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Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence

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The majority of neural stem cells (NSCs) in the adult brain are quiescent, and this fraction increases with aging. Although signaling pathways that promote NSC quiescence have been identified, the transcriptional mechanisms involved are mostly unknown, largely due to lack of a cell culture model. In this study, we first demonstrate that NSC cultures (NS cells) exposed to BMP4 acquire cellular and transcriptional characteristics of quiescent cells. We then use epigenomic profiling to identify enhancers associated with the quiescent NS cell state. Motif enrichment analysis of these enhancers predicts a major role for the nuclear factor one (NFI) family in the gene regulatory network controlling NS cell quiescence. Interestingly, we found that the family member NFIX is robustly induced when NS cells enter quiescence. Using genome-wide location analysis and overexpression and silencing experiments, we demonstrate that NFIX has a major role in the induction of quiescence in cultured NSCs. Transcript profiling of NS cells overexpressing or silenced for *Nfix* and the phenotypic analysis of the hippocampus of *Nfix* mutant mice suggest that NFIX controls the quiescent state by regulating the interactions of NSCs with their microenvironment.

[*Keywords*: epigenetics; genomics; NFIX; neural stem cells; nuclear factor one; quiescence; transcription factor]

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Cellular quiescence is a reversible state of growth and proliferation arrest that can be adopted by many types of cells, from bacteria and yeast to cultured mammalian fibroblasts and adult tissue stem cells (Coller et al. 2006; Valcourt et al. 2012). It is an active state that involves important changes in cell physiology, including energy metabolism and cell adhesion (Venezia et al. 2004; Coller et al. 2006; Fukada et al. 2007; Pallafacchina et al. 2010; Lien et al. 2011; Brohl et al. 2012; Valcourt et al. 2012).

Quiescence is essential to prevent the premature exhaustion of long-lived self-renewing stem cell populations (Orford and Scadden 2008). The balance between neural stem cell (NSC) proliferation and quiescence in the adult brain is regulated by diverse physiological stimuli, and disruption of this balance is thought to contribute to the cognitive decline of old age (Lee et al. 2011; Faigle and Song 2013). However, the cell-intrinsic mechanisms that mediate these effects and control NSC quiescence and activity remain poorly understood.

Stem cells are present in two regions of the postnatal and adult brain: the subependymal zone (SEZ) adjacent to the lateral ventricles and the dentate gyrus (DG) of the hippocampus, where they continuously generate new neurons that integrate into neuronal circuits of the olfactory bulb and hippocampus, respectively (Temple 2001; Fuentealba et al. 2012). In contrast to the highly proliferative stem cells of the embryonic neural tube, NSCs in the postnatal and adult brain are relatively quiescent (Temple 2001; Niu et al. 2011; Fuentealba et al. 2012). Adult NSCs are stimulated to divide by diverse physiological stimuli, including physical exercise and cognitive stimulation, while conversely, stress, anxiety, and old age suppress their divisions (Fabel and Kempermann 2008; Ma et al. 2009; Lucassen et al. 2010). Seizures stimulate

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NSC divisions in aged mice, suggesting that this cell cycle arrest is reversible (Lugert et al. 2010). Adult stem cells inhabit specialized niches that produce signals controlling their lifelong self-renewal and production of differentiated progeny (Fuchs et al. 2004; Riquelme et al. 2008; Fuentealba et al. 2012; Faigle and Song 2013). In particular, Notch and BMP signaling, activated by ligands presented by differentiating neural precursors in the neurogenic niches, provide negative feedback signals that maintain the quiescent state of SEZ and hippocampal stem cells (Bonaguidi et al. 2008; Ables et al. 2010; Ehm et al. 2010; Imayoshi et al. 2010; Mira et al. 2010).

Although progress has been made in identifying transcription factors (TFs) that regulate different steps of neurogenesis in the postnatal and adult brain (Hsieh 2012), the nature of the factors that mediate the activity of extrinsic signals and determine cell-intrinsically the quiescent or proliferating state of NSCs is still largely unknown. Elucidating these transcriptional mechanisms is essential to understand how the physiology and behavior of NSCs is regulated and, in the longer term, develop therapeutic interventions; e.g., to counteract the decline of neurogenesis in the aging brain. The FoxO proteins are currently the best-studied TFs promoting quiescence in NSCs. In mice mutant for FoxO1, FoxO3, and FoxO4 or for FoxO3 alone, an initial excess of NSC proliferation is followed by a depletion of the NSC pool and a decline in neurogenesis (Paik et al. 2009; Renault et al. 2009). Whether other TFs act downstream from quiescence-promoting signals and regulate common or distinct aspects of the physiology of quiescent NSCs is not known.

The main obstacles to studying NSC quiescence are the difficulty of isolating these cells in sufficient numbers from highly complex adult neurogenic niches (Beckervordersandforth et al. 2010; Fuentealba et al. 2012) and the lack of a well-characterized cell culture model. NSCs are routinely maintained in culture (NS cell cultures) in the presence of high concentrations of mitogens and are highly proliferative (Pastrana et al. 2009; Ehm et al. 2010; Mira et al. 2010; Sun et al. 2011). However, BMP ligands have recently been shown to promote cell cycle arrest in adherent cultures of mouse and rat NS cells (Mira et al. 2010; Sun et al. 2011). In this study, we examined in detail BMP-treated, embryonic stem cell-derived NS cells and demonstrated that they have characteristic features of quiescent cells. We also showed that entry into quiescence involves major changes in the transcriptional profile of these cells and particularly in their expression of cell adhesion and extracellular matrix (ECM) molecules.

We used this NS cell quiescence model to identify TFs that participate in the gene regulatory network (GRN) that governs the quiescent state in NSCs. For this, we characterized regulatory elements that are active in quiescent NS cells by genome-wide mapping of the enhancer-associated histone mark H3 Lys 27 acetylation (H3K27ac) and coactivator p300 (Heintzman et al. 2009; Creyghton et al. 2010; Rada-Iglesias et al. 2011; Rada-Iglesias et al. 2012). We found that proteins of the nuclear factor one (NFI) family bind to a very large fraction of these enhancers and that family member NFIX is required for the establishment of a significant portion of the gene expression program of quiescent NS cells and the suppression of a significant part of the gene expression program of proliferating NS cells. Finally, we show that mutation of the *Nfix* gene results in loss of quiescence in a significant fraction of hippocampal NSCs in vivo.

Together, this study shows that establishing a cell culture model of NSC quiescence has allowed us to characterize fundamental aspects of the biology of NSCs and identify a key TF that plays an essential role in implementing the quiescent NSC gene expression program.

Results

BMP4-treated NS cells are quiescent

To model NSC quiescence in culture, we replaced the mitogen EGF with BMP4 in the culture medium of NS cells, which also contains FGF2 (Conti et al. 2005; Mira et al. 2010; Sun et al. 2011). We monitored cell proliferation by staining for the proliferation marker Ki67 and measuring incorporation of the thymidine analog EdU. We observed that NS cells had stopped proliferating 24 h after addition of BMP and remained cell cycle-arrested when maintained in the presence of BMP for 3 d and up to 28 d (Fig. 1A–E; data not shown). The cell cycle arrest was due to exposure to BMP, since removing EGF from the culture medium without adding BMP4 did not block proliferation (Supplemental Fig. S1A), and adding the BMP signaling inhibitor Noggin to the BMP4-containing medium prevented NS cells from exiting the cell cycle or caused cell cycle re-entry when cells had previously been exposed to BMP4 for 3 d (Supplemental Fig. S1A). Flow cytometry analysis revealed that BMP-treated cells were arrested with a 2N DNA content; i.e., in the G1 or G0 phase of the cell cycle (Supplemental Fig. S1B). Antibody staining confirmed that the cell cycle-arrested cells maintained expression of the NSC markers Sox2, Nestin, and BLBP and did not express the astrocyte marker S100β or the neuronal marker βIII-tubulin, while expression of the NSC/astrocyte marker GFAP was increased and expression of EGFR, a marker of activated NSCs (Pastrana et al. 2009), was suppressed by the BMP treatment (Supplemental Fig. S1C).

To determine whether the proliferation arrest of BMP-treated NS cells is reversible, a defining property of quiescent cells, we removed the BMP4-containing medium after 3 or 28 d and returned the cells to EGF-containing medium (Fig. 1A). After 3 d in proliferation medium, NS cells had resumed proliferation at a rate similar to that of control NS cells (Fig. 1B–E; Supplemental Fig. S1D) and had also retained their neuronal differentiation potential (Fig. 1F–H). To confirm that the effect of BMP4 is fully reversible, we used expression microarrays to compare the transcript profile of NS cells cultured sequentially in EGF medium, BMP medium for 3 d, and EGF for 6 d (called "EBE cultures" below and in Fig. 1) with the transcriptome of NS cells cultured continuously in EGF medium ("E cultures") and with that of NS cells cultured in EGF and then BMP for 3 d ("EB cultures") (Fig. 1A). Only 49 genes were significantly

