects during neurogenesis on the epigenetic modification state and expression of genes that promote neuronal commitment and differentiation, we observed no change in the number of β -III tubulin-positive cells or the overall phenotype of these cells with or without Gem OE (data not shown). Corroborating this observation, Gem-mediated repression of neuronal genes was still observed at day 10 but did not

persist to day 14, by which time the majority of the cells are terminally differentiated (Fig. 7A and B). This was seen either when Dox-induced Gem overexpression was performed from day 3 through day 14 or upon transient Gem overexpression from day 10 to 14 (Fig. 7A and B and data not shown). These results suggest that while Gem is capable of antagonizing neuronal gene expression in neural precursor cells, its activity is not sufficient to prevent differentiation under conditions that promote neuronal differentiation.

To further assess Gem's activities in neural precursor cells, we isolated neural stem cells (NSCs) from embryonic day 12.5 (E12.5) mouse forebrain (telencephalon) and tested whether Gem

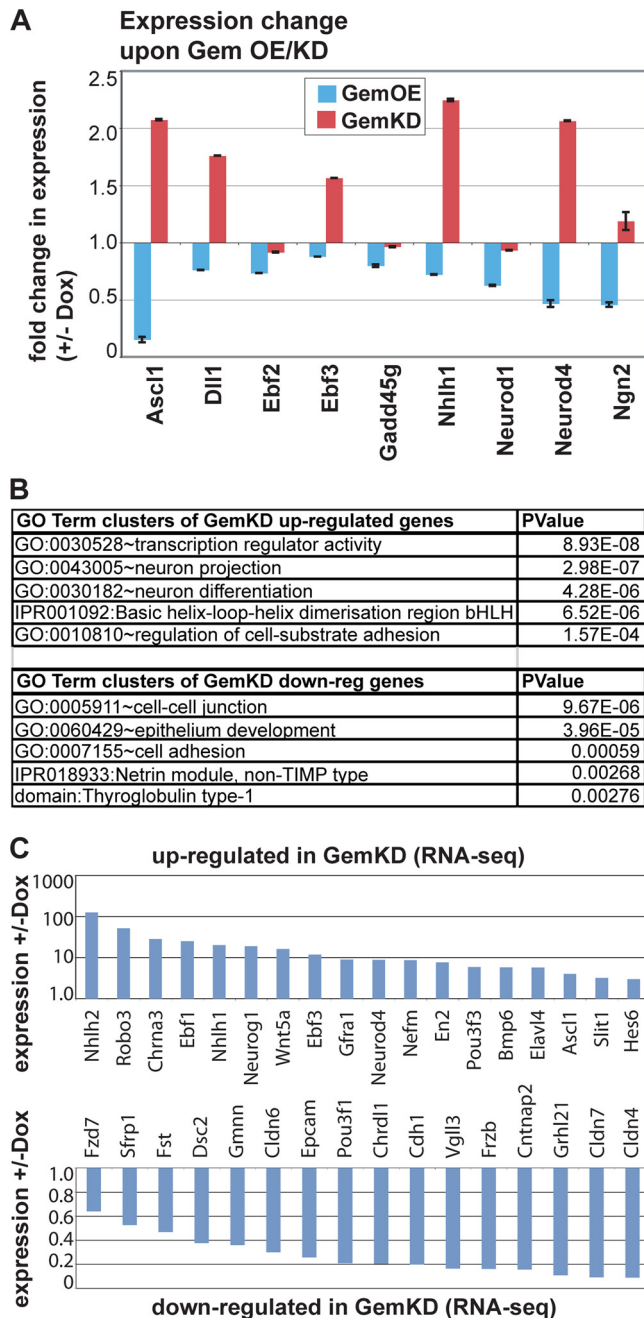


FIG 5 Geminin restrains the expression of genes that promote neuronal commitment and differentiation in ES cells undergoing neurogenesis. (A) Dox-mediated overexpression (OE) (blue) or knockdown (KD) (red) was performed during days 3 to 7 of neurogenesis in N2B27 monolayer culture, and relative expression of the genes indicated was determined by qRT-PCR (expressed as fold change in expression with and without Dox). Gem OE suppressed and Gem KD enhanced expression of neurogenesis-promoting genes. (B and C) Transcriptome profiling of ES-derived neuronal precursor cells (day 5) following Gem knockdown from day 3 to 5 was performed by RNA-seq. (B) Enriched Gene Ontology (GO) term clusters for the gene sets that had increased (upregulated) and decreased (downregulated) expression upon Gem knockdown are shown (terms and *P* values were determined using the DAVID Bioinformatics Suite). (C) Examples of genes with significantly changed expression upon Gem knockdown in ES-derived neuronal precursor cells (RNA-seq analysis). Gem KD enhanced expression of genes associated with neurogenesis and neuronal differentiation and function.

regulates NSC maintenance or ability to undergo terminal differentiation into neurons or glia (see Materials and Methods). We transduced NSCs with a lentiviral construct for expression of an shRNAmir directed against Gem, under the control of the cytomegalovirus (CMV) promoter (see Materials and Methods); this resulted in ~95% knockdown of Gem at the protein level (Fig. 7C). As controls, NSCs were transduced with a scrambled