HDAC3 controls gap 2/mitosis progression in adult neural stem/progenitor cells by regulating CDK1 levels

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The maintenance of the resident adult neural stem/progenitor cell (NSPC) pool depends on the precise balance of proliferation, differentiation, and maintenance of the undifferentiated state. Identifying the mechanisms that regulate this balance in adult hippocampal NSPCs can provide insight into basic stem cell self-renewal principles important for tissue homeostasis and preventing tumor formation. Pharmacological inhibition of histone deacetylases (HDACs), a class of histone-modifying enzymes, have promising effects in cancer cells, yet the specific roles of individual HDACs in stem cell proliferation is unclear. Here using conditional KO (cKO) mice and in vitro cell culture, we show that histone deacetylase 3 (HDAC3) is required for the proliferation of adult NSPCs. Detailed cell cycle analysis of NSPCs from Hdad3 cKO mice reveals a defect in cell cycle progression through the gap 2/mitosis (G2/M) but not the S phase. Moreover, HDAC3 controls G2/M phase progression mainly through posttranslational stabilization of the G2/M cyclin-dependent kinase 1 (CDK1). These results demonstrate that HDAC3 plays a critical role in NSPC proliferation and suggest that strategies aimed at pharmacological modulation of HDAC3 may be beneficial for tissue regeneration and controlling tumor cell growth.

adult hippocampal neurogenesis | epigenetic | acetylation | ubiquitination | malignancy

Adult hippocampal neural stem cells, also called radial glial-like (RGL) cells or type 1 cells, self-renew and give rise to transit-amplifying progenitors (TAPs) before differentiating into granule neurons and astrocytes in the dentate gyrus subgranular zone (SGZ) (1, 2). Self-renewal of RGLs is tightly regulated to promote proliferation as well as maintain the undifferentiated state, which is important for homeostasis and lifelong neurogenesis. Cell cycle regulators have been extensively studied in cell cycle progression in the context of cancer cells (3). However, whether these genes merely control cell proliferation in adult neural stem/progenitor cells (NSPCs) or even control cell fate decisions, such as remaining quiescent or undergoing differentiation, is not entirely clear. Recent studies reveal the roles of G1 phase regulators E2F3 and cyclin D not only in cell proliferation, but also in cell fate commitment (4, 5), suggesting a possible role of cell cycle regulators in mediating self-renewal and differentiation of adult NSPCs.

Our previous work demonstrated HDAC inhibitors decreased adult NSPC proliferation and promoted neuronal differentiation (6). This raises the question of the role of specific HDACs involved in adult NSPC maintenance and neurogenesis. HDAC1 and HDAC2 appear to function redundantly to control the progression of neural precursors to neurons during brain development (7). In adult brain, HDAC2 is involved in neuronal differentiation and survival (8). Although HDAC3 is highly expressed in the brain (9) and has documented roles in learning and memory associated with cocaine-seeking behavior (10), its role in adult NSPCs is largely unknown.

HDAC3 is best known for its actions as an epigenetic regulator of gene expression through deacetylation of histone tails (11). In recent years, a role of HDAC3 in cell cycle progression has also been uncovered. In human colon cancer cells, HDAC3 levels are elevated, which have been suggested to control cells in both S and gap 2/mitosis (G2/M) phase (12). Loss of HDAC3 in hematopoietic progenitor cells results in only S-phase progression defects (13), whereas in HeLa cells, a G1/S transition defect was observed after knockdown of HDAC3 (14). However, the underlying mechanisms are still unclear; in particular, which stage(s) of cell cycle HDAC3 control(s) in NSPCs.

Here, we investigate the function and mechanism of HDAC3 in adult NSPCs. From HDAC3 loss of function experiments in vitro and in vivo, we identified that HDAC3 is required for the proliferation of adult NSPCs. We further determined that HDAC3 controls G2/M phase progression in adult NSPCs by blocking ubiquitination and degradation of cyclin-dependent kinase 1 (CDK1). These data reveal previously unidentified insights regarding the key role of HDAC3 in controlling adult neurogenesis by regulating cell proliferation and cell cycle progression.

Results

HDAC3 Is Required to Promote the Proliferation of Adult NSPCs in Vitro. To rapidly determine whether HDAC3 is required for adult NSPC proliferation in vitro, we first designed a shRNA plasmid to knockdown HDAC3 (Fig. S1L4). The HDAC3 shRNA was packaged into a lentivirus vector expressing internal ribosome entry site (IRES)-EGFP and used to infect a line of previously characterized adult rat hippocampal neural progenitor cells (HCN cell line) (15). Expression of HDAC3 shRNA-IRES-EGFP resulted in fewer GFP+ HCN cells incorporating BrdU, compared with control IRES-EGFP infected cells (Fig. L4), suggesting HDAC3 is required for NSPC proliferation.