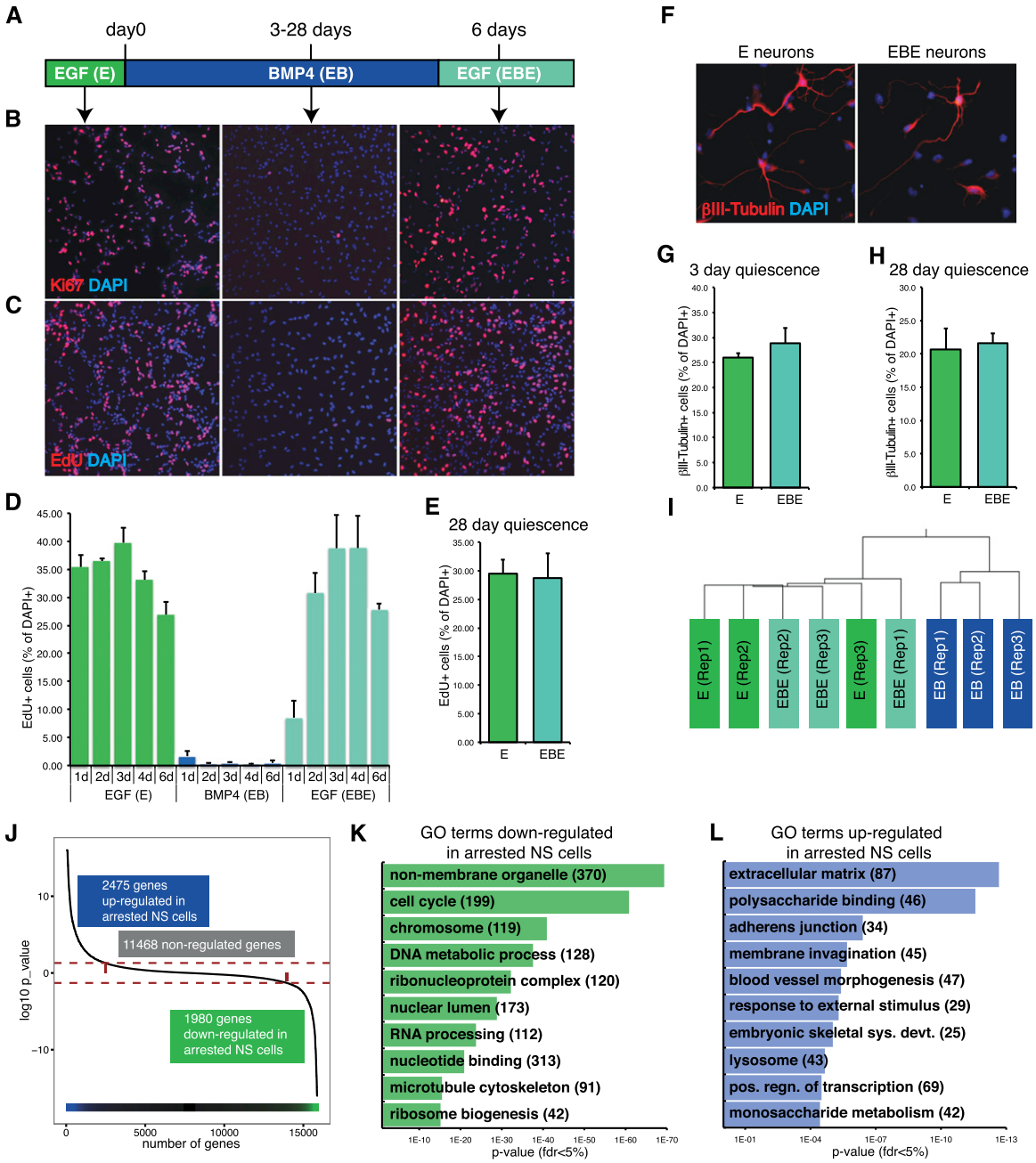


Figure 1. Characterization of cell cycle-arrested NS cell cultures. (*A*) Time course of experimental treatments. E cultures were maintained continuously in EGF-containing medium. EB and EBE cultures were first transferred into BMP4-containing medium for 3 d and then replated into either BMP4-containing (EB) or EGF-containing (EBE) medium for 1–6 d. (*B*,*C*) Analysis of proliferation by Ki67 immunostaining (*B*, red) and EdU detection (*C*, red) after 4-h exposure of NS cells in E, EB, or EBE cultures as indicated. Cells were counterstained with DAPI (blue). (*D*) Percentages of EdU-positive NS cells in E, EB, and EBE cultures. EB and EBE cultures were replated in BMP4 or EGF, respectively, for 1–6 d, as indicated. Cell cycle-arrested cells promptly resume proliferation when EGF replaces BMP4. Error bars represent the standard deviation ($n = 3$ biological replicates). (*E*) Percentages of EdU-positive cells in cultures exposed to BMP4 for 28 d and to EGF for another 3 d (*right*) or maintained in EGF for the same period (*left*). BMP-induced cell cycle arrest remains fully reversible even after a prolonged exposure. (*F*) Expression of the neuronal-specific gene βIII-tubulin in E and EBE cultures (exposed to BMP4 for 3 d) switched to a neuronal differentiation medium for 10 d. (*G*,*H*) Percentages of βIII-tubulin$^+$ cells in E and EBE cultures maintained in BMP medium for 3 d (*G*) or 28 d (*H*) before being switched back to EGF medium for 6 d and neuronal differentiation medium for 10 d. Prolonged exposure to BMP4 does not affect the differentiation potential of NS cells. (*I*) Hierarchical clustering of normalized expression values from gene microarray analysis of NS cells in E, EB, and EBE cultures. Cycling NSCs (E cultures) cluster separately from cell cycle-arrested NS cells (EB) but together with reactivated NS cells (EBE). Three independent samples were hybridized to microarrays for each condition (Rep1–Rep3). (*J*) Comparison of transcript levels in cycling and cell cycle-arrested NS cells by RNA-seq. Genes are ranked along the *X*-axis according to the statistical significance ($\log_{10}$ *P*-value) of difference in normalized expression levels (FPKM [fragment per kilobase transcriptome per million mapped reads]) between proliferating NS cells (E cultures) and arrested NS cells (EB cultures). Transcripts down-regulated in arrested NS cells are given a negative value. The horizontal dotted line represents the $P = 0.05$ significance threshold. The corresponding heat map is shown at the *bottom*. (*K*, *L*) GO analysis of genes down-regulated (*K*) and up-regulated (*L*) in cell cycle-arrested NS cells. The *X*-axis values correspond to DAVID *P*-values. All terms reported have a false discovery rate (FDR) < 5%. The number of genes belonging to each category is shown in brackets. See also Supplemental Figure S1 and Supplemental Table S1.

deregulated in EBE cultures compared with E cultures (17 down-regulated and 32 up-regulated more than twofold; $P < 0.05$). Moreover, cluster analysis of the microarray data showed that EBE cultures clustered together with E cultures and separately from EB cultures, thus suggesting that they had reverted to a transcriptional state indistinguishable from that of cells that had proliferated continuously (Fig. 1I). We thus conclude that exposure of NS cells to BMP4 for 3–28 d induces a state of cell cycle arrest that is entirely reversible.

To further examine the changes in gene expression associated with BMP4-induced cell cycle arrest, transcripts from cell cycle-arrested and proliferating NS cells were compared by RNA sequencing (RNA-seq). We found that 2475 genes were up-regulated and 1980 genes were down-regulated in arrested NS cells compared with proliferating NS cells ($P < 0.05$) (Fig. 1J). The quality of this data set was assessed by quantitative PCR (qPCR) analysis, which confirmed the regulation of a selection of up-regulated and down-regulated genes in BMP4-treated cells (Supplemental Fig. S1F). Gene ontology (GO) analysis using DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov) showed that down-regulated mRNAs were mostly involved in the cell cycle (e.g., GO terms: "cell cycle" and "chromosome") and DNA and RNA metabolism ("DNA metabolic process" and "RNA processing"), as expected for a cell cycle-arrested cell population (Fig. 1K). Other down-regulated genes were associated with protein translation ("ribonucleotide complex" and "ribosome biogenesis"), which is reminiscent of the reduction in protein synthesis associated with quiescence in many mammalian cells as well as yeast and bacteria (Valcourt et al. 2012).

Conversely, up-regulated genes included the cyclin-dependent kinase inhibitor *Cdkn2b/p15/INK4B* (fold change = 17.5; $P = 6.56 \times 10^{-7}$) as well as many cell cycle inhibitors induced in other types of quiescent cells (Venezia et al. 2004; Coller et al. 2006; Fukada et al. 2007; Lien et al. 2011). However, the most significantly enriched up-regulated gene categories in cell cycle-arrested NS cells were associated with the ECM ("extracellular matrix" and "polysaccharide binding") and cell–cell adhesion ("adherens junction") (Fig. 1L), including a large number of ECM genes (15 collagens, three laminins, and one spondin), receptors for ECM proteins (nine integrins), and cell adhesion molecules (four cadherins, two protocadherins, six cell adhesion molecules [CAMs], and four claudins) (Supplemental Table S1). All of these classes of gene are known to control the interaction of stem cells with their niche and signaling environments (Chen et al. 2013).

We then used gene set enrichment analysis (GSEA) (Subramanian et al. 2005) to directly compare the genes up-regulated and down-regulated in arrested NSCs with genes induced in published microarray profiling studies of different types of quiescent cells, including hematopoietic stem cells (Venezia et al. 2004), skeletal muscle stem cells (Fukada et al. 2007), hair follicle stem cells (Lien et al. 2011), and fibroblasts (Coller et al. 2006). All gene sets expressed in these quiescent cell populations were highly enriched in transcripts up-regulated in arrested NS cells (Fig. 2A–D), and several of the GO terms associated with quiescence-enriched gene sets in other cell types were also associated with cell cycle-arrested NS cell genes (Fig. 2F,G; Supplemental Fig. S2A,B; Beckervordersandforth et al. 2010). It is noteworthy that although many genes up-regulated in arrested NS cells were enriched in one or two other quiescent cell types, there was no common gene signature shared by all of the quiescent cells analyzed (Fig. 2E). GSEA also showed strong enrichment among the genes induced in arrested NSCs; in genes expressed in adult SEZ NSCs, which are mostly in a quiescent state (Beckervordersandforth et al. 2010); and in genes induced in neurosphere cultures by the quiescence-promoting factor FoxO3 (Supplemental Fig. S2C,E; Renault et al. 2009). Conversely, genes expressed by non-NSC astrocytes (Beckervordersandforth et al. 2010) were not significantly enriched, suggesting that BMP-treated NS cells in culture are more similar to SEZ NSCs and other quiescent adult stem cell populations than to differentiated parenchymal astrocytes (Supplemental Fig. S2D). Collectively, these results indicate that BMP4 induces in cultured NS cells a state of reversible cell cycle arrest and a transcriptome profile that are characteristic of quiescent cells.

Identification of active enhancers in quiescent and proliferating NS cells

To identify components of the GRN that control the quiescent state in NS cells, we characterized the enhancer elements that recruit TFs in these cells. To identify putative active enhancers in quiescent NS cells, we performed chromatin immunoprecipitation (ChIP) coupled to high-throughput DNA sequencing (ChIP-seq) to locate the histone acetlytransferase p300 and the histone modification H3K27ac in the genome of these cells. We defined an active enhancer as a genomic region located >2 kb from a gene transcription start site (TSS) where a ChIP-seq peak for p300 occurred within an island of H3K27ac, in agreement with recent reports (Rada-Iglesias and Wysocka 2011; Rada-Iglesias et al. 2012). Using this definition, we identified 16,810 active enhancers in the genome of quiescent NS cells (Fig. 3A; Supplemental Table S2). The large majority of these enhancers was located <20 kb (38%) or between 20 and 100 kb (42%) of the nearest genes (Supplemental. Fig. S3A).