target sequence (nonspecific [NS]), and another shRNAmir construct directed against a distinct targeting sequence in Gem was also used to control for off-target effects. Gem knockdown NSCs could be propagated for multiple generations in the same manner as the parental NSC cells (data not shown). Wild-type (NS-transduced) and Gem KD NSCs also formed neurospheres of similar size, suggesting that Gem knockdown did not impair NSC proliferation or self-renewal (Fig. 7D). We also assessed changes in the expression of two neurogenesis-regulatory genes (Ebf2/Neurod1) during neuronal differentiation. Expression of these genes increased to a similar extent during induction of neuronal differentiation in NS or Gem KD NSCs (Fig. 7E). This suggested that Gem loss did not alter neural gene expression changes in NSCs during neuronal differentiation. Therefore, Gem loss in embryonic NSCs does not alter their capability to self-renew or their expression of neuronal fate-promoting genes.

As Gem knockdown did not appear to alter NSC self-renewal, we wanted to assess whether forced overexpression of Gem in NSCs could affect their ability to self-renew and differentiate into neuronal or glial lineages. For this purpose, we developed neural stem cell lines from our stable ES cell lines that enable Dox-dependent induction of Gem overexpression (referred to here as NSC Gem OE) (Fig. 7F) (see Materials and Methods). The Gem OE NSC cells proliferated in an EGF/Fgf2-dependent manner on laminin-coated dishes and showed no change in their overt morphology or proliferative potential when Gem OE was induced by Dox treatment (data not shown). These cells were further differentiated into either neuronal or glial cells (see Materials and Methods) for 3 days, with or without Dox treatment, and we measured the ability of Gem OE to alter cell differentiation and gene expression. At a morphological level, Dox-dependent Gem OE did not appear to affect the ability of NSCs to differentiate into neuronal or glial derivatives (Fig. 7G). We also monitored neuronal differentiation by measuring the expression of the *Ascl1*, *Chrna4*, and *Gad1* neuronal marker genes and glial fate by measuring the expression of *Gfap*, *Plp*, and *S100B*. As shown in Fig. 7H and I, no changes in expression of these genes were observed upon Gem OE in either differentiation scheme, correlating with the lack of morphological difference between neuronal and glial cells differentiated with versus without Gem OE. Therefore, these results further confirm our observations that while Gem can transiently repress neuronal genes during mammalian neurogenesis, manipulation of Gem levels is not sufficient to alter the differentiation potential of these cells.