As a complementary approach, we obtained an HDAC3-selective inhibitor (HDAC3i) to test in HCN cells (16). HDAC3i shows 10 times higher selectivity against HDAC3 relative to HDAC1 and HDAC2 (16). It has also been used to investigate the role of HDAC3 in cocaine-seeking behavior in mice (10). To determine the optimum dosage for HDAC3i in HCN cells, we performed the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay to determine its cytotoxicity

Significance

Cell cycle regulation is one of the most fundamental mechanisms to control various biological processes, including the proliferation of neural stem/progenitor cells (NSPCs) in adult mouse brain. This study shows that histone deacetylase 3 (HDAC3), a well-studied epigenetic factor, is required for the proliferation of neural stem cells. We also demonstrate that HDAC3 controls gap 2 and mitosis phase of cell cycle through stabilization of cell cycle protein cyclin-dependent kinase 1. These findings suggest an important role for HDAC3 in NSPC proliferation, and modulation of HDAC3 activity pharmacologically may be beneficial for tissue regeneration and cancer therapy.
hippocampal NSPC line that the major effect of deletion or inhibition of HDAC3 is a proliferation defect in vitro.

Adult NSPCs Lacking HDAC3 Display Specific G2/M Phase Defects. Previous studies showed that HDAC3 could regulate the G1/S phase transition in HeLa cells (14), but was only required for S-phase progression in mouse embryonic fibroblast (MEF) cells (18), suggesting that HDAC3 has distinct roles on cell cycle progression in different cell types. To gain further insight into the role of HDAC3 in NSPC proliferation, specifically, in regulating cell cycle progression, we performed cell cycle analysis following propidium iodide staining of WT and Hdad3 floxed neurospheres. After infection with Ad-Cre-GFP, HDAC3 deleted neurospheres showed decreased percentage of cells in S phase and increased percentage of cells in G2/M phase compared with WT neurospheres (Fig. 2 A and B).

To further confirm the role of HDAC3 in cell cycle progression in adult NSPCs, we treated HCN cells with HDAC3i for 1 d before cell cycle analysis. Compared with cells treated with vehicle (DMSO), HDAC3i-treated cells exhibited a decrease in S phase and an increase in G2/M phase (Fig. 2C). To determine whether decreased S phase and increased G2/M phase was a

as well as performed Western blotting against acetyl-histone H4 lysine 12 at different concentrations as a biological readout of its activity (Fig. S1 B and C). We found that 10 μM minimized cytotoxicity and maximized biological activity. Thus, 10 μM HDAC3i was used to treat HCN cells for 1 d (Fig. 1B). We performed immunostaining of BrdU after adding BrdU for 1 h before fixing the cells. Consistent with the effects of knocking down HDAC3 with an shRNA, HCN cells treated with an HDAC3i also showed significant defects in proliferation as indicated by the decreased number of BrdU+ cells.

To confirm that HDAC3 is required for NSPC proliferation in multiple rodent species, we also used established primary NSPCs (neurospheres) from the hippocampus and subventricular zone of 1-mo-old Hdad3 WT (HDAC3 WT) or floxed (HDAC3 F/F) mice (17). First, we confirmed that adenovirus (Ad) Cre-GFP infection of Hdad3 floxed neurospheres could efficiently KO HDAC3 levels (Fig. S1D). Using BrdU exposure (1 h) after 2 d of Ad-Cre-GFP infection, we observed a reduction of proliferation in Hdad3 floxed neurospheres as indicated by fewer BrdU+ and GFP double-positive cells (Fig. 1C). Moreover, we did not observe a significant effect on differentiation, at least under neuronal differentiation conditions (Fig. S1E). Taken together, we showed in primary mouse NSPC cultures and in an adult rat

Fig. 1. Loss of HDAC3 results in decreased proliferation in vitro. (A) Immunocytochemical staining of BrdU followed by 1 h BrdU labeling in HCN cells infected with control (Cont) or HDAC3-shRNA-IRES-EGFP lentivirus for 3 d. Quantification of GFP+BrdU+ cells out of the total GFP+ cells is shown. (B) BrdU staining of 1 h BrdU labeling in HCN cells treated with either vehicle (DMSO) or HDAC3i for 1 d. Quantification of BrdU+ cells out of the total DAPI+ cells is shown. (C) HDAC3 WT or F/F neurospheres were infected with Ad-Cre-GFP virus for 2 d. Immunocytochemical staining of GFP and BrdU following 1 h BrdU labeling revealed a decrease in the percentage of BrdU+GFP+ double-positive cells out of the total GFP+ cells in HDAC3 F/F neurospheres. In A–C, *p < 0.05. (Scale bars: 200 μm in A and B, 100 μm in C.) All experiments were performed at least three times independently.

Fig. 2. HDAC3 controls G2/M phase progression. (A and B) Flow cytometry analysis of HDAC3 WT and F/F neurospheres infected with Ad-Cre-GFP virus for 2 d before being harvested and fixed in 70% ethanol. Quantification of the percentage of cells in S and G2/M phase is shown. (C) Cell cycle analysis of HCN cells treated with HDAC3i or VPA for 1 d. The percentage of each cell cycle stage from one representative experiment is shown in the bar graph. (D) HCN cells were synchronized in the G1/G0 phase by adding BMP4 to proliferation medium for 1 d before switching to proliferation medium containing either DMSO or HDAC3i. Cells were collected at 6 and 12 h and analyzed by flow cytometry. The percentage of each cell cycle stage from one representative experiment is shown in the bar graph. (E) HCN cells were synchronized in M phase by addition of nocodazole in proliferation medium for 16 h before switching to proliferation medium containing either DMSO or HDAC3i. Cells were collected at 6 and 12 h and analyzed by flow cytometry. The percentage of each cell cycle stage from one representative experiment is shown in the bar graph. In A and B, *p < 0.05. All cell cycle experiments were replicated at least three times independently.
general property of blocking HDACs, we treated HCN cells with the broad HDACi valproic acid (VPA) for 1 d. Interestingly, we observed an increase in G1/G0 phase and a decrease in S phase, with no change in G2/M phase (Fig. 2C), suggesting that individual HDACs have divergent effects on cell cycle progression.