We next asked whether enhancers identified in quiescent NS cells were present in all NS cells regardless of their cell cycle status or whether they were specific for the quiescent state. We examined the location of p300 and the H3K27ac mark in the genome of proliferating (EGF-treated) NSCs by ChIP-seq and identified 10,270 active enhancers in these cells using the same definition as above (Fig. 3A; Supplemental Fig. S3B; Supplemental Table S2). As expected from the large differences in transcript profiles between quiescent and proliferating NSCs (Fig. 1J), the majority of genomic regions with active enhancer features in quiescent NS cells did not have these features in proliferating cells (9157 quiescence-specific enhancers), while a smaller proportion of enhancers active in proliferating NS cells was not found in quiescent cells (3098

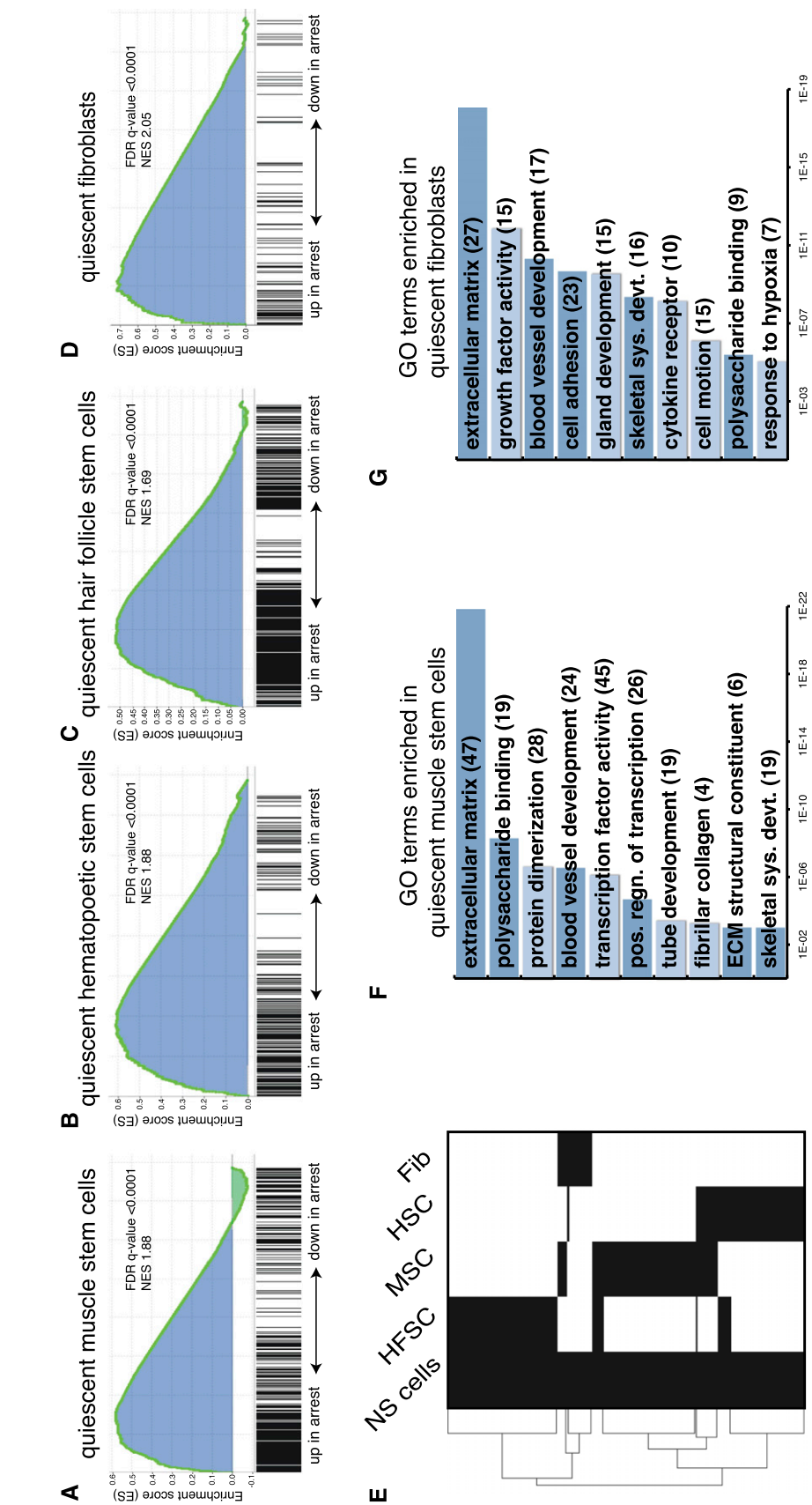


Figure 2. Cell cycle-arrested NS cells share transcriptomic features with various types of quiescent cells. (*A–D*) GSEA analysis shows that genes that are up-regulated in cell cycle-arrested NS cells (ranked by expression along the *X*-axis) are highly enriched in gene sets that are up-regulated in quiescent muscle stem cells (*A*), hematopoetic stem cells (*B*), hair follicle stem cells (*C*), and quiescent fibroblasts (*D*). (NES) Normalized enrichment score. (*E*) Hierarchical clustering of the 455 genes up-regulated in cell cycle-arrested NSCs, quiescent muscle stem cells (MSCs), hematopoetic stem cells (HSCs), hair follicle stem cells (HFSCs), and quiescent fibroblasts (Fib). (*F*,*G*) GO analysis of genes expressed by quiescent muscle stem cells (*F*) and fibroblasts (*G*). GO terms also enriched in cell cycle-arrested NSCs (see Fig. 1L) are indicated by darker blue bars. See also Supplemental Figure S2.

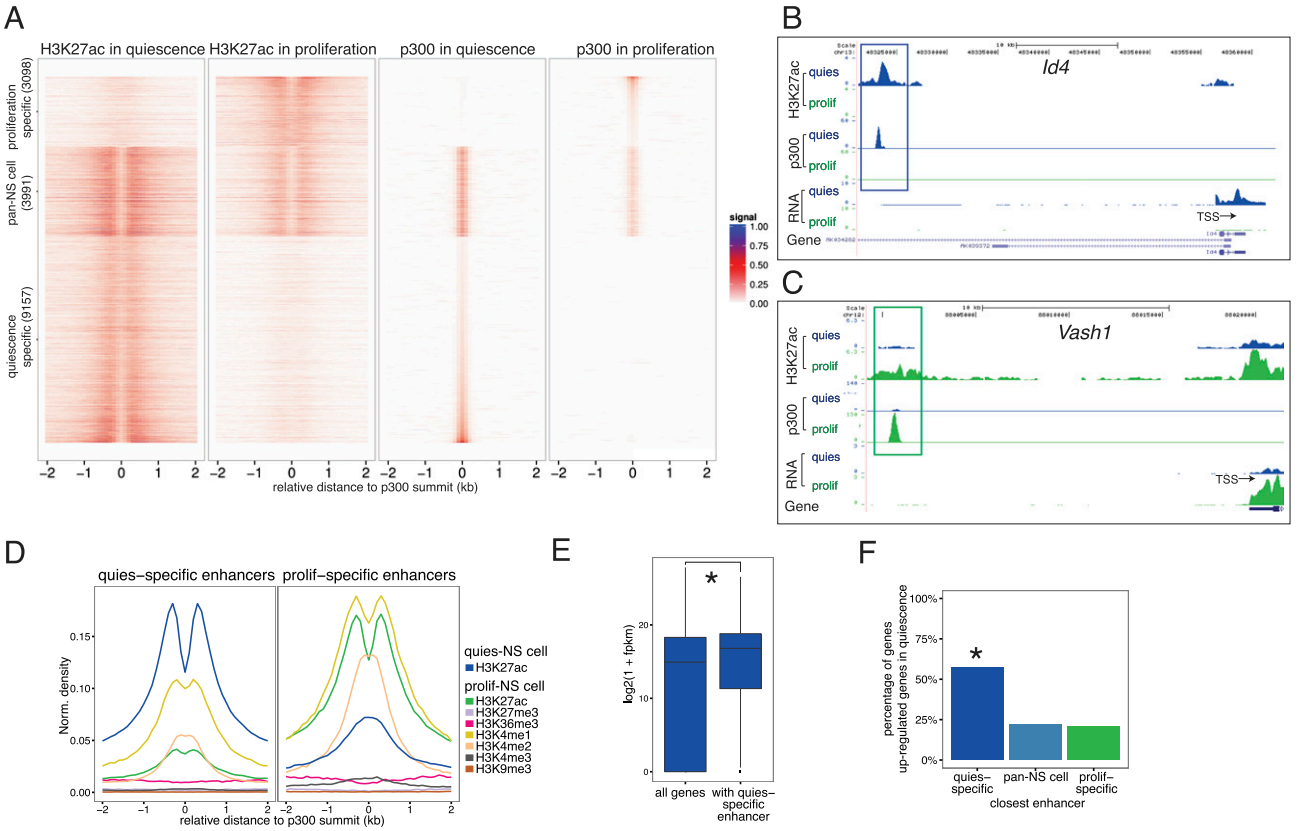


Figure 3. Identification of active enhancers in quiescent NS cells. (*A*) Heat map representation of the density of ChIP-seq reads for H3K27ac and p300 ±2 kb relative to the midpoint of enriched regions at 16,246 active enhancers in NS cells. This panel represents the merger of data obtained in proliferating and quiescent NS cells. A large fraction of the regions displayed presents active enhancer features only in proliferating NS cells or only in quiescent NS cells, and a smaller fraction presents these features in both cellular states. Intensity of color represents the normalized statistical significance of the signal versus input control sequences. (*B*,*C*) H3K27ac and p300 ChIP-seq signal and RNA expression level (FPKM) in quiescent (blue) and proliferating (green) NS cells in the vicinity of Id4 and Vash1, two representative genes that are up-regulated in quiescent and proliferating NS cells, respectively. Regions defined as quiescent and proliferating NS cell-specific enhancers are indicated by blue and green rectangles, respectively. ChIP-seq peak height corresponds to SICER *P*-value for H3K27ac and MACS *Q*-value for p300. (*D*) Average ChIP-seq signal profile for H3K27ac in quiescent (blue line) and proliferating (green line) NS cells and several other epigenetic marks in proliferating NS cells at regions defined as quiescent (*left*) and proliferating (*right*) NS cell-specific enhancers. Plots are centered on the p300 summit. Quiescent NS cell-specific enhancers show strong signals for the enhancer-associated H3K4me1 mark and weak signals for the open chromatin-associated H3K4me2 and H3K27ac marks in proliferating NS cells, consistent with these regions being marked as enhancers but minimally active in proliferating NS cells. Proliferating NS cell-specific enhancers have strong signals for H3K27ac, H3K4me1, and H3K4me2 but not the other nonenhancer-associated epigenetic modifications. Note the dip in the enrichment profile for H3K27ac, indicative of a localized depletion of nucleosomes characteristic of enhancers (Heintzman et al. 2007; Bonn et al. 2012). (*E*) Box plots of normalized transcript counts (FPKM) for all genes expressed in quiescent NS cells (*left*) and genes associated with quiescent NS cell-specific enhancers (*right*). The latter are expressed at higher levels than the transcriptomic average (Wilcoxon test, $P < 2.2 \times 10^{-16}$). (*F*) Fraction of genes up-regulated in quiescent NS cells whose closest enhancer is quiescent NS cell-specific (*left*), pan-NS cell (*middle*), or proliferating NS cell-specific (*right*). Asterisk denotes significant *P*-value (Wilcoxon test). See also Supplemental Figure S3.

proliferation-specific enhancers), and the remaining enhancers were equally active in either both quiescent and proliferating NS cells (3991 pan-NS cell enhancers) or an intermediate activity state (Fig. 3A–C; Supplemental Table S2).

To validate the enhancers that we identified in NS cells, we compared our p300 and H3K27ac profiles with published data sets on the distribution of histone marks in proliferating neural precursor cells (Mikkelsen et al. 2007; Meissner et al. 2008). We found that proliferating NS cell-specific enhancers were strongly enriched for the histone modifications H3K4me1 and H3K4me2, previously associated with enhancers in many cell types (Fig. 3D; Heintzman et al. 2007; Ernst et al. 2011). Quiescent NS cell-specific enhancers were also enriched in H3K4me1 and H3K4me2 in proliferating neural precursors but to a much lower degree. There was also some residual signal for H3K27ac, although it lacked the "valley" shape that is characteristic of active enhancers (Fig. 3D; Heintzman et al. 2007; Bonn et al. 2012). These observations suggest that these quiescence-specific enhancers exist in a primed but less active state in proliferating NS cells (Creyghton et al. 2010; Zentner et al. 2011; Bogdanovic et al. 2012),

although they lack the H3K27me3 mark that some studies have found to be characteristic of "poised" enhancers (Rada-Iglesias et al. 2011). Furthermore, both proliferation-specific and quiescence-specific enhancers showed little or no enrichment for histone modifications associated with promoters (H3K4me3), gene transcription (H3K36me3), or repression (H3K27me3 and H3K9me3) (Fig. 3D).