DISCUSSION

Geminin affects the epigenetic state and expression of neurogenesis-regulatory genes in neural precursor cells. Previously, we found that Gem blocks terminal neuronal differentiation during *Xenopus* neurogenesis (20). Here, we demonstrated that Gem represses a major fraction of the transcriptional program driven by neural bHLH transcription factors during *Xenopus* primary neurogenesis. This could account, at least in part, for Gem's ability

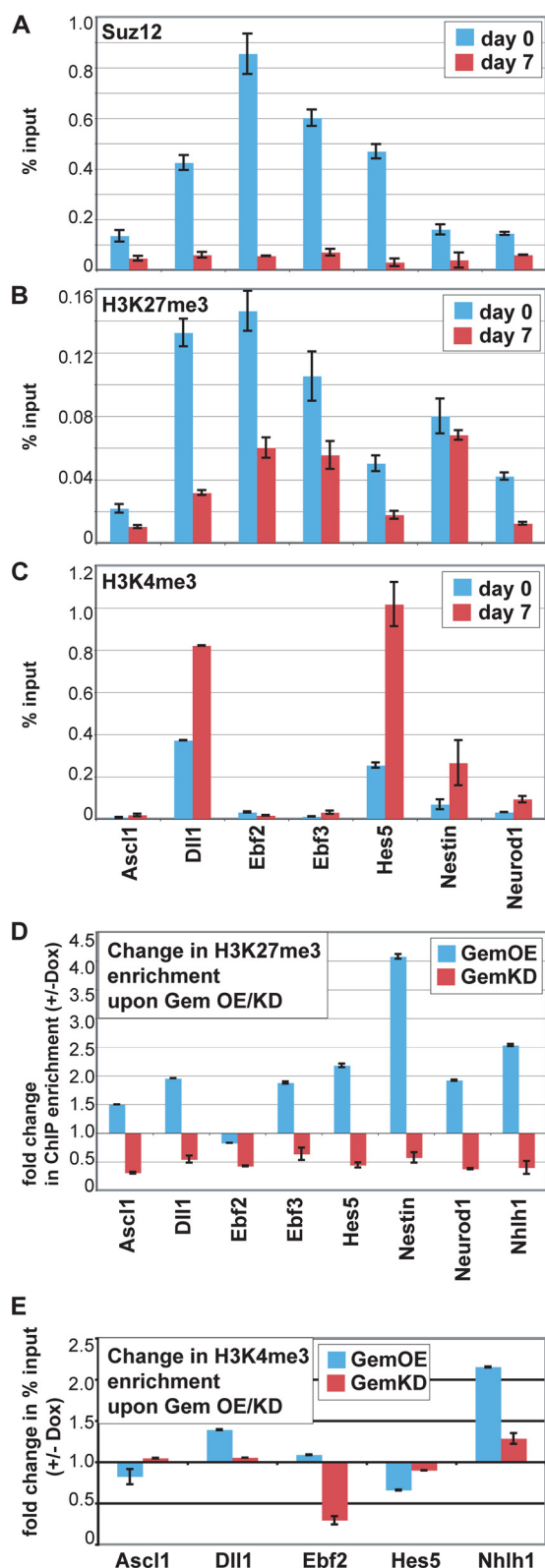


FIG 6 Geminin regulates the epigenetic state of neurogenesis-regulatory genes. (A to C) Enrichment was defined by ChIP flanking the promoters of the *Ascl1*, *Dll1*, *Ebf2*, *Ebf3*, *Hes5*, *nestin*, and *Neurod1* loci. These neurogenesis-associated genes show a 90% reduction in Suz12 enrichment (A), a 60 to 80% reduction of H3K27me3 enrichment (B), and a 1.5- to 5-fold increase in H3K4me3 enrichment (C) by day 7 of neurogenesis, compared

to block neuronal differentiation. We also determined that Gem can repress bHLH-driven transactivation of many of these same target genes during mammalian neurogenesis, using both P19 EC and ES-derived neural precursor cells that were driven to undergo neuronal commitment and differentiation. Therefore, Gem contributes to the molecular mechanisms restraining neurogenesis in both *Xenopus* and mammals. Previously, we showed that Gem also plays a conserved role in promoting the initial acquisition of neural fate from pluripotent cells (9, 11, 30). These findings are compatible with Gem's expression in both *Xenopus* and mammalian systems: Gem is highly expressed in pluripotent stem cells and early neural precursors, both *in vivo* and *in vitro*, but its expression is downregulated as proneural bHLH activities increase and the later aspects of neurogenesis, neuronal commitment, and differentiation occur (20, 23).