To determine the primary cause of the observed cell cycle defects after HDAC3 inhibition, we performed cell cycle synchronization in HCN cells using various molecules to enrich cells in either G1/G0 or G2/M phase. We synchronized HCN cells in G1/G0 phase after addition of bone morphogenic protein 4 (BMP4) to proliferation medium (fibroblast growth factor 2) for 1 d (19) (Fig. 2D and Fig. S2C). To release HCN cells from G1/G0, BMP4 was withdrawn and cells were treated with either HDAC3i or DMSO in proliferation medium. To confirm HCN cells were effectively enriched in G1/G0 and could be released back into cell cycle, we performed Western blotting with known cell cycle proteins (Fig. S2C). We observed that both DMSO- and HDAC3i-treated HCN cells entered into S phase with no lag at 12 h after withdrawal of BMP4, indicating that HDAC3 is not required for G1/S phase transition and entry into S phase (Fig. 2D).

To examine whether HDAC3 is required for M-phase progression, we enriched HCN cells in M phase with nocodazole treatment for 16 h before releasing them into HDAC3i or DMSO-containing proliferation medium (Fig. 2E and Fig. S2D). To confirm HCN cells were effectively enriched in G2/M and could be released back into cell cycle, we performed Western blotting with known cell cycle proteins (Fig. S2D). Twelve hours after release from nocodazole, most of the cells treated with HDAC3i were still in G2/M phase, whereas control-treated cells had entered into G1/G0 phase, similar to cells in proliferation conditions (Fig. 2E). These data suggest that the proliferation defect observed after HDAC3 inhibition is primarily due to an impairment of G2/M phase progression.

**CDK1 Is Selectively Degraded After Loss or Inhibition of HDAC3 in Adult NSPCs.** To understand the mechanism by which HDAC3 controls G2/M phase progression, we performed Western blotting to detect G2/M phase-enriched cyclins and CDKs in both primary neurospheres and HCN cells. First, we observed decreased HDAC3 levels and increased acetylated histone H4 as expected after Ad-Cre–mediated deletion of HDAC3 in HDAC3 F/F neurospheres (Fig. 3A). We also observed a decrease in the levels of G2/M CDKs and cyclins, such as CDK1, phospho-CDK1, cyclin B1, but no change in the level of the G1/S cyclin A (Fig. 3A). Treatment with HCN cells with HDAC3i also resulted in decreased CDK1, phospho-CDK1, and cyclin B1 (Fig. 3B). Interestingly, in both HDAC3 F/F neurospheres and HCN cells, we found an increase in the mitosis marker phosphohistone H3 serine 10 (p-H3S10) after HDAC3 deletion or inactivation (Fig. 3A and B), consistent with the increased percentage of cells in G2/M phase.

To gain further mechanistic insight into HDAC3, we next examined whether the decline of CDK1 and cyclin B1 levels after HDAC3 deletion or inactivation was due to its roles as an epigenetic repressor of gene transcription. Thus, we used quantitative PCR (qPCR) to investigate a panel of cell cycle genes and found that only cyclin B1 mRNA levels were reduced after deletion of HDAC3 in neurospheres (Fig. 3C). Besides deacetylation of nuclear histone tails, HDAC3 has been described to shuttle between the nucleus and cytoplasm and may also have nonhistone protein targets (20). To examine whether reduced CDK1 levels after loss of HDAC3 occurred through posttranslational mechanisms to control protein stability, we measured CDK1 levels by treating HCN cells with HDAC3i and a protein synthesis inhibitor cycloheximide (CHX) (Fig. 3D and E). Compared with CDK1 levels in cells treated with HDAC3i alone which decreased by 1 d, we observed decreased CDK1 levels at 12 h, suggesting that CDK1 has a shorter half-life after HDAC3 inhibition and that control of CDK1 levels by HDAC3 occurred at the posttranslational level.

Ubiquitination is the most common posttranslational modification to control protein stability (21). To explore whether CDK1 levels in adult NSPCs is subjected to ubiquitin-mediated protein degradation, we first tried to block the proteasome pathway by treating HCN cells with a proteasome inhibitor carboxenzyo-Leu-Leu-leucinal (MG132) together with HDAC3i for 1 d. We observed selective loss of CDK1 after HDAC3i treatment in HCN cells, which was prevented by MG132 treatment, without a change in cyclin A or CDK4 levels (Fig. 3F). Interestingly, in non-NSPCs such as human HEK 293T and...
Inkn4a/Arf KO mouse glioma cells (SS05 cell line) (22), we did not observe down-regulation of CDK1 after HDAC3i treatment (Fig. S3A), suggesting that the HDAC3/CDK1 ubiquitination pathway may be selective for NSPCs. To examine CDK1 ubiquitination in NSPCs, we first treated HCN cells with MG132 and DMSO or HDAC3i, followed by immunoprecipitation of CDK1 and Western blotting against ubiquitin. We observed heavily ubiquitinated bands in HDAC3i-treated cells, indicating that CDK1 can be ubiquitinated in HCN cells (Fig. 3G). These results suggested that CDK1 might be degraded through the ubiquitination pathway after loss or inhibition of HDAC3 in NSPCs.