We also examined whether epigenomically defined regulatory elements displayed enhancer activity in a luciferase reporter assay. Six out of the seven quiescence-specific enhancers analyzed drove significant reporter gene activity in quiescent NS cells, and all of these regions were silent in proliferating cells, as expected (Supplemental Fig. S3C,D). We also obtained a good validation rate for proliferation-specific enhancers, with five out of seven enhancers analyzed showing activity in proliferating NS cells. Other recent studies have shown similar, or slightly lower, validation rates for epigenomically defined enhancers in luciferase assays (Zentner et al. 2011; Ostuni et al. 2013). Unexpectedly, we observed that all of the proliferation-specific enhancers were active in quiescent NS cells, suggesting that epigenetic factors that are absent from the transfected reporter constructs are required to silence proliferation-specific enhancers in NS cell quiescence (Supplemental Fig. S3C,D).

Altogether, these results confirm that the genomic elements identified by the coincidence of p300 and H3K27ac signals have an overall epigenetic signature of active enhancers (Heintzman et al. 2007, 2009; Ernst et al. 2011; Rada-Iglesias and Wysocka 2011; Rada-Iglesias et al. 2012).

Quiescent NS cell enhancers are associated with highly expressed genes

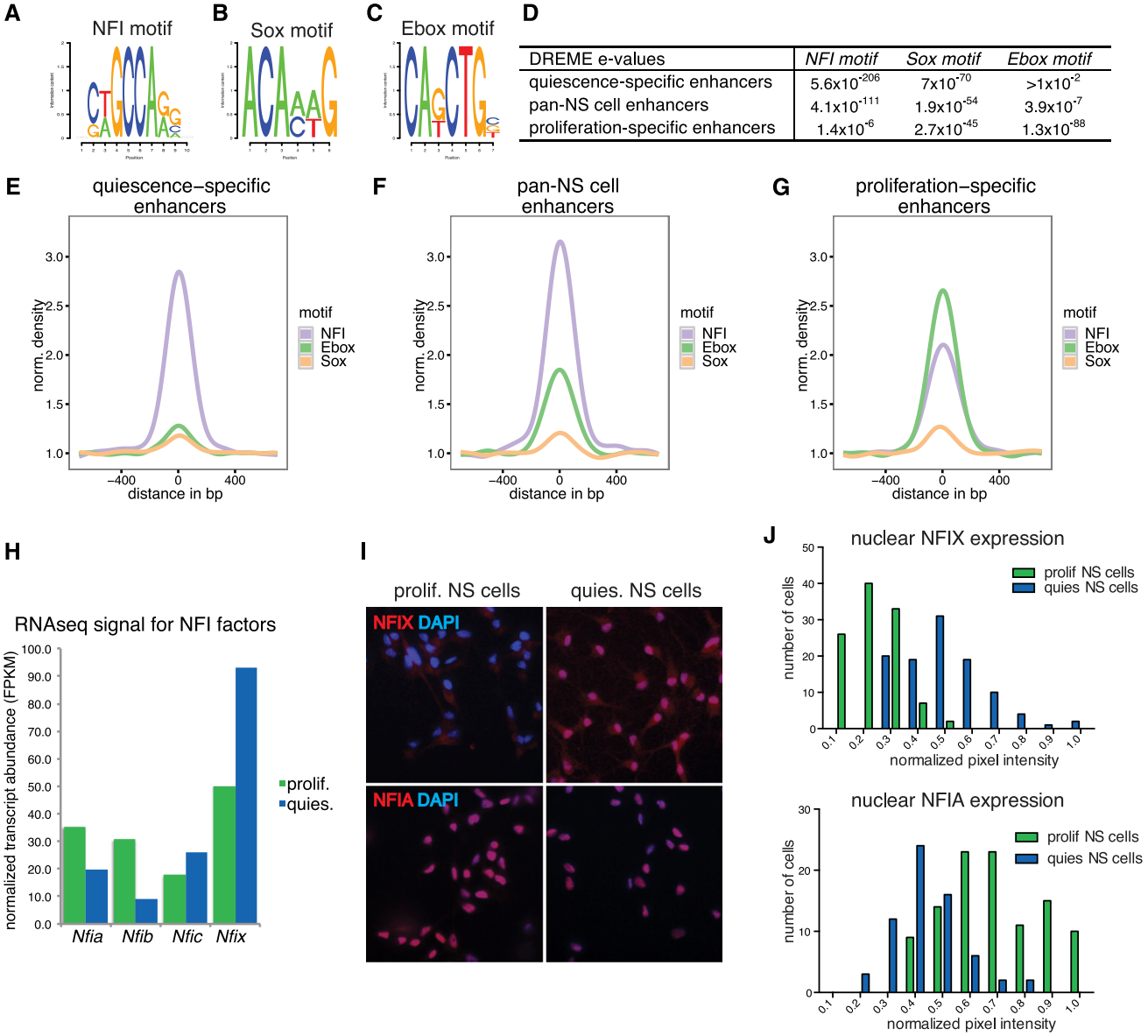
When we assigned enhancers to their nearest gene, we found a strong positive correlation between quiescence-specific enhancers and genes highly expressed in quiescent NS cells (Wilcoxon test, $P < 2.2 \times 10^{-16}$) (Fig. 3E). To investigate the functions of these enhancer-associated genes, we used the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al. 2010). We found that genes associated with quiescence-specific enhancers are highly overrepresented for generic stem cell terms ("stem cell development") and terms associated with quiescent NS cell-enriched genes ("cell junction assembly") (Fig. 1G; Supplemental Fig. 3E).

We then examined the reciprocal association of quiescent NSC-enriched genes with the different types of NS cell enhancers and found, as predicted, that genes up-regulated in quiescent NS cells are more likely to have a quiescence-specific element than a proliferation-specific or pan-NS cell element as their nearest enhancer (Wilcoxon test, $P < 2.2. \times 10^{-16}$) (Fig. 3F). The strong association between quiescent NS cell-enriched genes and quiescence-specific enhancers is also observed if one considers not just the nearest element, but an aggregate score of all enhancers present in the intervals between the genes and their two neighbors (see the Materials and Methods). Genes with a higher aggregate enhancer score in quiescent NS cells than in proliferating NS cells were significantly more likely to be induced in quiescence than expected by chance ($P = 1.24 \times 10^{-6}$). Altogether, our definition of enhancers succeeds in identifying genomic regions that have the epigenetic characteristics of active enhancers and are associated with genes that are highly expressed in quiescent NS cells and have functions relevant to the quiescent NS cell state.

Widespread binding of NFI TFs to quiescent NS cell enhancers

We hypothesized that TFs that play major roles in the GRN of quiescent NS cells should bind to a large fraction of enhancers in these cells. Quiescence-specific enhancers should therefore be enriched in the DNA-binding motifs of these TFs. De novo motif searches using the algorithms GADEM (Li 2009), MEME-chip (Machanick and Bailey 2011), and RSAT (Thomas-Chollier et al. 2012) consistently recovered three distinct motifs that were significantly enriched, specifically around the summit of p300 binding, in all three categories of NS cell enhancers (Fig. 4A–G; data not shown). These motifs closely resemble consensus binding sites for NFI (Fig. 4A), Sox factors (Fig. 4B), and basic helix–loop–helix (bHLH) factors (Fig. 4C). When taking into account the frequency of random occurrence of these motifs in the genome, the NFI motif was by far the most strongly enriched in quiescence-specific enhancers (Fig. 4D,E) and was also more prevalent in pan-NS cell enhancers (Fig. 4D,F), while the bHLH motif (E-box) was the most abundant in proliferation-specific enhancers (Fig. 4D,G). These results suggest that members of the NFI TF family bind to a large fraction of enhancers in quiescent NS cells and may therefore contribute significantly to the GRN that operates in these cells.

To address whether motif enrichment predicts TF binding and examine the function of enhancer-bound TFs, we chose to focus on the NFI family, since the NFI motif was the most prevalent in quiescent NSC enhancers, and these factors were not previously known to regulate NSC biology, whereas the functions of Sox and bHLH factors have already been extensively studied in these cells (Bylund et al. 2003; Ligon et al. 2007; Scott et al. 2010; Castro et al. 2011). Our RNA-seq, immunocytochemistry, and Western blot data showed that the four members of the NFI family (NFIA, NFIB, NFIC, and NFIX) are expressed in both proliferating and quiescent NSCs, but NFIX is up-regulated when NS cells become quiescent (the nuclear protein ratio in quiescent cells/proliferating cells is 226%), while NFIA, NFIB, and NFIC are down-regulated or unchanged (64%, 51%, and 87%, respectively) (Fig. 4H–J; Supplemental Fig. S4A–C). To examine NFI protein binding to the NS cell genome, we performed a ChIP-seq analysis with an antibody that specifically recognizes the four NFI factors, which are closely related in sequence (Mason et al. 2009; Pjanic et al. 2011). NFI factors bound to 25,807 high-confidence sites in quiescent NS cells (Fig. 5A,B). As expected, de novo analysis identified the consensus NFI-binding motif as the most overrepresented in NFI-bound regions (Supplemental Fig. S4D,E). NFI-binding events were found in a very



DREME e-values	*NFI motif*	*Sox motif*	*Ebox motif*
quiescence-specific enhancers	5.6×10^{-206}	7×10^{-70}	$>1\times10^{-2}$
pan-NS cell enhancers	4.1×10^{-111}	1.9×10^{-54}	3.9×10^{-7}
proliferation-specific enhancers	1.4×10^{-6}	2.7×10^{-45}	1.3×10^{-88}

Figure 4. The NFI motif is most strongly overrepresented in quiescent NS cell enhancers. (*A–C*) DNA sequence motifs matching consensus binding sites for NFI (*A*), Sox (*B*), and bHLH (*C*, E-box) TFs are found overrepresented in quiescence-specific, pan-NS cell, and activity-specific enhancers by de novo motif searches. (*D*) Enrichment values (*E*-values) of NFI, Sox, and E-box motifs in quiescence-specific, pan-NS cell-specific, and activity-specific enhancers as reported by DREME. (*E–G*) Observed frequency of motif occurrence around the summit of p300 binding in quiescence-specific, pan-NS cell, and activity-specific enhancers. The NFI motif is the most overrepresented in quiescence-specific and pan-NS cell enhancers. (*H*) RNA-seq shows that all four NFI genes are transcribed in NS cells, with transcript levels of *Nfix* increasing sharply in quiescent cells, while those of *Nfia*, *Nfib*, and *Nfic* decrease or remain unchanged. (*I,J*) Immunocytochemistry shows that NFIX protein is strongly induced in quiescent NS cells, while NFIA expression is reduced. See also Supplemental Figure S4.

large fraction of the enhancers in quiescent NS cells (12,222; 73%), thus confirming that the strong enrichment of NFI motifs is a useful predictor of the widespread binding of these factors in quiescent NS cell enhancers (Fig. 5A,B). Conversely, an unusually high fraction of the NFI-binding sites (12,323; 48%) mapped within an active enhancer, while a further 2675 binding sites (10.3%) mapped in promoter regions (Fig. 5B; Supplemental Fig. S4F). Furthermore, within quiescent NS cell enhancers, the significance of the NFI-binding peaks correlated strongly with that of p300 peaks (Fig. 5C), and the peak summits mapped closely to each other (Fig. 5D), suggesting that NFI factors have a central role in enhancer activity in quiescent NS cells.