In the current study, we observed that Gem's activities in neural precursor cells correlate with its ability to alter the epigenetic modification state of genes whose expression increases during neurogenesis and neuronal differentiation. Specifically, Gem enhanced enrichment of PcG proteins and of the PcG activity-associated histone modification H3K27me3 at genes that encode activities promoting neuronal commitment and differentiation. Gem was previously shown to interact with members of the PcG PRC1 complex, including *Scmh1*, and to functionally cooperate with PcG activity in *Hox* gene repression during rostrocaudal patterning of the embryo (12). During the earliest aspects of fate acquisition of pluripotent embryonic cells in *Xenopus*, Gem also repressed nonneural (mesodermal, epidermal, and endodermal) fate acquisition through mechanisms involving functional cooperativity with PcG (11). Therefore, our results here suggest that neurogenesis represents another cellular context in which Gem enhances retention of PcG-mediated repressive modifications to restrain gene expression.

Interestingly, we found that Gem increased enrichment levels of both the repressive histone modification H3K27me3 and histone modifications that promote gene transactivation, H3K4me3 and H3K9ac. The coenrichment of target genes by H3K27me3 and H3K4me3 represents a "bivalent" signature used to retain genes that promote lineage-specific commitment programs in a poised but repressed state in stem and precursor cells, including lineage-restricted, undifferentiated neural precursor cells (16). By promoting a bivalent chromatin status, Gem may contribute to maintenance of the neural precursor cell state by restraining the expression of genes that promote cell progression through neurogenesis and initiation of neuronal differentiation. Gem can directly interact with multiple chromatin-modifying complexes, includ-

with enrichment levels at each promoter in undifferentiated ES cells (day 0). (D) Stable clonal lines of ES cells underwent neurogenesis for 7 days in N2B27 medium, with or without Gem overexpression or knockdown by Dox treatment from day 3 through 7. The fold change in ChIP enrichment for H3K27me3 at the promoters of the genes shown was determined following Gem overexpression (blue) or knockdown (red). Gem OE enriched, while Gem KD diminished, enrichment levels of H3K27me3 at the promoter. (E) ChIP analysis was conducted to detect H3K4me3 enrichment at neurogenesis-regulatory genes upon Dox-inducible Gem overexpression or knockdown during days 3 to 7 of neurogenesis in N2B27 monolayer culture. Minor changes in H3K4me3 enrichment occurred in a gene-dependent manner, with no specific trend across the gene set. Fold change in enrichment at the promoter with (+Dox) versus without (−Dox) induced Gem overexpression and knockdown is shown for five neural genes.