To further examine whether HDAC3 controls ubiquitination of CDK1, we overexpressed HA-tagged CDK1 and Flag-tagged ubiquitin with or without a cDNA overexpressing full-length HDAC3 in HCN cells (Fig. 3H). Following immunoprecipitation of CDK1 and Western blotting against ubiquitin, we observed reduced CDK1 ubiquitination after HDAC3 overexpression, indicating that HDAC3 is sufficient to prevent ubiquitination of CDK1 in adult NSPCs.

Two possible mechanisms by which HDAC3 regulates ubiquitination and stability of CDK1 are through regulation of ubiquitin-related genes (23) or through physical interaction and ubiquitination and stability of CDK1 are through regulation of ubiquitin-related genes (23) or through physical interaction and ubiquitin-related genes (23) or through physical interaction and ubiquitin-related genes (23) or through physical interaction and ubiquitin-related genes (23) or through physical interaction and ubiquitin-related genes (23) or through physical interaction and ubiquitin-related genes (23) or through physical interaction.

**HDAC3 Is Broadly Expressed in Adult NSPC and Neuronal Populations.**

We have shown that HDAC3 is required for the proliferation of NSPCs in vitro. To begin to investigate the role of HDAC3 during the course of adult neurogenesis in vivo, we determined the expression pattern of HDAC3 in adult mouse brain, particularly within the SGZ of the dentate gyrus. We performed immunohistochemical (IHC) staining using an antibody recognizing HDAC3 together with stage-specific markers. RGLs can be identified using a set of immunohistological markers and morphological criteria (24). We observed HDAC3 expression within glial fibrillary acidic protein (GFAP)+Sox2+ RGL and GFAP+Sox2+ TAP stem/progenitor cells (Fig. 4A). We quantified the percentage of GFAP+Sox2+ RGL cells that expressed HDAC3 and found HDAC3 is expressed in a large proportion of RGLs (94.1 ± 3.6%). We also detected HDAC3 in doublecortin (DCX)+ immature dentate granule cells as well as in postmitotic NeuN+ neurons throughout the hippocampus and cortex (Fig. 4B and Fig. S4). Moreover, HDAC3 was found in MCM2+ proliferating cells in SGZ, consistent with its possible role in regulating proliferation in vivo as it does in vitro (Fig. 4C). Due to the broad expression of HDAC3 in adult NSPC and neuronal populations and because HDAC3 null mice are reported to die at embryonic stage 9.5 due to gastrulation defects (17), we decided to use an inducible conditional KO (cKO) strategy to determine the cell-autonomous requirement of HDAC3 in adult NSPCs in vivo.

cKO of HDAC3 Results in Reduced Proliferation of Adult NSPCs and Decreased Neurogenesis. To evaluate the impact of deleting HDAC3 in adult NSPCs and their progeny in vivo, we crossed Hdc3 floxed mice with a tamoxifen (TAM)-inducible Nestin-CreER<sup>22</sup> allele (Hdc3 cKO mice) and killed mice at various time points after TAM (Fig. 5A). These mice were also bred to a R26R-YFP reporter allele so YFP<sup>+</sup>-recombined cells can be used as surrogate markers for cells recombined and deleted for HDAC3 (25). To validate the efficiency of HDAC3 recombination after TAM treatment in adult Hdc3 cKO mice, we performed PCR of the Hdc3 genomic region from primary hippocampal neurospheres isolated from TAM-injected adult Hdc3 floxed mice and were unable to detect Hdc3 gene expression consistent with an efficient recombination and deletion of Hdc3 (Fig. S5A).

To examine the potential ability of HDAC3 to control proliferation in vivo, we killed Hdc3 cKO at an early time point, such as 10 d post-TAM (dpt). We observed a significant reduction in the number of YFP<sup>+</sup>ki67<sup>+</sup> and YFP<sup>+</sup>MCM2<sup>+</sup>-proliferating cells in the SGZ of Hdc3 cKO mice compared with WT littermates (Fig. 5B and Fig. S5B). Moreover, further analysis of YFP<sup>+</sup> cells with cell type-specific markers revealed that both the percentage of proliferating YFP<sup>+</sup>GFAP<sup>+</sup>ki67<sup>+</sup> RGL cells out of the total YFP<sup>+</sup> cells and the number of YFP<sup>+</sup>ki67<sup>+</sup>DCX<sup>+</sup> TAPs were reduced in Hdc3 cKO mice compared with WT mice at 10 dpt (Fig. 5B and C), indicating that HDAC3 is required for the proliferation of RGL cells as well as TAPs.