Functional annotation of NFI-bound enhancers using GREAT also supported an important role for these enhancers in regulating the quiescent NS cell state. Genes linked to quiescent-specific enhancers that are bound by NFI factors are particularly enriched for the processes of

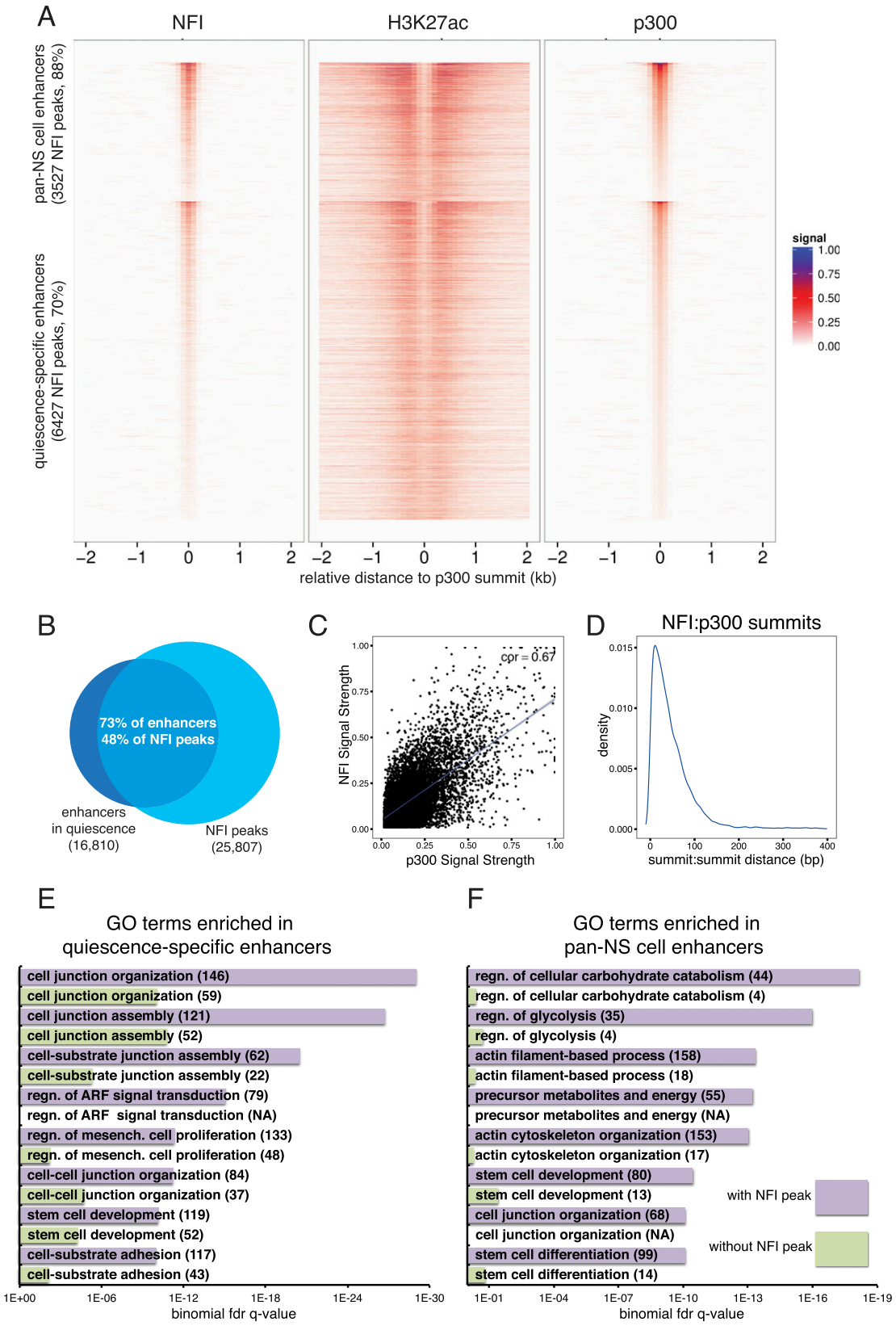


Figure 5. NFI TFs bind to the majority of quiescent NS cell enhancers. (*A*) Heat map representation of all enhancers active in quiescent NS cells sorted into quiescent-specific and pan-NS cell enhancers showing ChIP-seq signal for NFI TFs, H3K27ac, and p300. (*B*) Venn diagram showing the large overlap of enhancers in quiescent NS cells with regions of significant NFI TF binding. (*C*,*D*) Strong correlation of the strength of ChIP-seq signals for p300 and NFI in enhancers (*C*; correlation coefficient = 0.67) and close proximity of their summits (*D*; median intersummit distance = 35 base pairs [bp]), consistent with p300 recruitment by NFI TFs. (*E*,*F*) Functional annotation of quiescence-specific (*E*) and pan-NS cell (*F*) enhancers bound by NFI TFs by GREAT according to GO biological process. Enhancers bound by a NFI factor (purple) and those that are not significantly bound (green) were examined separately. The *X*-axis values represent the binomial FDR *Q*-values; the numbers in parentheses are the number of binomial region hits.

cell junction organization and assembly, while genes associated with pan-NS cell enhancers bound by NFI are mostly involved in carbohydrate metabolism (Fig. 5E,F). These activities are specifically associated with the quiescent NS cell state (Fig. 1L), which argues for a central role of NFI proteins in the GRN operating in quiescent NS cells.

NFIX is both required and sufficient to induce aspects of quiescence in NS cells

To directly address the role of NFI factors in quiescent NS cells, we focused on NFIX, as it is the only family member whose expression is up-regulated when cells enter quiescence. NS cells were transduced with a lentivirus encoding an shRNA for *Nfix* that reduced *Nfix* mRNA and protein levels to 37%–30% and 51%–30% of controls, respectively (Fig. 6A; Supplemental Fig. S5A–D; Messina et al. 2010). EdU incorporation and flow cytometry analysis showed that in proliferation conditions, *Nfix* shRNA-expressing and control cells proliferated at the same rate, suggesting that *Nfix* does not play a major role in NS cell proliferation (Supplemental Fig. S5E). In contrast, the entry into quiescence of *Nfix* shRNA-expressing cells was delayed, and a significant fraction of the cells remained proliferative even after 3 d of exposure to BMP (Fig. 6B,C; Supplemental Fig. S5E). Conversely, overexpressing NFIX in proliferating NS cells resulted in a rapid cell cycle arrest of electroporated cells without induction of markers of astrocytic (S100β) or neuronal (βIII-tubulin) differentiation (Fig. 6D,E; Supplemental Fig. S5F), suggesting that NFIX is sufficient and to some extent required for NS cells to exit the cell cycle.

To further examine the cellular states induced by *Nfix* silencing and overexpression, we analyzed the transcriptome of NS cells expressing *Nfix*, *Nfix* shRNA, or control vectors with microarrays. Silencing *Nfix* in quiescent NS cells resulted in the up-regulation or down-regulation of 1677 genes, while its overexpression in proliferating cells resulted in the regulation of 2565 genes, and 628 genes were regulated in both experiments (i.e., 37% of the genes regulated by *Nfix* loss of function and 24% of the genes regulated by *Nfix* gain of function) (Supplemental Fig. S5G–I). Remarkably, 69% of the 3634 genes regulated by *Nfix* in either of the two conditions were part of the set of genes that is regulated when BMP-treated NS cells enter quiescence, and *Nfix*-regulated genes represented 48% of all of the quiescence-regulated genes (Figs. 1J, 6F). Among all *Nfix*-regulated genes, 1713 genes were activated by *Nfix* (i.e., down-regulated by *Nfix* knockdown and/or up-regulated by *Nfix* overexpression), and 44% of these were part of the gene expression program induced in quiescent NS cells (hypergeometric test, $P < 2.2 \times 10^{16}$), including genes involved in vasculature development, morphogenesis, cell adhesion, and ECM that were also overrepresented in the quiescence program (Figs. 1J,L, 6G; Supplemental Fig. S5H). Two-thousand-three-hundred -forty-nine genes were repressed by *Nfix* (i.e., up-regulated by *Nfix* knockdown and/or down-regulated by *Nfix* overexpression), and 38% of these were part of the program activated in proliferating NS cells (hypergeometric test, $P < 2 \times 10^{16}$), including, predictably, genes involved in the cell cycle, DNA metabolism, and protein translation (Fig. 6H; Supplemental Fig. S5I). Genes shown to be regulated by microarray were validated by qPCR (Supplemental Fig. S5J–M). A majority of *Nfix*-activated quiescence-specific genes (71%) were associated with NFI-binding events (Supplemental Fig. S5 H), suggesting that NFIX directly activates an important fraction of the genes in the quiescence program (534 genes corresponding to 20% of quiescence up-regulated genes). In contrast, NFI factors were only bound to a minority of *Nfix*-repressed proliferation-specific genes (43%), suggesting that its suppression of the proliferation program is more indirect (Supplemental Fig. S5 I).

To examine with an independent method the role of *Nfix* in activation of the quiescence gene expression program, we used a dominant-negative construct that interferes with the activity of all NFI family members (NFI-EnR or DN-NFI) (Supplemental Fig. S6A–C; Bachurski et al. 2003) Microarray analysis of NS cells electroporated with DN-NFI or a control vector and placed in BMP4-containing medium for 18 h, showed that 58% of 638 genes down-regulated by DN-NFI were part of the quiescence program induced by BMP4 in NSCs (hypergeometric test, $P < 2.2 \times 10^{-16}$). These 368 NFI-induced genes represented 19% of the quiescence program and were mostly involved in cell adhesion and the ECM. Conversely, 43% of 518 genes up-regulated by DN-NFI were part of the proliferation-specific program and were predominantly involved in protein translation (Supplemental. Fig. S6D–G).

Together, this functional analysis demonstrates that NFIX is both required and sufficient to activate a very significant portion of the gene expression program of NS cell quiescence and suppress an important part of the program of NS cell proliferation.

NFIX is required for NSC quiescence in the postnatal brain

We next asked whether our finding that NFIX regulates part of the quiescence program in cultured NS cells was predictive of a role for this factor in NSCs in vivo. We examined the expression of NFIX in the adult brain and found that it was expressed by NSCs in the two adult neurogenic regions (Fig. 7; data not shown). In the DG of the hippocampus, NFIX was expressed by Nestin$^+$, GFAP$^+$ radial NSCs, including both quiescent (MCM2-negative, 49%) and proliferating cells (MCM2-positive, 66.6%) (Fig. 7A–E). Thus, NFIX is expressed by a subset of quiescent NSCs in vivo and might play a similar role in regulating their gene expression programs, as we observed in cultured quiescent NS cells.