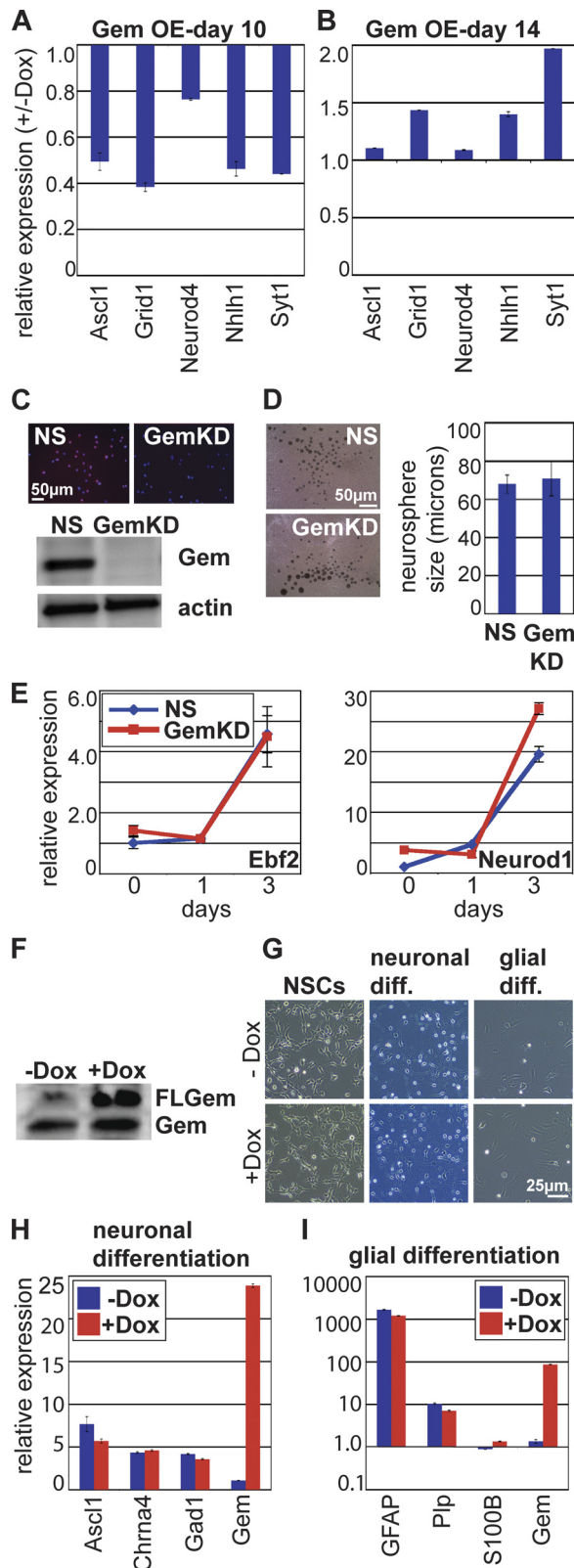


FIG 7 Geminin is dispensable for neural stem cell maintenance and control of neuronal or glial differentiation potential. (A and B) Clonal ES lines were induced to undergo neuronal differentiation with (+Dox) or without (–Dox) inducible overexpression of Gem from day 3 to day 10 (A) or day 14 (B). Expression levels of five neuronal genes were determined by qRT-PCR and are

ing the PcG repressor complex (potentially via Scmh1) and the SWI/SNF chromatin-remodeling complex (through its catalytic subunit Brg1). Gem can also modulate the acetylation status of neural genes during initial neural fate acquisition of pluripotent ES cells (12, 20, 30). Gem's ability to interact with and alter the activities of multiple chromatin-modifying complexes could enable Gem to perform distinct, context-dependent activities. We found that Gem promotes a bivalent epigenetic modification state, characterized by the presence of both activating and repressive epigenetic modifications, at neurogenesis-regulatory genes in neural precursor cells. These data provide a molecular correlate for prior phenotypic observations, where Gem was suggested to restrain terminal neuronal differentiation and promote maintenance of neural progenitor cells (20).

Geminin's ability to block neuronal differentiation during primary neurogenesis in the *Xenopus* embryo was dependent on its interaction with the SWI/SNF catalytic subunit Brg1 (20). Brg1 can interact with neural bHLH transcription factors, and, in the context of *Xenopus* primary neuronal differentiation, SWI/SNF activity is required for proneural bHLH-mediated transactivation of target genes that regulate neuronal differentiation. In contrast, it is not clear whether Gem's ability to interact with Brg1 is functionally relevant to mammalian neurogenesis. Deletion of a Gem carboxy-terminal motif required for Gem-Brg1 interaction did not affect Gem's ability to promote neural gene expression during initial neural fate acquisition in mammalian cells (30). In addition, neural bHLH-dependent target gene transactivation does not appear to be strictly dependent on SWI/SNF activity during neurogenesis in mammals. For example, in P19 cells, we found that overexpression of a dominant negative form of Brg1 (7) did not affect the ability of Ngn2 to transactivate expression of target genes that promote neurogenesis (Neurod1 and Ebf2 genes) (data not shown). During mammalian neurogenesis, the role of SWI/SNF remodeling complexes appears to be complex: the transition from neural progenitor cells to postmitotic neurons is accompanied by an exchange of paralogous forms of several SWI/SNF complex subunits. This subunit switching alters the functional properties of the complex, such that one SWI/SNF complex is involved in maintenance of neural progenitors, while a distinct complex controls neuronal properties in postmitotic neurons (10). Distinct SWI/SNF complexes likewise play complex and context-dependent roles in other aspects of mammalian develop-