Finally, one prediction of a defect in NSPC proliferation and cell cycle progression due to conditional deletion of Hdc3 is a change in neurogenesis over time. Thus, we examined the number of immature and mature dentate neurons in Hdc3 cKO mice between 30 and 90 dpt by quantification of immature and mature dentate neurons in Hdc3 cKO mice compared with WT mice at 10 dpt (Fig. 5B and C), indicating that HDAC3 is required for the proliferation of RGL cells as well as TAPs. To examine this, we immunoprecipitated CDK1 and performed Western blotting with the pan-acetyl-lysine antibody (Fig. S3D), suggesting that HDAC3 is not necessary for CDK1 acetylation. Taken together, these results suggest that HDAC3 plays a role in stabilizing CDK1 by regulating ubiquitin levels for proper G2/M phase progression in adult NSPCs.
Interestingly, although the number of YFP\(^+\)GFAP\(^+\)Sox2 RGLs decreased between 60 and 90 dpi in WT mice as expected, we did not observe a significant difference in the number of YFP\(^+\)GFAP\(^+\)Sox2\(^+\) RGLs in Hdac3 cKO mice compared with WT at 60 and 90 dpi (Fig. S5D). These results suggest that the decreased neurogenesis found in Hdac3 cKO mice could be due to the decreased proliferation of RGLs and TAPs and not from a change in the total number of RGLs. Together these results support Hdac3’s major role in regulating adult NSPC proliferation and cell cycle progression to ensure the continuous generation of adult-born neurons over time.

Discussion

The immense cellular heterogeneity within the brain represents a formidable challenge for the development of HDAC inhibitors as potential therapeutics to treat brain disorders. Moreover, the function of various HDAC members in different progenitor, neuronal, or glial cell types—some having redundant properties—provides rationale for uncovering the roles of specific HDACs in the nervous system. In this study, we showed that Hdac3 is required for the proliferation of adult NSPCs (both RGLs and TAPs), ultimately resulting in decreased generation of mature dentate neurons. Surprisingly, we did not observe a significant difference, although there was a trend toward reduction of immature YFP\(^+\)DCX\(^+\) cells at 60 dpi, suggesting that DCX\(^+\) neuroblasts may increase their proliferation to compensate for the early reduction of YFP\(^+\)Ki67\(^+\) cells. We also demonstrated that Hdac3 is important in regulating CDK1 levels for proper G2/M phase progression. We propose that in WT NSPCs Hdac3 stabilizes CDK1 to promote normal cell cycle progression, whereas in NSPCs lacking or inactive for Hdac3, CDK1 is degraded through the ubiquitin–proteasome pathway, resulting in G2/M phase progression defects. Our results suggest that inhibition of Hdac3 may be beneficial for preventing brain malignancies as well as treating certain neurological diseases, such as epilepsy, which may arise from abnormal proliferation of NSPCs after seizures.

**Hdac3 Plays a Role in Adult NSPC Proliferation, Specifically in G2/M Phase Progression.** Previously, we and others showed that application of pharmacological inhibitors of class I HDACs leads to enhanced neuronal differentiation within the adult hippocampus and developing forebrain (6, 26). However, some reports indicate that neurogenesis in certain brain regions is decreased after HDAC inhibitor treatment (27). These studies indicate there may be context-dependent differences based on the development stage and/or brain region and provide a rationale for additional studies of the individual roles of HDACs in neurogenesis. Hdac1 and Hdac2 appear to function redundantly during neurodevelopment to promote cell survival and neuronal migration (7), whereas deletion of Hdac2 alone in adult brain is sufficient to drive cell death and defective neuronal maturation (8). In contrast to Hdac1 and Hdac2, which are enriched in neural progenitors and mature neurons, respectively (8, 28)—we observed that Hdac3 is broadly expressed in adult brain, including hippocampal NSPCs, neuroblasts, and mature neurons. Deletion of Hdac1 and Hdac2 in fibroblasts blocks the cell cycle in G1 phase by increasing the mRNA levels of CDK inhibitors p21 and p57 (29). In adult NSPCs, p21 does not increase after removal of Hdac3, which is consistent with our observation that G1/S phase progression is not primarily affected. We have shown that Hdac3 specifically controls G2/M phase progression in adult NSPCs. In other cell types, such as hematopoietic progenitor cells and MEFS, loss of Hdac3 results in S-phase progression and DNA replication defects (13). The discrepancies in cell cycle defects after loss of Hdac3 may reflect alterations in the level of Hdac3 at different stages of the cell cycle. For instance, in HeLa cells, the levels of Hdac3 are reduced in metaphase due to proteasome degradation (14). However, in NSPCs, we observed consistently high levels of Hdac3 throughout the cell cycle (Fig. S2B). Therefore, we do not believe that Hdac3 control of cell cycle progression in mainly due to changes in Hdac3 levels, at least in adult NSPCs.