Nfix-null mutant mice present severe morphological defects in both the SEZ and DG at postnatal stages as well as defects in progenitor cell differentiation in the DG at birth (Campbell et al. 2008; Heng et al. 2012b). They die at 3 wk of age, which precludes an analysis of hippocampal NSCs by label retention, but postnatal hippocampal NSCs can also be identified by their radial morphology and the coexpression of Nestin and GFAP. Analysis of the DG of *Nfix* mutant mice soon before they die revealed

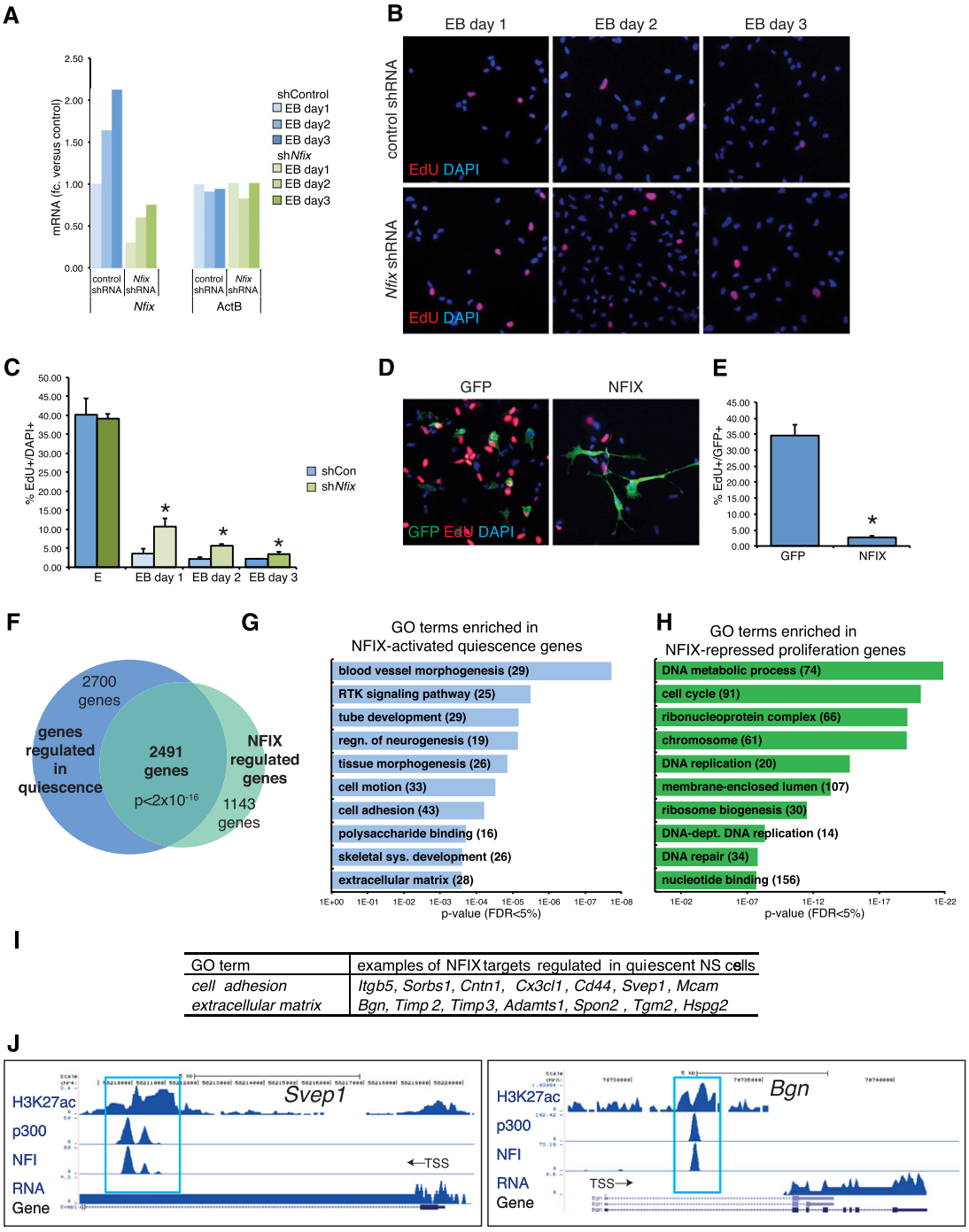


Figure 6. NFIX is both required and sufficient to induce aspects of quiescence in NS cell cultures. (*A*) Efficiency of *Nfix* silencing in NS cells exposed to BMP to induce quiescence at the time of shRNA electroporation analyzed by qPCR 1, 2, and 3 d after shRNA transfection and BMP exposure. A scrambled shRNA was used in the control experiment, and expression of the gene ActB is analyzed for comparison. Note that Nfix transcript levels increase progressively between days 1 and 3 as cells enter quiescence in both control and *Nfix* knockdown experiments. (*B*) Analysis of proliferation by EdU immunostaining after 4 h of exposure in NS cell cultures following 1, 2, and 3 d of BMP exposure as indicated. Cells are counterstained with DAPI (blue). (*C*) Percentages of EdU-positive NS cells in *Nfix* shRNA transfected and control cultures. The BMP-induced cell cycle arrest is delayed by *Nfix* silencing. The progressive reduction in cell proliferation of *Nfix* shRNA-treated cultures between days 1 and 3 might be due to the progressive increase in *Nfix* expression during this period (shown in *A*). Error bars represent the standard deviation ($n = 3$ biological replicates). (*D*) Analysis of proliferation by EdU immunostaining in NS cell cultures transfected 18 h earlier with a *Nfix* expression construct and GFP or with GFP alone. (*E*) Percentages of EdU-positive cells in NS cell cultures transfected with GFP or GFP and *Nfix*. *Nfix* efficiently promotes cell cycle arrest. (*F*) Venn diagram showing the large fraction of genes regulated in quiescent NS cells that are also regulated by *Nfix*. GO analysis of Nfix-activated genes that are also induced in quiescent NS cells (*G*) and Nfix-repressed genes that are up-regulated in proliferating NS cells (*H*). (*I*) Representative examples of putative NFI direct target genes (associated with a NFI-bound enhancer/promoter and activated by *Nfix*) induced in quiescent NS cells and belonging to functionally important GO categories. (*J*) ChIP-seq signal for H3K27ac, p300, and NFI and RNA-seq signal (FPKM) for *Svep1* and *Bgn*, two representative NFI direct target genes up-regulated in quiescent NSCs. Significant NFI binding within enhancer regions is indicated by pale blue rectangles. Peak height corresponds to SICER *P*-value for H3K27ac and MACs *Q*-value for p300 and NFI. See also Supplemental Figure S5 and Supplemental Table S2.

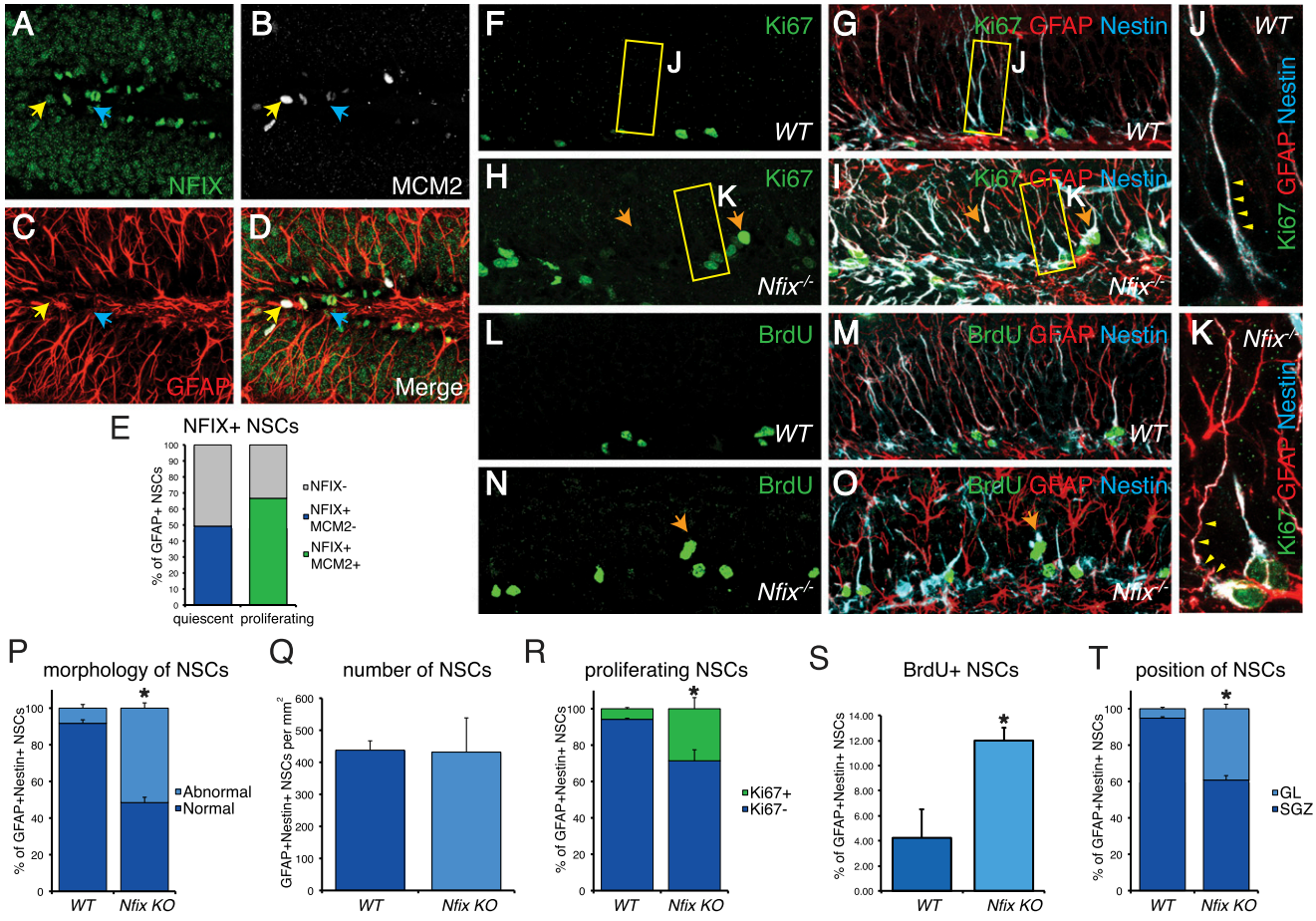


Figure 7. NFIX is required for NSC quiescence in the postnatal brain. (*A–D*) NFIX expression in NSCs in the postnatal day 90 (P90) mouse DG subgranular zone (SGZ). NSCs express GFAP and have a distinctive radial morphology. Proliferative NSCs express MCM2. (*E*) NFIX is expressed in 49% of quiescent and 66% of proliferative NSCs. Increased proliferation, reduced quiescence, and abnormal morphology and position of NSCs in the P20 *Nfix*$^{-/-}$ DG (*H,I,K,N,O*) compared with wild-type (WT) littermates' DG (*F,G,J,L,M*). (*Q*) Quantification of the number of NSCs in the wild-type and *Nfix*$^{-/-}$ DG reveals no difference in the density of NSCs. An increased proportion of *Nfix*$^{-/-}$ NSCs exhibit abnormal morphology (*P*) and position within the DG (*T*). (GL) Granule cell layer. Examples are indicated with orange arrows in *H*, *I*, *K*, and *N*. An increased number of *Nfix*$^{-/-}$ NSCs are in a proliferative state compared with wild-type littermates, as measured by expression of Ki67^{+} (*R*) and incorporation of BrdU (*S*). In *A–D*, yellow arrows highlight examples of proliferating NSCs, and blue arrowheads highlight examples of quiescent NSCs. In *J*, yellow arrowheads demonstrate the typical radial orientation of wild-type DG NSC processes, while *K* shows an *Nfix*$^{-/-}$ NSC with an abnormally oriented main process. Values in *P–T* are the mean plus standard deviation from counts in three mice. Asterisks in *P–T* indicate statistical significance of difference between wild type and *Nfix* mutants (*t*-test, $P < 0.05$; $n = 3$ independent mice). See also Supplemental Figure S6.