represented as relative expression with (+Dox) versus without (–Dox) Gem overexpression. Gem represses neuronal gene expression at day 10, but this repressive activity is lost by day 14. (C) NSCs derived from the forebrain of a 12.5-day embryo (see Materials and Methods) were transduced with a pGIPZ control (scrambled shRNA, nonspecific [NS]) or with a pGIPZ-Gem shRNA-expressing lentivirus (denoted GemKD) (OpenBiosystems). Gem knockdown was confirmed using immunocytochemistry (Gem, red; 4',6'-diamidino-2-phenylindole [DAPI]-stained nuclei, blue) and Western blotting. (D and E) Gem KD in primary NSCs did not affect neurosphere size (D) or changes in expression levels of Neurod1 or Ebf2 (E) during 3 days of neuronal differentiation of NSCs. (F) NSCs were derived from doxycycline-inducible ES cell lines for overexpression of FLAG-tagged Gem. Endogenous and FLAG (FL)-tagged Gem are shown by Western blotting after 2 days of Dox treatment. (G to I) Dox-dependent induction of Gem overexpression in NSCs resulted in significant increases in Gem expression but did not alter the ability of NSCs to differentiate into neuronal or glial lineages, as assayed by morphology (G) and by expression of neuronal (H) or glial (I) genes. Gene expression was measured by qRT-PCR after 3 days of neuronal and glial differentiation with or without Gem overexpression.

ment, such as pluripotency maintenance, acting as both activators and repressors of transcription (6, 32). Therefore, the requirement for Gem-Brg1 interaction during primary neurogenesis in *Xenopus*, versus the apparent lack of a similar requirement in mammals, may reflect evolutionary divergence of these activities, where additional regulatory complexity appears to be utilized to control mammalian neurogenesis.

Geminin is not sufficient to prevent terminal neuronal differentiation of mammalian neural precursor cells. While Gem could block neuronal differentiation during *Xenopus* primary neurogenesis, results from mammalian work have been conflicting. Here, we found that while Gem transiently repressed the expression of neuronal genes during mammalian neurogenesis, Gem was not sufficient to alter the differentiation potential of lineage-restricted neural precursor cells. These results are consistent with the recent observation that Gem loss in mammalian NSCs in a conditional mouse model did not affect their capability to self-renew or their differentiation potential *in vivo* or *in vitro* (19). We also found that while Gem increases PcG enrichment and otherwise alters the epigenetic status of neuronal genes in neural precursor cells, it did not alter the progression toward terminal neuronal or glial differentiation at either a phenotypic or molecular level. Therefore, while Gem contributes to establishing the epigenetic modification state and transcriptional status of neurogenesis-related genes in neural precursor cells, in these mammalian contexts, other developmental cues exert significant and overriding effects to drive the terminal differentiation of these cells.

ACKNOWLEDGMENTS

RNA-seq was performed by the Genome Technology Access Center (GTAC) at Washington University School of Medicine. We thank Paul Clifton and Dana Oschwald of GTAC for sample preparation and assistance with data analysis. We are grateful to Matt Elitt, Anthony Crossland, and Laura Dieden for technical assistance. We thank Cathy Collins for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (GM66815), the March of Dimes, and the American Cancer Society (to K.L.K.).

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