**Hdac3 Mediates G2/M Progression Through Posttranslational Control of Cdk1 in Adult NSPCs.** How does Hdac3 specifically control G2/M progression? Because Hdac3 does not bind to chromatin during M phase (30), one possibility is that Hdac3 may have nonhistone substrates in adult NSPCs. Indeed, Hdac3 was previously reported to control mitosis by targeting A-kinase–anchoring proteins Akap96 and aurora B kinase in 293T cells (31). However, when Hdac3 is deleted in 293T cells, an increase in he percentage of G2/M phase is correlated with a decrease in levels of the aurora B kinase substrate, p-H3S10, which is in contrast to what we observed in NSPCs lacking Hdac3 (Fig. 3A). Thus, it is reasonable to postulate that Hdac3 controls G2/M phase in adult NSPCs through targets other than Akap96 and aurora B and, depending on the cell type, Hdac3 may have different targets to control the cell cycle.

Based on our observation that Hdac3 is required for maintaining Cdk1 levels, we postulate that Hdac3 regulates the stability of Cdk1, thus controlling G2/M progression. Our results also indicate that Hdac3 is required for preventing Cdk1 ubiquitination. The exact mechanism by which Hdac3 regulates ubiquitination of Cdk1 is unknown. In yeast, Cdk1 can be acetylated at lysine 33, which is essential for proper growth (32). Moreover, a recent study using HeLa cells showed that Hdac3 deacetylates and prevents the ubiquitination of cyclin A (14).
However, in our study, although we detected acetylated CDK1 in HCN cells, we did not observe any differences in CDK1 acetylation levels after HDAC3 inhibition or overexpression, possibly due to regulation by other HDACs. It would be interesting in future studies to examine the detailed mechanisms by which HDAC3 controls CDK1 ubiquitination.

**Therapeutic Implications of HDAC3**. Broad HDAC inhibitors (such as VPA) can mediate proneurogenic differentiation of NSPCs as well as spinal cord graft improvement (6, 33). Here we have shown that an HDAC3-specific blocker adult NSPC proliferation, instead of inducing neuronal differentiation. One potential advantage of HDAC3-selective inhibitors over broad HDAC inhibitors is their ability to pharmacologically regulate the proliferation of adult NSPCs, such as in postseizure aberrant neurogenesis, which is a fundamental problem in epilepsy (34). Moreover, hyperproliferation of cancer cells is correlated with neurogenesis, which is a fundamental problem in epilepsy (34). Thus, we speculate that inhibition of HDAC3 may decrease the proliferation of proliferating cancer cells as well as activated brain cancer stem cells, which may be a more promising strategy than targeting proliferating cells alone.

On the other hand, activation of HDAC3 may promote the proliferation of adult NSPCs. Consistent with this idea, overexpression of HDAC3 in transgenic mice was sufficient to induce cardiomyocyte proliferation (36). Our results suggest that development of HDAC3 activators may help in the pharmacotherapy of neurodegenerative diseases. Sequential use of an HDAC3 activator to boost the endogenous NSPC pool and then treatment with a broad HDAC inhibitor to promote neuronal differentiation may be more effective in spinal cord repair while avoiding possible immunological complications from exogenous stem cell transplantation. Thus, combined use of HDAC-selective activators and broad-acting inhibitors may extend the therapeutic applications of HDAC inhibitors.

In summary, we have identified a crucial role for HDAC3 in adult NSPC proliferation. Mechanistically, HDAC3 is essential for proper G2/M progression, at least in part through post-translational regulation of CDK1. Our work confirms that a detailed analysis of individual HDACs is important for translating HDAC inhibitors in the clinic.

**Materials and Methods**. All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (UTSW). Mice were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care at UTSW on a 12 h light–dark cycle. Nestin-CreERT2, R26R-YFP, and Hdc3a floxed mice, and their genotyping have been described previously (17, 25). Hdc3a KO mice carrying HDAC3flox/ and Nestin-CreERT2, and R26R-YFP alleles were given TAM (i.p. daily for 6 d [150 mg/kg dissolved in 10% [vol/vol] ethanol and 90% [vol/vol] sunflower oil] and then killed for analysis at indicated time points after the last injection. Additional experimental procedures are provided in SI Materials and Methods and Table S1.

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Hippocampal neural anti-TBS for 1 h. The following SDS HDac3 anti-Tuj1 TBS three times and incubated in μ anti- doublecortin pfu per mL stock; University of Iowa, Iowa anti-cyclin D1 (1:1,000; Cell Signaling), – Mice were TBS and then blocked with 3% normal donkey μ anti-CDK4 (1:1,000; Sigma), and chicken anti-phosphohistone H3 Ser-10 (1:1,000; Cell Signaling), Hdac3 of5 – Mice were used to maintain growth of the cells. Mouse primary neural stem/progenitor (HCN) cells were synchronized in G1/G0 phase by adding bone morphogenic protein 4 (50 ng/mL; R&D Systems) to proliferation medium for 1 d, and in M phase by addition of nocodazole (2 μg/mL; Sigma) to proliferation medium for 16 h, before releasing in either HDAC3i or DMSO containing proliferation medium. Cells were then collected, centrifuged, washed, and resuspended in 70% ethanol for fixation. For DNA content analysis, cells were stained with propidium iodide (20 μg/mL; Sigma) and RNase A (200 μg/mL; Invitrogen). The cell cycle was monitored on a FACSCalibur flow cytometer (BD Biosciences) with 10,000 events per determination and analyzed with FlowJo software (Tree Star Inc.).