a marked reduction in the number of GFAP^{+}, Nestin^{+} NSCs with a typical radial morphology compared with wild-type control mice as well as a concomitant increase in the number of GFAP^{+}, Nestin^{+} NSCs with a polarized but abnormal morphology, presumably due to a deleterious effect of loss of NFIX on NSC integrity (Fig. 7P). When grouping together GFAP^{+}, Nestin^{+} NSCs with radial and abnormal morphologies, their number per area unit was not significantly different in *Nfix* mutant and wild-type control mice, indicating that NFIX is not essential for the maintenance of NSCs (Fig. 7Q). However, for both radial and abnormal GFAP^{+}, Nestin^{+} NSCs, the fraction of cells that divide, as marked by Ki67 expression, was dramatically increased in *Nfix* mutants (28.6% ± 6%) compared with control mice (5.8% ± 0.6%; *t*-test, $P = 0.02$; $n = 3$) (Fig. 7F–J,R). We obtained similar results by quantifying proliferating NSCs with BrdU after a 2-h BrdU incorporation (12.0% ± 0.6% BrdU^{+} NSCs in *Nfix* mutant mice and 4.2% ± 1.3% in control mice; *t*-test, $P = 0.006$; $n = 3$) (Fig. 7L–O,S). Thus, Nfix is essential to maintain hippocampal NSCs in a quiescent state. Moreover, the distribution of proliferating NSCs in the *Nfix* mutant DG was highly abnormal, with a large fraction of these cells ectopically located in the granular layer of the DG (Fig. 7T). This suggests that *Nfix* mutant NSCs are not properly anchored to the subgranular layer of the DG, and therefore, similar to NFI factors in cultured NSCs, NFIX regulates the cell adhesion properties of hippocampal NSCs, which in turn might directly influence the quiescent status of NSCs.

Discussion

Our study illustrates how modeling stem cell physiology in culture can be used in combination with epigenomic profiling to identify major TFs regulating stem cell states. Here, we characterized and validated a cell culture model of NSC quiescence. We used this model to demonstrate that proteins of the NFI family bind to a large fraction of enhancer elements active in quiescent NS cells and that NFIX regulates a gene expression program controlling multiple aspects of the quiescent NSC phenotype and in particular their cell adhesion properties. We discuss below our findings on the quiescent state in NSCs and the role and mode of action of NFIX in these cells.

An in vitro model of NSC quiescence

In contrast to embryonic NSCs that are highly proliferative, NSCs in neurogenic regions of the postnatal and adult brain are relatively quiescent, with the vast majority resting in G0, and only a small fraction actively progressing through the cell cycle (Temple 2001; Fuentealba et al. 2012). To understand how adult NSCs select between proliferative and quiescent states and how this selection is biased by a variety of physiological and pathological stimuli, it is necessary to characterize the transcriptional mechanisms that control the quiescent state in these cells. However, NSCs are intermingled with other cell types in the neurogenic niches of the brain and are difficult to purify in significant numbers, thus precluding a systematic study of the mechanisms regulating gene expression in NSCs in vivo (Pastrana et al. 2009; Beckervordersandforth et al. 2010). Homogenous NS cells have been established from embryonic stem cells and embryonic and adult brain tissue (Conti and Cattaneo 2010). NS cells are highly proliferative and therefore have been used to investigate mechanisms controlling cell proliferation, fate specification, and differentiation (Conti and Cattaneo 2010; Castro et al. 2011). In contrast, the quiescent state of NS cells and the GRN inducing and maintaining this cellular state have been poorly studied.

Our extensive characterization of embryonic stem cell-derived NS cells cultured in the presence of BMP4 and FGF2 has shown that these cells have hallmarks of quiescent stem cells. Different types of quiescent cells share few characteristic properties beyond reversible cell cycle arrest, but gene expression profiles can be used as a representation of the unique physiology of cellular quiescence (Coller et al. 2006). Analysis of the gene expression profile of cell cycle-arrested NS cells showed that these cells are very significantly enriched for genes induced by fibroblasts and different types of adult tissue stem cells when they enter quiescence, thus suggesting that different cell types, including NS cells, employ overlapping quiescence gene expression programs. In contrast, these cells were not significantly similar to parenchymal astrocytes, in agreement with a recent report showing that while BMP signaling alone promotes terminal astrocytic differentiation, exposure to both BMP and FGF2 maintains the stem cell character of NS cells (Sun et al. 2011).

Beside the down-regulation of genes related to the cell cycle and protein translation, entry of NS cells into quiescence is accompanied by the up-regulation of many genes encoding ECM proteins, receptors for ECM molecules, and cell–cell adhesion molecules. This suggests that quiescence involves a profound change in the attachment of NSCs to the ECM and neighboring cells, as previously suggested for NSCs in the adult SEZ (Kazanis et al. 2010; Kokovay et al. 2010, 2012) and for other types of adult stem cells (Venezia et al. 2004; Fukada et al. 2007; Pallafacchina et al. 2010; Brohl et al. 2012). For example, entry of hematopoietic stem cells into quiescence involves homing to their cellular niche, which is mediated by integrins and the transmembrane glycoprotein endoglin (which are significantly induced in quiescent NS cells) (Supplemental Table S1; Venezia et al. 2004). Similarly, it has been proposed recently that NSCs in the adult SEZ move from an ependymal niche to a vascular niche as they become activated (Kokovay et al. 2010). Interestingly, α6 integrin, a factor that we found expressed by proliferating NSCs and down-regulated when cells enter quiescence (Supplemental Table S1), is required for the binding of NSCs to endothelial cells in the SEZ (Shen et al. 2008). Expression of different repertoires of adhesion molecules, ECM proteins, and ECM receptors by quiescent and activated NSCs is therefore likely to promote or facilitate their interactions with different niche cells and hence play an important role in their exposure to different signaling environments as well as influence how these cells respond to such signals (Kerever et al. 2007; Riquelme et al. 2008; Hynes 2009).

BMP signaling and quiescence

BMP signaling promotes quiescence in not only NS cell cultures (Mira et al. 2010; Sun et al. 2011; this study), but also the adult hippocampus, where perturbation of BMP signaling results in excessive proliferation and eventual depletion of hippocampal stem cells (Mira et al. 2010). BMPs have also been implicated in the quiescent state of other types of adult stem cells, including hair follicle, intestinal, and hematopoietic stem cells (Kobielak et al. 2007; Li and Clevers 2010; Lien et al. 2011). Surprisingly, Smads, the main transcriptional effectors of BMPs, do not seem to play a major role in regulating gene expression in BMP-treated quiescent NS cells, since the consensus Smad-binding motif is not significantly overrepresented in enhancers active in quiescent NS cells (data not shown). Genes of the inhibitor of differentiation (Id) family are major targets of BMP signaling in many tissues, including the embryonic nervous system (Nakashima et al. 2001; Samanta and Kessler 2004; Vinals et al. 2004). The four family members, and particularly Id1 and Id4, are highly up-regulated in quiescent NS cells and may therefore contribute significantly to the quiescence-inducing activity of BMP. Id proteins inhibit the activity of bHLH TFs by disrupting dimerization with their E protein partners and preventing their binding to DNA (Massari and Murre 2000). Id proteins may contribute to the quiescent state by antagonizing bHLH factors that promote the proliferation

of neural progenitors, including Olig2 (Ligon et al. 2007) and Ascl1 (Castro et al. 2011). This idea is supported by the finding that consensus binding sites for bHLH proteins are much more prevalent in enhancers active in proliferating NSCs than in enhancers active in quiescent NSCs. However, induction of Id genes is unlikely to be the main mechanism by which BMP4 drives NS cell quiescence, since overexpression of Id1 in NS cells is sufficient to arrest their divisions but not to induce the broad changes in gene expression observed in BMP-treated quiescent NS cells (B Martynoga, unpubl.). Hes1 is another gene induced by BMP signaling in both the embryonic brain and cultured NS cells (Nakashima et al. 2001; this study) and inhibits progenitor cell proliferation (Baek et al. 2006; Yu et al. 2006). Moreover, Hes1 is essential for the maintenance of fibroblast quiescence (Sang et al. 2008). However, Hes1-binding motifs are not strongly overrepresented in NS cell enhancers (data not shown), arguing against an important role of Hes factors in these cells. Our analysis shows instead that NFIX has a major role in the GRN operating in quiescent NS cells, as discussed below. How BMP signaling regulates NFI proteins is currently not known, and further investigations are required to address this important question.

Widespread enhancer binding of NFI factors in quiescent NS cells

To identify TFs with important roles in the regulation of NS cell quiescence, we first annotated enhancer elements that are active in these cells, defined as genomic regions that recruit the coactivator p300 and harbor H3K27ac (Heintzman et al. 2009; Creyghton et al. 2010; Rada-Iglesias and Wysocka 2011; Rada-Iglesias et al. 2012). We found a larger number of enhancers in quiescent NSCs (16,810) than in proliferating NSCs (10,270), in keeping with the greater number of genes that are up-regulated in quiescent NS cells (2475) than in proliferating NS cells (1980) and with the fact that quiescence is an active state that involves both large-scale induction and suppression of gene expression (Coller et al. 2006).

We interrogated quiescent NS cell enhancers for enriched DNA sequence motifs in order to predict TFs that regulate the quiescent state (Rada-Iglesias et al. 2012). Finding the NFI-binding motif as the most overrepresented in these enhancers was unexpected, since the NFI gene family previously had no known function in NSC biology. Location analysis demonstrated that NFI factors are indeed bound to a remarkable 73% of enhancers in the quiescent NSC genome. Reciprocally, half of NFI-binding sites are located within epigenomically defined enhancers. In contrast, a recent study showed that despite strong enrichment of its motif, only 10% of TFAP2A TF ChIP-seq peaks mapped within enhancers in cultured human neural crest cells, and just 30% of all active enhancers in these cells were TFAP2A-bound (Rada-Iglesias et al. 2012). This, together with the strong correlation observed between NFI- and p300-binding strengths, argues for a central role for NFI factors in regulating gene expression in quiescent NS cells.