Histology and Immunohistochemistry on Brain Tissue. Mice were perfused, sectioned, and immunohistochemically stained as described before (1). Immunohistochemistry (IHC) staining was performed on free-floating sections. On average, we used four to five sections per mouse for the staining. Sections were washed twice with 1x TBS and then blocked with 3% normal donkey serum and 0.3% Triton X-100 in 1x TBS for 1 h. The following primary antibodies were used for 2 d at 4 °C: rabbit–anti-HDAC3 (1:1,000; Santa Cruz Biotechnology), chicken–anti-GFP (1:2,000; Aves Lab), mouse–anti-NeuN (1:1,000; Millipore), rabbit–anti-Ki67 (1:500; Neomarkers), guinea pig–anti- doublecortin (DCX) (1:2,000; Millipore), mouse–anti-glial fibrillary acidic protein (GFAP) (1:4,000; Millipore), goat–anti-SOX2 (1:750; Santa Cruz Biotechnology), and goat–anti-MCM2 (1:500; Santa Cruz Biotechnology). Sections were washed with 1x TBS three times before incubation in fluorescence-conjugated secondary antibodies (1:1,000; Jackson ImmunoResearch) overnight at room temperature. Sections were then washed with 1x TBS three times, mounted on slides, and coverslipped with polyvinyl alcohol (PVA) DABCO (Sigma-Aldrich).

Cell Culture and Immunocytochemical Staining. The adult rat HCN cells were cultured in Dulbecco’s MEM/Ham’s F-12 (DMEM/F-12; Omega Scientific) supplemented with N2 (Invitrogen), glutamine (Omega Scientific), and Penicillin–Streptomycin–Fungizone (PSF) (Invitrogen). In all experiments with HCN cells, proliferation medium containing fibroblast growth factor 2 (FGF2) (20 ng/mL) was used to maintain growth of the cells. Mouse primary neural stem/progenitor cell (NSPCs) (neurospheres) from the hippocampus and lateral ventricle of P30 Hcn3 WT or floxed mice were used to maintain growth of the cells. Mouse primary neural stem/progenitor (HCN) cells were synchronized in G1/G0 phase by adding bone morphogenic protein 4 (50 ng/mL; R&D Systems) to proliferation medium for 1 d, and in M phase by addition of nocodazole (2 μg/mL; Sigma) to proliferation medium for 16 h, before releasing in either HDAC3i or DMSO containing proliferation medium. Cells were then collected, centrifuged, washed, and resuspended in 70% ethanol for fixation. For DNA content analysis, cells were stained with propidium iodide (20 μg/mL; Sigma) and RNase A (200 μg/mL; Invitrogen). The cell cycle was monitored on a FACSCalibur flow cytometer (BD Biosciences) with 10,000 events per determination and analyzed with FlowJo software (Tree Star Inc.).

Coimmunoprecipitation and Western Blot. Cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with a mixture of protease (Roche) and phosphatase inhibitors (Sigma). After protein quantification by the bicinchoninic acid colorimetric assay system (Thermo Scientific), 500 μg protein samples were used for coimmunoprecipitation. Five micrograms of HDAC3 or CDK1 antibodies were added to the protein samples and incubated at 4 °C overnight, followed by 1 h incubation with 50 μL protein G or A Sepharose (GE Healthcare) beads. The beads were rinsed with lysis buffer five times and boiled in 2× SDS loading buffer for 5 min. After brief centrifugation, supernatants were loaded onto 4–12% SDS PAGE gels for Western blotting. Western blotting was performed using standard protocols with these primary antibodies: mouse–anti-CDK1 (1:1,000; Santa Cruz Biotechnology), rabbit–anti-phospho-CDK1 Tyr15 (1:1,000; Cell Signaling), rabbit–anti-cyclin B1 (1:1,000; Cell Signaling), mouse–anti-cyclin A (1:1,000; Cell Signaling), rabbit–anti-CDK4 (1:1,000; Cell Signaling), mouse–anti-cyclin D1 (1:1,000; Cell Signaling), rabbit–anti-phosphohistone H3 Ser-10 (1:1,000; Cell Signaling), rabbit–anti-acetyl-histone H4 (1:10,000; Millipore), rabbit–anti-acetyl-H4K12 (1:1,000; Millipore), rabbit–anti-histone H4 (1:10,000; Millipore), rabbit–anti-HDAC3 (1:1,000; Santa Cruz Biotechnology), secondary antibodies (1:200; Jackson ImmunoResearch). Cells were washed with 1x TBS three times, counterstained with DAPI (1:5,000; Roche), and coverslipped with PVA/DABCO. To delete HDAC3 in vitro, Hdac3 floxed neurons were infected with GFP (control) or Cre-GFP adenovirus (Ad) (1:10,000 of 1 × 10¹⁰ pfu per mL stock; University of Iowa, Iowa City, IA) on coated plates for 2 d. To determine the cell survival rate, the 3-(4,5-diimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed with HCN cells according to the manufacturer’s protocol (Promega).

Plasmids, Lentiviruses Production, Transfection, and Electroporation. An shRNA sequence that target mouse HDAC3 was cloned into the lentiviral plhu2g vector: 5’-TGAATGATCGTCTCAAGCCTTTCAGAGAAGGCATTGAACGATCATCTTTTTT3’-3’. Lentivirus was made as previously described (4). HCN cells were infected at a multiplicity of infection of 10 particles per cell in N2, glutamine, and PSF containing DMEM/F-12 medium with FGF2 for 3 d. Mouse Hdaec cDNA, HA-tagged cyclin-dependent kinase 1 (CDK1) cDNA, and Flag-tagged Ubiquitin were cloned into a retroviral pCAG-internal ribosome entry site-GFP vector and confirmed by DNA sequencing. HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Electroporation was performed with an Amaxa electroporator at a ratio of 5 μg DNA per 5 million HCN cells. Cells were maintained in proliferation medium (FGF2) and collected for Western blot or immunoprecipitation 3 d postelectroporation. The proteasome inhibitor MG132 (10 μM) was added 12 h before harvesting cells.

Supporting Information
Jiang and Hsieh 10.1073/pnas.1411939111
SI Materials and Methods
Cell Synchronization and Flow Cytometry. Hippocampal neural progenitor (HCN) cells were synchronized in G1/G0 phase by adding bone morphogenic protein 4 (50 ng/mL; R&D Systems) to proliferation medium for 1 d, and in M phase by addition of nocodazole (2 μg/mL; Sigma) to proliferation medium for 16 h, before releasing in either HDAC3i or DMSO containing proliferation medium. Cells were then collected, centrifuged, washed, and resuspended in 70% ethanol for fixation. For DNA content analysis, cells were stained with propidium iodide (20 μg/mL; Sigma) and RNase A (200 μg/mL; Invitrogen). The cell cycle was monitored on a FACSCalibur flow cytometer (BD Biosciences) with 10,000 events per determination and analyzed with FlowJo software (Tree Star Inc.).
mouse–anti-GAPDH (1:10,000; Millipore), mouse–anti-beta-Actin (1:1,000; Santa Cruz Biotechnology), rabbit–anti-acetyl-lysine (1:1,000; Cell Signaling), mouse–anti-HA (1:10,000; Sigma), mouse–anti-Flag (1:10,000; Sigma), and rabbit–anti-Ubiquitin (1:500; Santa Cruz Biotechnology). HRP-conjugated (Cell Signaling) or alkaline phosphatase-conjugated (Santa Cruz Biotechnology) secondary antibodies were used. Immunoblots were developed with an ECL-plus kit (GE Healthcare) or detected with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride phosphatase substrate (Kirkegaard & Perry Laboratories, Inc.). Some Western blots were quantified with Image J software (National Institutes of Health, Bethesda).

**Statistical Analysis.** Data are represented as means ± SEM. Statistical comparisons were assessed with unpaired, two-tailed Student t test. A P value <0.05 was considered significant.

Fig. S2. (A and B) Cell cycle analysis of WT and HDAC3 F/F neurospheres with and without Ad-GFP (controls) virus infection in proliferation medium. This analysis was replicated three times independently. (C) Western blot of lysates from HCN cells released from G1/G0 into proliferation medium (FGF2) for different lengths of time and probed with antibodies against HDAC3, G1/S-enriched cyclin D1, G2/M-enriched cyclin B1, and phosphohistone H3 serine (p-H3S10). (D) Western blot of lysates from HCN cells released from G2/M into proliferation medium (FGF2) for different lengths of time and probed with antibodies against G1/S-enriched cyclin D1, G2/M-enriched cyclin B1, and p-H3S10. GAPDH was used as a loading control. The Western blot experiments were done in replicates.

Fig. S3. (A) Western blotting against CDK1 for lysates from HEK293T or SS05 cells treated with MG132, HDAC3i, or HDAC3i+MG132. (B) Quantitative PCR (qPCR) of several ubiquitin-related genes in HDAC3 WT and F/F neurospheres infected with Ad-Cre-GFP virus. These qPCR assays were repeated three times independently. (C) Immunoprecipitation of CDK1 and Western blotting against HDAC3 in HCN cells. (D) Immunoprecipitation of CDK1 and Western blotting against acetyl-lysine in HCN cells treated with DMSO or HDAC3i or electroporated with HDAC3 overexpression plasmids. All of the Western blot experiments were replicated three times independently.
Fig. S4. Immunostaining of HDAC3 and NeuN (a marker of mature neurons) in the cortex, CA1, and CA3 in brain sections from P30 WT mice. (Scale bar: 50 μm.) One representative image from three independent stainings is shown.
Fig. S5. (A) Genomic PCR of Hdac3 in neurospheres isolated from tamoxifen (TAM)-injected WT and Hdac3 conditional KO (cKO) mice. (B) Representative immunostaining of YFP and MCM2 in Hdac3 WT (n = 3) and cKO mice (n = 3) at 10 days post-TAM (dpt). Arrows indicate YFP+MCM2+ transit-amplifying progenitor cells. (Scale bar: 50 μm.) (C) Quantification of YFP+DCX+ cells in Hdac3 WT and cKO mice at 30 and 60 dpt. (D) Quantification of YFP+GFAP+Sox2+ cells in Hdac3 WT and cKO mice at 60 and 90 dpt. *P < 0.05. ns, not significant. At least six mice of each genotype were used in C and D. DG, dentate gyrus.

Table S1. Primer information for quantitative PCR

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