NFIX targets cell adhesion and ECM genes to promote NS cell quiescence

Three of the four NFI genes—*Nfia*, *Nfib*, and *Nfix*—are widely expressed in the developing nervous system, including in progenitors, post-mitotic neurons, and glial cells (Mason et al. 2009; Heng et al. 2012a). These genes have been implicated in multiple aspects of neural development, including the specification, differentiation, and migration of both astrocytes and neurons (Shu et al. 2003; Deneen et al. 2006; Campbell et al. 2008). The four family members are expressed in cultured NS cells; however, NFIX is the only member whose expression is sharply up-regulated at the transition from proliferation to quiescence and is the most abundant in quiescent NS cells. We therefore focused our functional analysis on *Nfix* by overexpressing or silencing the gene in NS cells and examining *Nfix* mutant brains. Our results show that *Nfix* does indeed play an essential role in the regulation of the quiescent state of NS cells.

A prominent change in the transcriptome of NS cells when *Nfix* was induced or silenced was the regulation of a large number of ECM and cell adhesion molecules. The mislocalization of NSCs in *Nfix* mutant hippocampus suggests that *Nfix* controls the cell adhesion properties of NSCs in vivo. As already discussed, ECM and cell adhesion molecules constitute a large fraction of the gene expression programs of different types of quiescent cells, and adhesion of stem cells to their niche is thought to be important for maintenance of the quiescent state, particularly for hematopoietic stem cells (Hurley et al. 1995; Scott et al. 2003; Venezia et al. 2004). Since our results show that *Nfix* directly regulates only a few cell cycle arrest genes, the primary cause of the loss of quiescence and excess proliferation of NSCs in *Nfix* mutant hippocampus might be the loss of cell adhesion and disruption of interactions with their niche.

Previous analyses of *Nfi* mutant mice have shown that *Nfi* family members have divergent roles in brain development (Mason et al. 2009). *Nfix* mutants present an overexpansion of the embryonic brain and a delay of hippocampal progenitor differentiation that are not seen in other *Nfi* mutants (Driller et al. 2007; Campbell et al. 2008; Heng et al. 2012b). Moreover, manipulation of *Nfia* expression alongside that of *Nfix* in NS cells shows that the two genes have different activities in the cells, and *Nfia* does not have a prominent role in the regulation of quiescence (B Martynoga, unpubl.). Given the close structural similarity between NFI factors, it will be interesting to elucidate how these factors exert their divergent functions. Different NFI proteins might regulate different target genes by recognizing subtly different DNA motifs or may target the same genes but regulate them differently. *Nfi* genes also have distinct expression patterns, and it will be important to characterize the pathways that regulate their expression; e.g., the up-regulation of *Nfix* and the down-regulation of *Nfia* and *Nfib* in quiescent NS cells.

Together, our study establishes a platform to understand how the signaling environment of the niche influences

NSC physiology and decipher the regulatory networks that control the different NSC states.

Materials and methods

NS cells

NS5 cells were cultured according to standard methods (Conti et al. 2005) with the following minor modification: Cells were plated onto uncoated tissue culture plastic with the addition of 2 μg/mL laminin (Sigma) to the medium. To induce quiescence, 35,000–65,000 cells per square centimeter were plated into normal proliferation medium (EGF and FGF2, both at 10 ng/mL; Peprotech), and, after 16 h, fresh NSC medium was added without EGF and with 50 ng/mL BMP4 (R&D Systems) and 20 ng/mL FGF2. For reactivation, after at least 3 d in BMP4-containing medium, cells were passaged with Accutase (Sigma) and plated into proliferation medium at a density of 35,000–65,000 cells per square centimeter. Details of immunostaining, cell cycle analysis by FACS, qPCR, and induction of neurogenesis are described in the Supplemental Material.

shRNA knockdown of Nfix

Lentiviral particles encoding a control or *Nfix*-specific shRNA construct and a puromycin resistance cassette (Messina et al. 2010) were generated in 293T cells according to standard procedures. Proliferating NS cells were transduced with the lentiviruses, and, 24 h later, 2 μg/mL puromycin was added to select for shRNA-expressing cells. After a further 48 h, NS cells were assessed for proliferation or plated into quiescence medium for proliferation, RNA, and FACS analysis.

Transfection and cell sorting

Cells were electroporated with NFIX (isoform X2), NFIA (isoform A1), NFIC (isoform C2), or DN-NFI (NFI-enR) (Bachurski et al. 2003) cloned upstream of an IRES and NLS-tagged GFP under the control of the CAGGs promoter or GFP control, both in the pCAGGS expression vector with AAD-1011 nucleofector (Amaxa). NFIX transfected cells were plated into proliferation medium, trypsinized, and FACS-sorted 18 h later. DN-NFI transfected cells were plated into prewarmed proliferation medium for 8 h, and then medium was replaced with quiescence medium. After 24 h, cells were trypsinized and FACS-sorted for GFP expression directly into Trizol LS (Invitrogen) for RNA extraction and downstream analysis.

ChIP

NS cells were fixed sequentially with di(N-succimidyl) glutarate and 1% formaldehyde in phosphate-buffered saline and then lysed, sonicated, and immunoprecipitated as described previously (Castro et al. 2011) using material from ~5×10^6 cells per sample. Immunoprecipitations were with rabbit anti-H3K27ac (4 μg per ChIP sample; Abcam, ab4729), rabbit anti-p300 (3 μg per ChIP sample; Santa Cruz Biotechnology, sc-585), or goat anti-NFI (6 μg per ChIP sample; Santa Cruz Biotechnology, sc-30918).

ChIP-seq data generation and processing

DNA libraries were prepared from 10 ng of immunoprecipitated DNA according to the standard Illumina ChIP-seq protocol for quiescent NS cell H3K27ac ChIP, quiescent NS cell p300 ChIP, quiescent NS cell NFI ChIP, quiescent NS cell input DNA, proliferating NS cell H3K27ac ChIP, proliferating NS cell p300 ChIP, and proliferating NS cell input DNA. Libraries were sequenced with the Genome Analyzer IIx (Illumina). The raw reads for p300, H3K27ac, and NFI in quiescent NS cells and p300 and H3K27ac in proliferating NS cells were mapped to the mouse genome (mm9, including random chromosomes) with Bowtie version 0.12.5 (Langmead 2010). For each cell condition, an input chromatin sample was mapped in the same way. The number of uniquely mapped reads in quiescent NS cells was 28.0 million for p300, 38.0 million for H3K27ac, 24.9 million for NFI, and 27.3 million for input. In proliferating NS cells, 18.8 million p300, 33.5 million H3K27ac, and 31.0 million input unique reads were mapped.

H3K27ac data sets were processed further with SICER version 1.1 (Zang et al. 2009) to define islands of enrichment, and we used MACS version 2.0.9 (Zhang et al. 2008) to define bound regions for p300 and NFI. Further details of data processing are described in the Supplemental Material.

ChIP-seq data generated in this study have been deposited in the European Nucleotide Archive's Sequence Read Archive under accession number ERP002084 and are also available via ArrayExpress under accession number E-MTAB-1423.

The ChIP-seq data sets for H3K4me1, H3K4me2, H3K4me3, H3K27me3, H3K9me3, and H3K36me3 in neural progenitors were retrieved from Gene Expression Omnibus repository with accession numbers GSE8024 and GSE11172.

Definition of active enhancers

In order to define active enhancer regions in quiescent and proliferating NS cells, we used the p300 peaks as reference, selecting peaks whose summit is included within a H3K27ac island. We removed promoter-proximal peaks whose summit is closer than 2 kb to the TSS of any gene in ENSEMBL version 61 annotated as known protein-coding. We used the *Q*-value reported by MACS for each p300 peak as the enhancer score. We divided these enhancer sets into quiescence-specific, proliferation-specific, or pan-NS cells by considering the H3K27ac and p300 signal in both cell states. For H3K27ac, we used SICER to call differentially enriched regions. Quiescence-specific enhancers are defined as p300 peaks that are only called in quiescent NS cells or that fall within an H3K27ac island that is specifically enriched in quiescence and vice versa for proliferation-specific enhancers. Pan-NS cell enhancers are defined by the consistent presence of p300 peaks in the two cell states that fall within a nondifferentially enriched H3K27ac island.

Motif analysis

To identify motifs overrepresented in the active enhancer regions, we used three tools that are based on different approaches: MEME-ChIP (Machanick and Bailey 2011), GADEM (Li 2009), and RSAT peak motifs (Thomas-Chollier et al. 2012). Parameters and further motif analysis are described in the Supplemental Material.

Generation and analysis of microarray and RNA-seq data

For microarray analysis, RNA from three biological replicates per conditions was prepared and hybridized to Illumina Mouseref-8 version 2.0 bead chips according to the manufacturer's specifications. Normalization and statistical analysis were carried out with GeneSpring software (Agilent). Probes were considered deregulated if there was ≥1.5-fold differential expression with a Benjamini-Hochberg-corrected *P*-value < 0.05 (*t*-test). Generation of RNA-seq data will be described elsewhere (S Hadjur and

D Georgopoulou, in prep.). We obtained a total of 48.3 million pairs of 75-base-pair (bp) paired-end reads for quiescent NSCs and 98.8 million pairs for proliferating NSCs and processed with TopHat and the Cufflinks package (Trapnell et al. 2012).

Functional classification of gene lists and TF-bound genomic regions

GO analysis was conducted with DAVID using functional annotation clustering. Representative terms from the top-ranking clusters of GO terms are reported, all with $P < 0.05$ and false discovery rate (FDR) < 5%. GSEA was carried out with 1000 permutations. Functional classification of genes associated with enhancers was conducted with GREAT using default settings.

Mice and immunohistochemistry

To characterize expression patterns of NFI factors in DG NSCs, we used wild-type MF1 mice. To test the function of Nfix in postnatal neurogenesis, we used mice carrying an allele of *Nfix* that lacks exon 2 (Campbell et al. 2008). We demonstrated previously that these mice do not produce NFIX protein (Campbell et al. 2008). For BrdU analysis, BrdU was administered intraperitoneally 2 h prior to sacrifice. Postnatal pups were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) and then post-fixed in 4% PFA at 4°C, and 40-μm vibratome sections were cut for immunohistochemistry. Immunohistochemistry was performed on free-floating sections in 10% normal donkey serum and 0.1% Triton-X100 according to standard protocols. Antibodies used are described in the Supplemental Material. All work with laboratory mice was conducted according to the relevant national and international guidelines and regulations.

Quantification of NSCs in postnatal DG

For each image counted, seven to 10 1-μm confocal Z-stacks were merged for quantification. For all counts, images from at least three sections from two or three independent mice were quantified. For quantification of the number of NSCs in the wild-type DG expressing NFI factors and/or MCM2 or BrdU, only NSCs with a clear radial $GFAP^+$ process that could be confidently linked to a nucleus within the subgranular zone (SGZ) were considered. To quantify NSCs in the $Nfix^{-/-}$ DG, counts were made in the proximal blade of the DG, where gross morphology was more similar to wild-type DG. To estimate total density of NSCs, the number of $GFAP^+$, $Nestin^+$ processes was counted. Cells were deemed to reside in the granule cell layer (GL) if their main process terminated more than two nucleus widths from the bottom of the SGZ. Morphology of NSCs was considered abnormal if the angle of their primary process deviated by >30° from perpendicular to the SGZ surface or they exhibited more than one main process. For assessment of proliferation, only NSC processes that could be clearly associated with a DAPI-positive nucleus were considered.

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